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Sandeep Kumar
Pierluigi Barone
Michelle Smith *Editors*



Transgenic Plants

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John M. Walker

School of Life and Medical Sciences

University of Hertfordshire

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Transgenic Plants

Methods and Protocols

Edited by

Sandeep Kumar, Pierluigi Barone, and Michelle Smith

Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA

 **Humana Press**

Editors

Sandeep Kumar
Corteva Agriscience™
Agriculture Division of DowDuPont™
Johnston, IA, USA

Pierluigi Barone
Corteva Agriscience™
Agriculture Division of DowDuPont™
Johnston, IA, USA

Michelle Smith
Corteva Agriscience™
Agriculture Division of DowDuPont™
Johnston, IA, USA

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Preface

Advances in plant transgenic technology in the twentieth century led to the first commercially grown transgenic crop in 1996. In the creation of transgenic plants, the major hurdle for transfer of genetic material between species was overcome. This not only enabled fundamental insights into plant biology but also revolutionized commercial agriculture. Adoption of transgenic plants in industrial agriculture has reduced pesticide use, improved crop yields, and increased or preserved farm profitability. Targeted on meeting needs for greater efficiency and wider application of transgenic technology in plants, continuous technological advances over more than 30 years have paved the way for high-frequency transformation, but still of only a very small number of plant species; the majority of plants or their genotypes remain recalcitrant to transformation and regeneration. In addition, producing the complex traits that provide broad spectrum insect control, herbicide tolerance, and other agronomic traits desired by modern agriculture requires precise genome engineering. Recent developments in genome editing tools provide unique opportunities to create precise on-demand transgenic plants to enhance crop productivity. Combining genome engineering with new tools that overcome some of the limitations in plant transformation, there is clearly significant opportunity yet to explore in the area of transgenic plants.

We intend this book to provide thorough coverage of the topic of transgenic plants with methods capturing the current status of plant transformation, including precise genome engineering technology, biotechnological application of transgenic plants, and future developments needed to enable genome editing technology in crops. Grouped into sections focused on transformation of model and crop plants, genome engineering, and transgenic event characterization, the chapters primarily comprise detailed protocols as is typical in this series. We also sought review-style chapters to broaden the utility for readers, provide additional references for further understanding of this fascinating area of research, and especially to present the technology's potential for solving some of our most urgent global challenges in food security.

Johnston, IA

*Sandeep Kumar
Pierluigi Barone
Michelle Smith*

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Contributors

- AJITH ANAND • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- MAREN ARLING • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- SCOTT A. BEVAN • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- CHRISTOPHER R. BOOMSMA • *American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Madison, WI, USA*
- MYRON A. BRUCE • *Montana State University, Bozeman, MT, USA; Kansas State University, Manhattan, KS, USA*
- TOBIAS BRUEGMANN • *Thuenen Institute of Forest Genetics, Grosshansdorf, Germany*
- JORDAN BRUNGARDT • *Department of Plant Pathology, Kansas State University, Manhattan, KS, USA*
- M. BRUNGART-ROSENBERG • *Department of Plant Sciences, Sheridan Research and Extension Center, University of Wyoming, Sheridan, WY, USA*
- SI NIAN CHAR • *Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA, USA*
- QIU XIA CHEN • *Department of Horticulture, Plant Biotechnology Resource and Outreach Center, Michigan State University, East Lansing, MI, USA*
- YUEYING CHEN • *Department of Plant Pathology, Kansas State University, Manhattan, KS, USA*
- WEI CHEN • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- MARY-DELL M. CHILTON • *Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*
- CORY A. CHRISTENSEN • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- C. CLAFLIN • *Sheridan College, Sheridan, WY, USA*
- VLADIMIR A. DA COSTA • *Kemin Industries, Inc., Des Moines, IA, USA*
- BÁRBARA ANDRADE DIAS BRITO DA CUNHA • *Genetics and Biotechnology Laboratory, Embrapa Agroenergy (CNPAE), Brazilian Agricultural Research Corporation (EMBRAPA), Parque Estação Biológica, Brasília, DF, Brazil*
- JOHN P. DAVIES • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- ERIC A. DEAN • *Pairwise, Research Triangle Park, NC, USA; Department of Horticulture and Crop Science, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH, USA*
- KHIRA DEECKE • *Thuenen Institute of Forest Genetics, Grosshansdorf, Germany*
- TARLOCHAN SINGH DHADIALLA • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- S. A. DHEKNEY • *Department of Plant Sciences, Sheridan Research and Extension Center, University of Wyoming, Sheridan, WY, USA*
- SHUJIE DONG • *Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*

- SIVAMANI ELUMALAI • *Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*
- MARÍA PAULA FILIPPONE • *Instituto de Tecnología Agroindustrial del Noroeste Argentino (ITANOA), EEAOC-CONICET, Las Talitas, Tucumán, Argentina*
- JOHN J. FINER • *Department of Horticulture and Crop Science, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH, USA*
- MATTHIAS FLADUNG • *Thuenen Institute of Forest Genetics, Grosshansdorf, Germany*
- CHUNXIANG FU • *Qingdao Institute of BioEnergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, Shandong, China*
- DANIEL J. GACHOTTE • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- SATYALINGA SRINIVAS GAMPALA • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- WILLIAM GORDON-KAMM • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- D. J. GRAY • *Mid-Florida Research and Education Center, University of Florida/IFAS, Apopka, FL, USA*
- ANDIKA GUNADI • *Department of Horticulture and Crop Science, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH, USA*
- MANJU GUPTA • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- ANTHONY J. HARVEY • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- MATTHEW J. HAYDEN • *Department of Economic Development, Jobs, Transport and Resources (DEDJTR), Agribio, Bundoora, VIC, Australia; School of Applied Systems Biology, La Trobe University, Agribio, Bundoora, VIC, Australia*
- GEORGE HOERSTER • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- QINGZHEN JIANG • *Noble Research Institute, Ardmore, OK, USA*
- TODD JONES • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- PATRICIA KEEN • *The Boyce Thompson Institute, Ithaca, NY, USA*
- ADILSON KENJI KOBAYASHI • *Genetics and Biotechnology Laboratory, Embrapa Agroenergy (CNPAE), Brazilian Agricultural Research Corporation (EMBRAPA), Parque Estação Biológica, Brasília, DF, Brazil*
- RACHELLE A. LEFFERT-SORENSEN • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- BRIAN LENDERTS • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- Z. T. LI • *USDA-ARS, Kearneysville, WV, USA*
- RIQING LI • *Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA, USA*
- KEITH LOWE • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- TEJINDER MALL • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- DAVID G. J. MANN • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*

- POLYANA KELLY MARTINS • *Genetics and Biotechnology Laboratory, Embrapa Agroenergy (CNPAE), Brazilian Agricultural Research Corporation (EMBRAPA), Parque Estação Biológica, Brasília, DF, Brazil*
- JOHN MASON • *Department of Economic Development, Jobs, Transport and Resources (DEDJTR), Agribio, Bundoora, VIC, Australia; School of Applied Systems Biology, La Trobe University, Agribio, Bundoora, VIC, Australia*
- HUGO BRUNO CORREA MOLINARI • *Genetics and Biotechnology Laboratory, Embrapa Agroenergy (CNPAE), Brazilian Agricultural Research Corporation (EMBRAPA), Parque Estação Biológica, Brasília, DF, Brazil*
- EWEN MULLINS • *Department of Crop Science, Teagasc, Oak Park, Carlow, Co. Carlow, Republic of Ireland*
- MÓNICA NAVIA-URRUTIA • *Department of Plant Pathology, Kansas State University, Manhattan, KS, USA*
- JULIA NIETSCH • *CGS Crop Genetic Systems, Hamburg, Germany*
- STEPHEN NOVAK • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- MICHAEL PACHER • *Botanical Institute, Karlsruhe Institute of Technology, Karlsruhe, Germany*
- OLAF POLAK • *Thuenen Institute of Forest Genetics, Grosshansdorf, Germany*
- HOLGER PUCHTA • *Botanical Institute, Karlsruhe Institute of Technology, Karlsruhe, Germany*
- QIUDENG QUE • *Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*
- DHEERAJ SINGH RATHORE • *Department of Crop Science, Teagasc, Oak Park, Carlow, Co. Carlow, Republic of Ireland*
- KIMBERLY A. RICHEY • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- SARRIA RODRIGO • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- SAREENA SAHAB • *Department of Economic Development, Jobs, Transport and Resources (DEDJTR), Agribio, Bundoora, VIC, Australia*
- CARLA SCHMIDT • *Botanical Institute, Karlsruhe Institute of Technology, Karlsruhe, Germany*
- LORENA NOELIA SENDIN • *Instituto de Tecnología Agroindustrial del Noroeste Argentino (ITANOA), EEAOC-CONICET, Las Talitas, Tucumán, Argentina*
- S. K. SESSIONS • *Department of Plant Sciences, Sheridan Research and Extension Center, University of Wyoming, Sheridan, WY, USA*
- LIANG SHI • *Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*
- JESSICA L. SHOUP RUPP • *Montana State University, Bozeman, MT, USA; Kansas State University, Manhattan, KS, USA*
- GUO-QING SONG • *Department of Horticulture, Plant Biotechnology Resource and Outreach Center, Michigan State University, East Lansing, MI, USA*
- GERMAN SPANGENBERG • *Department of Economic Development, Jobs, Transport and Resources (DEDJTR), Agribio, Bundoora, VIC, Australia; School of Applied Systems Biology, La Trobe University, Agribio, Bundoora, VIC, Australia*
- VIBHA SRIVASTAVA • *Department of Crop, Soil & Environmental Sciences, University of Arkansas, Fayetteville, AR, USA; Department of Horticulture, University of Arkansas, Fayetteville, AR, USA*

- YI SUI • *The National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China*
- BIN TIAN • *Department of Plant Pathology, Kansas State University, Manhattan, KS, USA*
- MICHELLE TJAHHADI • *The Boyce Thompson Institute, Ithaca, NY, USA*
- HAROLD N. TRICK • *Department of Plant Pathology, Kansas State University, Manhattan, KS, USA*
- JOYCE VAN ECK • *The Boyce Thompson Institute, Ithaca, NY, USA; Plant Breeding and Genetics Section, School of Integrative Plant Science, Cornell University, Ithaca, NY, USA*
- TERENCE A. WALSH • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- NING WANG • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- ZENG-YU WANG • *Noble Research Institute, Ardmore, OK, USA*
- POHAO WANG • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- LAURA L. WAYNE • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- EMILY WU • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Johnston, IA, USA*
- CHUANYIN WU • *The National Key Facility for Crop Gene Resources and Genetic Improvement, Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China*
- BRYANT WULFBUHLER • *Corteva Agriscience™, Agriculture Division of DowDuPont™, Indianapolis, IN, USA*
- BING YANG • *Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA, USA*
- HENG ZHONG • *Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*
- EVELYN ZUNIGA-SOTO • *Department of Crop Science, Teagasc, Oak Park, Carlow, Co. Carlow, Republic of Ireland*

Part III

Utility of Transgenic Traits



Chapter 21

Overview of Biotechnology-Derived Herbicide Tolerance and Insect Resistance Traits in Plant Agriculture

Tejinder Mall, Manju Gupta, Tarlochan Singh Dhadialla, and Sarria Rodrigo

Abstract

Biotechnology has been central for the acceleration of crop improvement over the last two decades. Since 1994, when the first commercial biotechnology-derived tomato crop was commercialized, the cultivated area for genetically modified crops has reached 185.1 million hectares worldwide. Both the number of crops and the number of traits developed using biotechnology have accounted for this increase. Among the most impactful biotechnology-derived traits are insect resistance and herbicide tolerance, which have greatly contributed to the worldwide increase in agricultural productivity and stabilization of food security. In this chapter, we provide an overview of the history of the biotechnology-derived input traits, the existing genetically engineered commercial crop products carrying insect resistance and herbicide tolerance traits, as well as a perspective on how new technologies could further impact the development of new traits in crops. With the projection of the world population to increase to 9.8 billion by the year 2050 and reduction in available farmland, one of the biggest challenges will be to provide sustainable nourishment to the projected population. Biotechnology will continue to be the key enabler for development of insect resistance and herbicide tolerance traits to overcome that imminent challenge.

Key words Biotechnology, Insect resistance, Herbicide tolerance, Molecular characterization

1 Introduction

Over the last two decades, genetically engineered (GE) crops have been developed in multiple plant species (maize, soybean, cotton, canola, eggplant, etc.) to safeguard against insect pests, disease, weed, and abiotic stress damage to obtain higher yields. Up until 2016, these GE crops have been planted in 30 countries covering about 457 million acres and adopted by ~18 million farmers [1]. The numbers represent a very fast adoption of GE crops since 1996 when the crop acreages increased by more than 100-fold from only 4.2 million acres.

Currently, the major countries growing GE crops are the USA, Brazil, and Argentina. These crops have also been adopted in other

countries including other parts of North America, Latin America, China, India, Australia, the Philippines, Myanmar, South Africa, and Spain, and their adoption is expected to increase in additional countries in the near future [2]. So far the major focus for the development of GE crops has been in soybean, maize, canola, cotton, and sugar beet. However, GE traits have been produced and cultivated in other crops such as alfalfa, eggplant, papaya, potato, pineapple, squash, apple, plum, eucalyptus, and poplar [3].

It is projected that by 2050, the world population will increase to 9.8 billion [4] and this population growth is projected to outpace the current rate of crop yield increase (1.3% per year) due to declining land resources for agriculture and global warming adding to stressed environmental conditions for optimal crop productivity [5]. Hence, there is an urgent need to develop crops for stressed environments, e.g., abiotic, insect pests, weeds, and crop disease, that would also give higher crop yield in order to feed the ever-growing population. The grain yield for corn increased by 0.8 bushels/acre/year from 1937 to 1955, due to adoption of hybrid corn production. Subsequently, a second phase of yield increase of 1.9 bushels/acre/year occurred from 1955 until now due to continuous improvements in corn genetics, biotechnology-derived traits, and better agronomic production practices [6]. A potential third phase of yield increase is yet to come through the integration of new technologies beyond those used in currently commercialized GE crops.

In this chapter, we provide an overview of insect resistance (IR) and herbicide tolerance (HT) GE crops, including historical perspectives, commercialized crop products, and future perspectives. For more in-depth knowledge of the topics covered in this chapter, the reader is referred to excellent reviews by other authors [7–16].

2 Historical Perspective on Input Trait Biotechnology

2.1 *Herbicide Tolerance*

An important breakthrough came around the year 1983 when deliberate efforts were made to transform genes into crop plants [17–19]. It was not until over a decade later in 1996 that the first commercial transgenic crop—“Flavr Savr” fresh tomatoes—was marketed by Calgene (now Monsanto). These tomatoes were the first example of the commercial value and impact biotechnology could have in a wide variety of traits in plant agriculture. Biotechnology techniques were also used to introduce genes for herbicide tolerance (HT) into soybean, cotton, corn, and canola crops [20]. HT crops help farmers in reducing production costs and environmental impact of using herbicides for weed control. Genetically engineered HT crops also eliminate the need for manual removal of weeds that compete with the crop of interest (COI)

[21] and help to preserve topsoil microflora by encouraging the use of non-tillage agricultural practices [22]. A variety of HT traits have been developed for selective control of weeds using glyphosate, glufosinate, 2,4-D, and other herbicides maintaining other agronomic attributes of GE crops. A premise for the development of HT traits is the availability and understanding of the herbicide chemistry, their environmental impact, ease of use, cost-effectiveness, and their mode of action. Furthermore, it is also important to consider the type of trait stewardship program needed after the trait is commercialized to minimize environmental impact, achieve trait longevity, and avoid development of resistant weeds in specific geographies where the technology is used. Without doubt, the HT traits presently available provide farmers great convenience, increase crop productivity and management efficiency, and also provide environmental benefits that impact soil health and water quality in their farms.

2.2 Insect Resistance

To date most of the bioinsecticides and insecticidal proteins have come from the soil bacterium *Bacillus thuringiensis* (*Bt*). The first *Bacillus* bacterium (*Bacillus sotto*) was discovered and isolated from silkworms in 1901, and subsequently *Bt* was isolated from Mediterranean flour moth caterpillars after the dead caterpillars were found to be loaded with *Bt* spores and crystals. This observation led to the conclusion that *Bt* spores and crystals had insecticidal properties, which led Mattes [23] to isolate the strain for subsequent use in field trials against the European corn borer, *Ostrinia nubilalis*, with promising results. This work eventually led to the development of Sporeine, a commercial *Bt* insecticide for lepidopteran pest control, used initially in 1938 in France, and registered for use in the USA in 1961. Since then, through strain improvement and improved fermentation processes, a number of *Bacillus* bioinsecticides have been commercialized for control of lepidopteran (e.g., Biobit, Dipel, Thuricide, and Javelin), dipteran (e.g., Tekar, Bactimos, Vectolex GC, and Acrobe), and coleopteran (Novodor and Trident) pests [8]. The next stage in the evolution of *Bacillus* bioinsecticides was the production of hybrid *Bacillus* strains with increased toxicity and insecticidal specificity. This was achieved by either conjugation of two different strains with different insecticidal protein-encoding genes or direct transformation of one strain with insecticidal genes from another *Bacillus* strain [24, 25]. This practice would be equivalent to today's "pyramiding" of insecticidal genes for purposes of insect resistance management and broader target insect pest specificity. An example of a trans-conjugant commercial product included 'Foil', which was based on a strain EG2424 created at Ecogen for activity against lepidopteran and coleopteran insects and carried the *cryIAc* gene from *Bt* var. *kurstaki* (active against *O. nubilalis*) and the *cry3A* gene from *Bt* var. *tenebrionis* (active against Colorado potato

beetle, *Leptinotarsa decemlineata*) [26]. An example of a recombinant strain EG2348 commercialized under the name of Raven by Ecogen for control of lepidopteran larvae expressed a combination of endogenous (*cryIAc*) and transgenic (*cry3A* and *cry3Bb*) insecticidal proteins from different *Bacillus* strains.

Use of *Bacillus* strains or their spores directly as sprays to control certain insects was attractive in being specifically toxic to host pests, nontoxic to humans and mammals, and biodegradable, but had disadvantages too. Some of the major disadvantages were that *Bt* strains or spores were susceptible to rapid inactivation with UV light, heat, and extreme pH rendering them ineffective. Additionally, the *Bt* sprays were susceptible to protease inactivation by host plant leaf exudates and were also easily washed away by wind and rain. This meant that for effective control of target pests, *Bt* sprays had to be applied and reapplied frequently, which resulted in increased cost to control target insect pests. Additionally, the *Bt* sprays were effective against specific chewing insects (Lepidoptera or beetle larvae on plants) but not effective against sap-sucking insect pest stages of hemipteran and homopteran orders like aphids, whiteflies, and others [27].

A solution to circumvent the challenges for effective use of insecticidal *Bt* strains or spores was to isolate genes encoding the insecticidal proteins and express them directly within the COI through genetic engineering techniques. Using this approach, the insecticidal proteins would be expressed in the COI and be readily available for intoxication of target insect pests for immediate control. However, technological hurdles were discovered in expressing bacterial genes in plants. *Bt* or other insecticidal toxin-encoding genes eventually required codon optimization and use of appropriate promoters for effective *in planta* expression [28, 29] to deliver the desired insect resistance. It was also discovered that the full-length gene sequence of the insecticidal protein may not be required for plant transformation as only the domains responsible for insecticidal activity and protein expression stability may be enough for *in planta* pest control efficacy for certain insecticidal proteins. Hence, Hoffmann [30] genetically engineered tobacco plants using a truncated gene encoding the N-terminal portion of a *cryIA* gene from *Bt* var. *kurstaki* HD-73 under the control of cauliflower mosaic virus (CaMV) 35S promoter. The engineered tobacco plants were protected from damage with infested *Helicoverpa zea* larvae. This demonstration of effective control of an insect pest using transgenic tobacco, though never meant to be developed for commercialization, paved the way to develop other approaches to create GE insect resistance traits in other COI. One of the first crops targeted for commercial development and marketed was the potato NewLeaf variety carrying the *cry3A* from *Bt* var. *tenebrios* developed by Monsanto in 1995 for the control of the Colorado potato beetle. This potato variety received the first registration

from the Environmental Protection Agency (EPA) for a crop resistant to an insect pest. Subsequently a number of other transgenic crops providing tolerance to specific insect pests have been developed (Table 1).

3 Important Technologies in Input Trait Development

3.1 Genetic Engineering of Crops

Plant breeding had long been a method of crop improvement to develop new varieties with improved characteristics. Use of traditional plant breeding approaches has led to tremendous success in crop improvement. Major plant breeding achievements such as introduction of hybrid corn and improved wheat and rice varieties were among the most important advancements that resulted in dramatic increases in crop productivity [31].

However, traditional plant breeding methods have limitations in that genes can be transferred only within the same or closely related species, and often can have significant yield drag from other genes linked to a given trait. Moreover, as indicated above, in the case of IR and HT traits, most of the more impactful genes have originated from the non-plant sources, requiring the use of gene transfer technologies to move genes across noncompatible species. Therefore, the discovery, development, and use of *Agrobacterium*- and gene gun-mediated gene transfer methods delivered additional tools and approaches for transfer of specific and desired segments of DNA from any organism into a wide range of crop species. Later, other methods of transformation were also discovered and overall these methods can be grouped under two broad categories: (1) indirect transformation methods (i.e., bacterium mediated) and (2) direct transformation methods (i.e., nonorganismic mediated by chemical and mechanical introduction of DNA into cells). An overview of these methods is presented in another chapter of this book (Chap. 1).

3.2 Molecular Characterization of Transgenic Events

Molecular characterization of transgenic events (where an event is a plant that contains the transgene(s) and expresses the inserted gene phenotype) is foundational to the advancement of high-quality events in the commercial trait development process. Hundreds of events are created in the beginning of the trait development process using any of the plant transformation methods described above, and eventually one event is selected for commercialization [32]. Molecular characterization encompasses multiple parameters for evaluation of transgenic events including identification of inserted gene copy number, number of insertion sites, integrity and location of inserts in the target genome, identification of “junction” sequences at both termini of a transgene(s) cassette, unintended DNA presence including transformation vector backbone, and stability of the insertions across generations [33]. All this

Table 1
List of insect resistance and herbicide-tolerance transgenic crops, product names, transgenes used, phenotype expressed, and company that produced the product

Crop	Product	IR/HT/IR_HT	Gene	Phenotype	Company
Soybean	Enlist Soybean	HT	aad-12, pat	Glufosinate tolerance	Dow AgroSciences
Corn	Herculex I Insect Protection × Roundup Ready Corn 2	IR/HT	cry34Ab1, cry35Ab1, pat, cp4 epsps	Coleoptera resistance, glufosinate and glyphosate tolerance	DAS, DuPont Pioneer
Corn	Optimum Leptra	IR	cry1F, pat, cry1Ab, Vip3Aa19, cp4 epsps	Lepidoptera resistance, glyphosate tolerance	DuPont Pioneer
Corn	Herculex I Insect Protection	IR	cry1F, pat	Lepidoptera resistance, glufosinate tolerance	DuPont Pioneer
Corn	Optimum Intrasect	IR	cry1f, pat, cry1Ab, cp4 epsps		DuPont Pioneer
Corn	Optimum Intrasect Xtra	IR	cry1F, pat, cry1Ab, cry34Ab1, cry35Ab1 cp4 epsps	Lepidoptera and coleoptera resistance, glufosinate and glyphosate tolerance	DuPont Pioneer
Corn	Optimum TRIsect	IR	cry1Fa2, pat, mcry3A, cp4 epsps	Lepidoptera and coleoptera resistance, glufosinate and glyphosate tolerance	DuPont Pioneer
Corn	Qrome	IR	cry1F, cry34Ab1, cry35Ab1, pat, cry1Ab, mcry3A, cp4 epsps	Lepidoptera resistance, rootworm resistance, glyphosate and glufosinate tolerance	DuPont Pioneer
Corn	Herculex I × Roundup Ready Corn 2	IR/HT	cry1F, pat, cp4 epsps	Lepidoptera resistance, glufosinate and glyphosate resistance	Dow AgroSciences, DP
Corn	PowerCore	IR/HT	cry1A.105, cry2Ab1, cry1F, pat	Lepidoptera resistance, glufosinate resistance	Dow AgroSciences, Monsanto
Corn	Herculex I	IR	cry1F, pat	Lepidoptera resistance, glufosinate resistance	Dow AgroSciences

Corn	SmartStax	IR	cry1A.105, cry2Ab1, cry1F, cry3Bb1, cry34Ab1, cry35Ab1, cp4 epsps, pat, aad-1	Lepidoptera resistance, rootworm resistance, glyphosate/glufosinate tolerance	Dow AgroSciences, Monsanto
Corn	SmartStax [®] Enlist [™] Corn	IR/HT	cry1A.105, cry2Ab1, cry1F, cry3Bb1, cry34Ab1, cry35Ab1, cp4 epsps, pat, aad-1	Lepidoptera resistance, rootworm resistance, glyphosate/glufosinate/2,4-D tolerance	Dow AgroSciences
Corn	PowerCore Enlist	IR/HT	cry1A.105, cry2Ab1, cry1F, pat, aad-1	Lepidoptera resistance, glyphosate/glufosinate/2,4-D tolerance	Dow AgroSciences
Corn	Herculex XTRA	IR	cry1F, pat, cry34Ab1, cry35Ab1	Lepidoptera and coleopteran resistance, glufosinate tolerance	Dow AgroSciences, DP
Cotton	WideStrike	IR	cry1F, cry1Ac,	Lepidoptera resistance	Dow AgroSciences
Cotton	WideStrike × FLEX	IR/HT	cry1F, cry1Ac, vip3A	Lepidoptera resistance	Dow AgroSciences
Cotton	WideStrike × RR1445	IR/HT	cry1F, cry1Ac, cp4 epsps, bar	Lepidoptera resistance, glyphosate and glufosinate tolerance	Dow AgroSciences
Cotton	WideStrike 3 × RR Flex × Enlist Cotton	IR/HT	cry1F, cry1Ac, vip3A, cp4 epsps, pat, aad-12	Lepidoptera resistance, glyphosate and glufosinate tolerance, 2,4-D	Dow AgroSciences
Cotton	WideStrike 3 × RR Flex	IR/HT	cry1F, cry1Ac, vip3A, cp4 epsps, pat	Lepidoptera resistance, glyphosate and glufosinate tolerance	Dow AgroSciences
Cotton	Enlist Cotton	HT	aad-12, pat	2,4-D tolerance	Dow AgroSciences
Cotton	WideStrike 3 × Enlist Cotton	HT	cry1F, cry1Ac, vip3A, aad-12	Lepidopteran resistance, 2,4-D tolerance	Dow AgroSciences
Cotton	WideStrike × RR Flex × Vipcot	IR/HT	cry1Ac, vip3A(a), cry1F, cp4 epsps, pat	Lepidoptera and coleoptera resistance, glufosinate and glyphosate tolerance	Dow AgroSciences
Corn	Herculex RW	IR/HT	pat, cry34Ab1, cry35Ab1	Coleoptera resistance, glufosinate tolerance	Dow AgroSciences
Soybean	Roundup Ready Soybeans	HT	cp4 epsps	Glyphosate tolerance	Monsanto

(continued)

Table 1
(continued)

Crop	Product	IR/HT/IR_HT	Gene	Phenotype	Company
Soybean	Roundup Ready 2 Xtend Soybeans	HT	cp4 epsps	Glyphosate tolerance	Monsanto
Soybean	Genuity Roundup Ready 2 Yield Soybeans	HT	cp4 epsps, dmo	Glyphosate, dicamba tolerance	Monsanto
Soybean	Intacta RR2 PRO Soybeans	IR/HT	cp4 epsps, cry1Ac	Glyphosate tolerance, lepidopteran resistance	Monsanto
Soybean	Vistive Gold	HT	cp4 epsps	Glyphosate tolerance, high oleic acid	Monsanto
Corn	Roundup Ready Corn 2	HT	cp4 epsps	Glyphosate tolerance	Monsanto
Corn	Roundup Ready Corn 2/LibertyLink Maize	HT	cp 4 epsps, pat	Glyphosate/glufosinate tolerance	Monsanto
Corn	SmartStax	IR/HT	cry1A.105, cry2Ab1, cry1F, cry3Bb1, cry34Ab1, cry35Ab1, cp4 epsps, pat	Lepidoptera resistance, rootworm resistance, glyphosate/glufosinate tolerance	Monsanto/DAS
Corn	YieldGard Corn Borer Corn	IR	cry1Ab	Lepidoptera resistance	Monsanto
Corn	YieldGard Corn Borer with Roundup Ready Corn 2	IR/HT	cry1Ab, cp4 epsps	Lepidoptera resistance, glyphosate tolerance	Monsanto
Corn	YieldGard Plus	IR	cry1Ab, cry3Bb1	Lepidoptera resistance, rootworm resistance	Monsanto
Corn	YieldGard Plus with Roundup Ready Corn 2	IR/HT	cry1Ab, cry3Bb1, cp4 epsps	Lepidoptera resistance, rootworm resistance, glyphosate tolerance	Monsanto

Corn	YieldGard Rootworm Corn	IR	cry3Bb1	Rootworm resistance	Monsanto
Corn	YieldGard Rootworm with Roundup Ready Corn 2	IR/HT	cry3Bb1, cp4 epsps	Rootworm resistance, glyphosate tolerance	Monsanto
Corn	YieldGard VT PRO	IR	cry1A.105, cry2Ab1,	Lepidoptera resistance	Monsanto
Corn	YieldGard VT Rootworm	IR	cry3Bb1, cp4 epsps	Rootworm resistance, glyphosate tolerance	Monsanto
Corn	YieldGard VT Triple	IR/HT	cry1Ab, cry3Bb1, cp4 epsps	Lepidoptera and rootworm resistance, glyphosate tolerance	Monsanto
	YieldGard VT triple PRO	IR/HT	cry1A.105, cry2Ab1, cry3Bb1, cp4 epsps	Lepidoptera and rootworm resistance, glyphosate tolerance	Monsanto
Cotton	Bollgard 3 Roundup Ready Flex	HT	cry1Ac, cry2Ab, vip3A, cp4 epsps	Lepidoptera resistance and herbicide tolerance	Monsanto
Cotton	Bollgard 3 XtendFlex cotton	IR/HT	cry1Ac, cry2Ab, vip3A, cp4 epsps, pat, DMO*	Lepidoptera resistance and herbicide tolerance each	Monsanto
Cotton	Bollgard Cotton	IR	cry1Ac	Lepidoptera resistance	Monsanto
Cotton	Bollgard II XtendFlex	IR	cry1Ac, cry2Ab, cp4 epsps, pat, DMO	Lepidoptera resistance and herbicide tolerance	Monsanto
Cotton	Genuity Bollgard II Cotton	IR	cry1Ac, cry2Ab	Lepidopteran resistance	Monsanto
Cotton	Genuity Bollgard II with Roundup Ready Flex Cotton	IR/HT	cry1Ac, cry2Ab, cp4 epsps	Lepidoptera resistance, glyphosate tolerance	Monsanto
Cotton	Genuity Roundup Ready Flex Cotton	HT	cp4 epsps	Glyphosate tolerance	Monsanto
Cotton	Roundup Ready Cotton	HT	cp4 epsps	Glyphosate tolerance	Monsanto

(continued)

Table 1
(continued)

Crop	Product	IR/HT/IR_HT	Gene	Phenotype	Company
Cotton	Roundup Ready with Bollgard Cotton	IR/HT	cp4 epsps, cry1Ac	Lepidoptera resistance, glyphosate tolerance	Monsanto
Cotton	XtendFlex Cotton	HT	epsps, pat, DMO	Herbicide tolerance	Monsanto
Canola	Genuity Roundup Ready Canola	HT	cp4 epsps	Glyphosate tolerance	Monsanto
Alfalfa	Roundup Ready Alfalfa	HT	cp4 epsps	Glyphosate tolerance	Monsanto
Alfalfa	HarvXtra with Roundup Ready	HT	ccomt(inverted repeat), cp4 epsps	Glyphosate tolerance	Monsanto
Sugarbeet	Roundup Ready Sugarbeets	HT	cp4 epsps	Glyphosate tolerance	Monsanto
Corn	Agrisure 3000GT	IR/HT	cry1Ab, mcr3A, pat, mepsps	Lepidoptera and coleoptera resistance and glyphosate tolerance	Syngenta
Corn	Agrisure 3120	HT	cry1Ab, cry1F, pat, mepsps	Lepidoptera resistance, herbicide tolerance	Syngenta
Corn	Agrisure 3122	iR/HT	cry1Ab, cry1Fa2, pat, mepsps, mcr3A, cry34Ab1, cry35Ab1	Lepidoptera and coleoptera resistance, glufosinate and glyphosate resistance	Syngenta
Corn	Agrisure CB/LL	iR/HT	cry1Ab, pat	Lepidoptera resistance, glufosinate tolerance	Syngenta
Corn	Agrisure CB/LL/RW	iR/HT	cry1Ab, pat, mcr3A	Lepidoptera and coleoptera resistance, glufosinate tolerance	Syngenta
Corn	Agrisure Duracade E-Z Refuge 5122	iR	ecry3.1Ab (synthetic/chimeric)	Lepidoptera resistance	Syngenta

Corn	Agrisure Duracade E-Z Refuge 5222	iR/HT	ecry3.1Ab, mcrv3A (synthetic/ chimeric), cry1Ab, pat, cry1Fa2, mepsps, vip3A20	Lepidoptera resistance, glufosinate and glyphosate tolerance	Syngenta
Corn	Agrisure GT	HT	mepsps	Glyphosate tolerance	Syngenta
Corn	Agrisure GT/CB/LL	IR/HT	cry1Ab, pat, mepsps	Lepidoptera resistance, glufosinate and glyphosate tolerance	Syngenta
Corn	Agrisure GT/RW	IR/HT	mcrv3A, mepsps	Coleoptera resistance, glyphosate tolerance	Syngenta
Corn	Agrisure RW	IR	mcrv3A	Coleoptera resistance	Syngenta
Corn	Agrisure Viptera	IR	vip3A20	Lepidoptera resistance	Syngenta
Corn	Agrisure Viptera 3110	IR/HT	cry1Ab, vip3Aa20, pat, mepsps	Lepidoptera resistance, glufosinate and glyphosate tolerance	Syngenta
Corn	Agrisure Viptera 3111	IR/HT	cry1Ab, vip3Aa20, mcrv3A, pat, mepsps	Lepidoptera and coleoptera resistance, glufosinate and glyphosate tolerance	Syngenta
Corn	Agrisure Viptera 3220	IR/HT	cry1Ab, cry1Fa2, vip3Aa20, pat, mepsps	Lepidoptera resistance, glufosinate and glyphosate tolerance	Syngenta
Corn	Enogen/Agrisure 3000GT	OT/IR/HT	amy797E, cry1Ab, pat, mepsps, mcrv3A	Amylase, lepidoptera, and coleoptera resistance, glufosinate and glyphosate resistance	Syngenta
Corn	Knockout Insect resistance corn	IR/HT	cry1Ab, bar	Lepidoptera resistance and glufosinate tolerance	Syngenta
Cotton	Bollgard III	IR	vip3A(a), cry1Ac, cry2Ab2	Lepidoptera resistance	Monsanto
Cotton	Vipcot Roundup Ready Flex	IR/HT	vip3A(a), cry1Ab, cp4 epsps	Lepidoptera resistance and glyphosate tolerance	Syngenta
Cotton	Vipcot Roundup Ready Flex	IR	vip3A(a), cry1Ab, cp4 epsps	Lepidoptera resistance	Syngenta
Soybean	LibertyLink Soybeans	HT	pat	Glufosinate tolerance	Bayer Crop Sciences

(continued)

Table 1
(continued)

Crop	Product	IR/HT/IR_HT	Gene	Phenotype	Company
Corn	LibertyLink Maize	HT	pat	Glufosinate tolerance	Bayer Crop Sciences
Cotton	LibertyLink Cotton	HT	bar	Glufosinate tolerance	Bayer Crop Sciences
Cotton	Liberty Link × Bollgard II Cotton	IR/HT	cry1Ac, cry2Ab2, bar	Lepidoptera resistance, glufosinate tolerance	Bayer Crop Sciences
Cotton	GlyTol Cotton	HT	2mepsps	Lepidoptera resistance, glufosinate and glyphosate tolerance	Bayer Crop Sciences
Cotton	GlyTol × Liberty Link	HT	2mepsps, bar	Herbicide tolerance	Bayer Crop Sciences
Canola	LibertyLink Canola	HT	bar	Herbicide tolerance	Bayer Crop Sciences
Canola	SeedLink Canola	HT	bar	Herbicide tolerance	Bayer Crop Sciences

information is used in determining proper trait functionality and stability, and for safety assessment of the commercial event(s) for regulatory approval [34, 35] leading to event deregulation. The technologies used for the molecular characterization of transgenes are discussed in another chapter in this book (Chap. 24).

3.3 Protein Detection and Quantitation

Gene expression analysis is a crucial component of trait product development for selection of transgenic events that express genes at desired levels, in specific tissues and temporally in the COI. The first step of gene expression analysis can be performed at the messenger RNA (mRNA) level. However, quantitative protein expression analysis is of utmost importance due to posttranscriptional and posttranslational modifications that can affect the correlation of mRNA levels with the final protein quantity and quality and the resulting phenotypic expression. This is a key step in the evaluation of gene expression for input traits. Immunoassays, e.g., western blots and enzyme-linked immunosorbent assays (ELISA), have been the primary analytical approaches for detection and quantitation of proteins. However, these assays require the development of antibodies that can take several months, may exhibit cross-reactivity to other protein(s), and have high development cost for routine use. More recently, significant progress has been made in the development of liquid chromatography in conjunction with tandem mass spectrometry (LC-MS/MS) as the next-generation protein detection technology toward solving the challenges posed by immunoassays [36]. The basis of the LC-MS/MS technology is the detection of specific signature peptides as surrogates representing the target proteins. It has significant advantage over immunoassays in that assay development requires only a few days as compared to multiple months needed for immunoassays. These technologies are also reviewed in detail in another chapter in this book (Chap. 25).

4 Currently Available Insect Resistance and Herbicide Tolerance Genes in GE Crops

4.1 Insect Resistance Traits: Gene Sources and Approaches

4.1.1 *Bacillus thuringiensis*-Derived Traits

Although it was realized in the early 1900s that *Bt* strains have insecticidal spores and crystals, it was not until 1986 when the first *Bt* gene encoding a lepidopteran active insecticidal protein was isolated, cloned, and used to protect tobacco plants from damage by *Helicoverpa zea* [30]. Since then a number of *Bt* genes, encoding insecticidal proteins, have been isolated, cloned, and screened for insecticidal properties against a range of pests. In addition to the insecticidal proteins in *Bt* crystals produced from *cry* (crystal) genes, *Bt* also produce cytolytic (*cyt*) insecticidal proteins and soluble insecticidal proteins, which are produced during the vegetative growth phases. Genes from *Bt*'s vegetative growth phase called *vips* (vegetative insecticidal proteins), either alone or in combination

with *cry* or *cyt* genes, have been isolated and used to develop transgenic crops with protection against insect damage.

Despite a wealth of knowledge of *Bt* insecticidal proteins and their genes, reviewed by Crickmore et al. and Narva et al. [37, 38], only a handful of the genes (*cry1A*, *cry2A*, *cry1F*, *cry3A*, *cry3B*, *cry34A*, *cry35A*, and *vip3A* and their variants) have been used to genetically engineer commercial crops for resistance to lepidopteran and coleopteran pests. Initially, GE crops were commercialized using a single insect resistance (IR) gene products, although in several instances IR traits were developed in combination with the HT traits. Another approach has been to stack insecticidal proteins with different modes of action targeting a specific order of insect pests to delay or prevent the development of resistance in the insects. Agricultural companies have mostly discovered homologs of *Bt cry* genes, and in very few cases produced synthetic variants of those *Bt* genes to create novel gene versions, like in the case of the mutated *mcry3A* gene for resistance to coleopteran pests and the synthetic chimeric gene, *ecry3.1Ab*, that also confers resistance to coleopteran pests (Table 1). Transgenic insect control traits have been widely beneficial and their commercial success is demonstrated by their widespread use by farmers in many industrialized and developing countries [39].

4.1.2 Non-Bacillus thuringiensis-Derived Traits

While *Bacillus thuringiensis* strains continue to provide a rich source of insecticidal proteins, there is a need to look for alternative sources or approaches for developing crops for IR traits to broaden the spectrum of modes of action available given the diverse pests that threaten commercial crops. The need for this is not only to increase the spectrum of activity to control primary and secondary insect pests using multiple modes of action of the insecticidal proteins, but also to delay the development of resistance by susceptible insects. However, after 20 years of *Bt* crop products in the market, there have been very few confirmed cases of insect pests developing resistance to the *Bt* gene-carrying crops [40]. This is mainly due to the implementation of strict insect resistance management strategies like inclusion of certain percentage of non-transgenic seed (refuge plants) along with the trait-containing transgenic seed provided. Also more recently, the industry has moved to stacked insecticidal products with more than one mode of action against key insect pests to help slow the development of resistance. However, it is expected that insects will eventually become resistant to the existing transgenic traits and in anticipation of this there have been numerous reports of alternative approaches being developed and insecticidal proteins being evaluated that are not derived from *Bt* sources.

4.1.2.1. Non-Bacillus thuringiensis Bacterial Gene Sources

Certain other bacteria have been the focus of extensive research as potential sources of insecticidal proteins. Such examples are the insect pathogenic bacteria, *Photorhabdus* and *Xenorhabdus* species, both of which are symbionts of the insect pathogenic nematode, *Heterorhabditis* [41]. These bacteria are nonpathogenic to the nematode itself. However, in insects, once the bacteria enter the insect host blood system or hemocoel, it becomes very pathogenic to the insect and rapidly overcomes the insect immune system resulting in insect mortality. Extensive research has been carried out to biochemically and molecularly characterize a complex (multiple proteins needed for insecticidal activity) of insecticidal *Photorhabdus* and *Xenorhabdus* toxin proteins, and attempts made to produce transgenic crops using these insecticidal proteins. Although much is known of the structure and function of the toxin complex proteins [42], little progress has been made in developing transgenic crops using genes encoding these proteins, possibly due to host plant phytotoxicity issues. It is possible that future research will allow the engineering of toxin complex genes with high insect control efficacy. Alternatively, other bacterial sources can be used to identify proteins with insecticidal activity.

4.1.2.2. Other Sources for Enhancing Insect Resistance in Plants

Production of proteases in the insect gut or in saliva is important for efficient protein digestion that sustains amino acid and metabolite pools essential for insect growth and development. Herbivorous insects feed either by chewing the plant tissues, as in the case of Lepidoptera and Coleoptera, or by piercing or rasping and sucking intra- or intercellular fluids from plants, as in the case of hemipteran and thysanopteran insects, respectively. Although not always effective, plants use proteinase inhibitors (PIs) as a common defense mechanism to defend from insect attack. When plant-produced PIs are ingested in artificial diets or overexpressed in plants, increased mortality and reduced growth and development have been reported for lepidopteran, coleopteran, or orthopteran immature stages [43–47].

Another approach explored for insect control is the expression of proteins that inhibit function and stability of α -amylases. Insects and other animals use α -amylases for digestion of starch ingested in their diet. Proteins that inhibit α -amylases are found widely in the plant kingdom. Such inhibitory proteins are directed specifically for the inhibition of α -amylase activity from insects and microorganisms without affecting the plant enzymes. Multiple examples of the use of α -amylase inhibitors have been reported with mixed results. The α -amylase inhibitor 1 gene from common bean (*Phaseolus vulgaris*) was expressed in pea (*Pisum sativum*) plants and it provided complete protection against the coleopteran pest, *Bruchus pisorum* L., commonly known as pea weevil. However, the α -amylase inhibitor 2 when expressed in pea plants only resulted

in delayed maturation of pea weevil larvae [48]. Some bifunctional PIs have been identified that simultaneously inhibited both proteinases and α -amylases [49]. These bifunctional PIs offer the benefit of controlling pests via two different modes of action, perhaps enhancing their effectiveness against insect pests.

Other types of plant proteins have also been identified with potential but with varying levels of efficacy for insect control. Lectins are a class of multivalent carbohydrate-binding proteins found in plants that have been tested against insects for growth inhibition and mortality. In a study by Shukle and Murdock [50], lectins from soybean seed were found to inhibit growth of tobacco hornworm larvae. Similar results have been obtained using wheat germ agglutinin and snowdrop lectin when tested against European corn borer [51] and tomato pinworm, *Lacanobia oleracea*, respectively [52]. Other non-plant proteins, chitinases, produced by insects, have also been tested for insect control and results demonstrated impact on insect growth inhibition by disruption of the peritrophic membrane lining in the gut of *Heliothis virescens* [53].

Although enhanced protection of plants against lepidopteran pests has been reported by expressing PIs, lectins, α -amylases, and chitinases [9, 54], there has yet to be a commercial product using PI-encoding genes in crops. Some of the challenges that may have prevented this success could be related to either the potency of the PI proteins expressed *in planta*, including their expression levels, or the insect's ability to use endogenous proteases that circumvent the plant-expressed PIs. This highlights the need to understand the physiology and temporal expression of proteases in the insect gut and salivary glands in response to different PIs. Other approaches may also be needed like the use of multiple and different PI genes in transgenic plants or even chimeric genes encoding bifunctional gene products, such as α -amylase/trypsin inhibitors [9], trypsin/carboxypeptidase A inhibitors [55], and cystatin/serine PI [56].

Among other strategies that have been used to identify new insecticidal toxins, the screening of small peptide toxins produced in venoms of predatory spiders was investigated [57]. The venoms of these predatory spiders contain a number of disulfide-rich small (3–4 kDa in size) peptides that are selectively toxic to insects upon injection into the insect hemocoel. However, one of the functional challenges that need to be circumvented first is the fact that these peptides are not very active when ingested orally. Promising results have been obtained in several studies where expression of venom proteins from the Australian funnel-web spider, *Atrax robustus*, in tobacco [58, 59] and cotton [60] showed significantly increased protection against *Helicoverpa armigera*. Results from these experiments offer the possibility to engineer improvements of the peptides for effective crop protection.

4.1.3 RNA-Mediated Technologies for Insect Control: dsRNA (RNAi)

Double-stranded RNA (dsRNA) to interfere with specific gene expression, also referred to as RNA *interference* (RNAi), has been used extensively to suppress or knock out specific genes in eukaryotic cells [61–63]. This approach takes advantage of a control mechanism that occurs naturally in eukaryotic cells whereby short stretches of diced mRNA or microRNA produced within a cell direct the degradation of complementary endogenous mRNA to suppress the expression of specific genes when required in that cellular environment. Similar effects can be achieved by design involving delivery and expression of an exogenous dsRNA molecule against a target transcript in cells of an organism. Timmons and Fire [64] and Timmons et al. [65] first demonstrated that ingestion of a dsRNA by the nematode, *Caenorhabditis elegans*, suppressed expression of a target gene. This work and many other investigations with insects [66–69] offered that a similar approach could be used for insect pest control, provided that (1) target genes critical for the survival or the growth and development of insects could be identified and (2) an effective method for delivery of dsRNA could be developed.

A number of insect target genes have been identified as critical for either insect growth and development or survival. An example of such genes is the vacuolar ATPase A subunit [62]. When a dsRNA against the vacuolar ATPase A was either fed mixed in artificial diet or injected into the hemocoel of coleopteran insect pests like corn rootworm and others, it affected larval development [70]. Baum et al. [67] also reported that western corn rootworm (WCR) larvae which fed on corn transformed with a dsRNA hairpin against the vacuolar ATPase A subunit gene, exhibited severe stunting of growth, and inhibited feeding damage to roots. Similarly, Mao et al. [71] demonstrated that *Arabidopsis* plants transformed with dsRNA hairpin that targets a cytochrome P450 monooxygenase gene in cotton bollworm led to decreased bollworm tolerance to the cotton sesquiterpene aldehyde, namely gossypol, thus decreasing insect damage.

While there has been significant progress either to identify target genes for dsRNA or in the delivery methods (e.g., via injection, in artificial diet, or through transgenic crop tissues), different levels of insect susceptibility to these approaches were observed. For example, the coleopteran insects, corn rootworm, Colorado potato beetle, and the canola flea beetle are sensitive to ingested dsRNA (e.g., V-ATPase subunit) with LC₅₀ values ranging from 1 to 10 ppb in both larval and adult stages [67, 72, 73]. Insects outside of the order Coleoptera (like Diptera, Lepidoptera, and Hemiptera) that have been tested with the V-ATPase subunit dsRNA are almost a 1000-fold less sensitive (LC₅₀ ≥ 10 ppm). Even within the order Coleoptera, not all coleopterans, like red flour beetle, are as sensitive to dsRNA as corn rootworm or Colorado potato beetle [67, 74]. Of the 290 gene targets identified by

Baum et al. [67], dsRNA was effective for only about 40% of the targets using a surface overlay diet bioassay with about 0.1 ppm for each target dsRNA, causing rootworm mortality or growth stunting. At the same time, when *H. armigera* and *Spodoptera exigua* larvae were fed transgenic crops expressing *H. armigera* ecdysone receptor dsRNA, the phenotypic effects (e.g., elevated mortality, growth inhibition, and suppression of ecdysone receptor transcripts over controls) on the larvae were similar [75].

Despite the tremendous gain in the understanding of gene targets that are essential for development and survival of insects, it has taken long to commercialize transgenic plants using the RNAi approach. In insects that are much less susceptible to sub-ppm dsRNA concentrations, the biggest barriers may be their gut anatomy that could hinder the effective delivery of dsRNA to target tissues and cells outside midgut. Therefore, challenges may remain in developing methods for improved delivery of dsRNA for effective adsorption in the insect gut of certain insects and delivery to target tissues.

The durability of RNAi-based approaches remains uncertain, mainly due to the lack of understanding of the mechanisms of resistance, i.e., whether there is a common mechanism of inactivation of dsRNA or multiple mechanisms. A single mechanism of resistance among target insect pests would severely reduce the durability of RNAi-based crop products.

Finally, the reader is referred to the volume in *Advances in Insect Physiology* [57], which covers many of the aspects discussed above in greater depth. Some other suggested readings covering the above topic extensively are [70, 76–81].

4.2 Herbicide Tolerance Traits and Weed Control

Weed control practices used by early farmers included hand or mechanical weed control, cultural practices like crop rotation, and even mechanical means through plowing and cultivation. These methods were often tedious and slow, and came with a high cost. Therefore, better, more efficient, and easier weed control methods were needed. The major breakthrough that came in the weed management practices was the discovery of synthetic auxin herbicides in 1940s which spawned an era of innovation yielding other selective and some nonselective herbicides in the intervening decades. Some important nonselective herbicides were glyphosate and glufosinate and selective herbicides including acetolactate synthase (ALS), acetyl coenzyme A carboxylase (ACCase), hydroxyphenylpyruvate dioxygenase (HPPD), phytoene desaturase (PDS), and protoporphyrinogen oxidase (PPO). Today, more than 200 herbicide-active ingredients are available that are classified in 29 different modes of action categories of which six modes of action cover more than 80% of the herbicide market [82].

Farmers that controlled weeds using selective herbicides needed to have a good understanding of weed identification in

order to choose appropriate herbicide applications and often multiple herbicides were required to control different categories of weeds. In addition to crop-selective herbicide use, another breakthrough in the field of weed control was the introduction of herbicide-tolerant crops, with the first set of crops developed tolerant to glyphosate and glufosinate (Table 1). Prior to the availability of HT crops, glyphosate was used to control weeds in non-crop areas and its use against weeds closely associated with crops was not possible due to its broad-spectrum activity which could damage the crops. In 1996, glyphosate-tolerant crops were introduced making weed control in crops much easier for the farmers, since a single application of glyphosate killed all weeds in the field while having no effect on the transgenic glyphosate-tolerant crops [83].

The glyphosate mode of action is based on its binding affinity to the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which is required for synthesis of essential aromatic amino acids in plants, and blocking EPSPS activity results in plant death [84]. In order to generate transgenic glyphosate-tolerant crops, three strategies have been attempted: (1) overexpression of the endogenous COI EPSPS enzyme to avoid glyphosate toxicity; (2) introduction of genes that metabolize and detoxify glyphosate in the COI; and (3) introduction of a glyphosate-insensitive form of the EPSPS enzyme t [85]. The first strategy using overexpression of the native EPSPS enzyme resulted in glyphosate tolerance of plant cells in culture and transgenic *Petunia* plants [86]. However, overall the strategy has not been very successful since application of glyphosate still affected the growth of the plants. Until now, no transgenic plants overexpressing a native EPSPS enzyme have been commercialized [85]. The second strategy for metabolic degradation of glyphosate has been in which glyphosate-metabolizing enzymes, like GOX (glyphosate oxidoreductase) and GAT (glyphosate N-acetyl transferase), have been engineered to impart glyphosate tolerance to plants [87]. The third strategy of using a glyphosate-insensitive form of the target enzyme and its variants has been the most effective and successfully used for the production of transgenic crops [82, 88]. Mutated forms of this enzyme, which are resistant to the action of glyphosate, have been introduced into crops resulting in commercial levels of tolerance in transgenic plants [89].

Another weed control system that became widely used was the production of genetically modified crops tolerant to glufosinate. Glufosinate inhibits the glutamine synthetase enzyme leading to accumulation of ammonia in the cells, which affects photosynthesis and eventually leads to plant death. Glufosinate is another nonselective herbicide but is faster acting than glyphosate. Glufosinate has gained greater adoption to help manage weeds that have developed glyphosate resistance [90]. In contrast to glyphosate, which is a systemic herbicide, glufosinate is a very effective contact

herbicide. It needs to be applied when plants are smaller to have a good leaf coverage. The gene that encodes the enzyme phosphinothricin acetyltransferase (PAT) detoxifies glufosinate by acetylation of the amino group and hence makes plants tolerant to glufosinate [91]. Using this technology, transgenic canola, corn, cotton, and soybean herbicide-tolerant crops were introduced into the market starting 1997 [92].

Glyphosate-tolerant soybean and canola were introduced and deregulated for commercialization in 1996, followed by glyphosate-tolerant cotton and maize in 1997 and 1998, respectively [83]. Within 10 years of the introduction, the adoption rate for these crops reached >90% in the USA [93]. Glyphosate- and glufosinate-resistant crops were initially widely adopted crops by farmers. First, the glyphosate- and glufosinate-resistant crops were introduced as single-trait crops. However, later with the discovery of insect-resistance traits, the herbicide-tolerance traits were stacked with insect-resistant traits. Moreover, multiple modes of action for insect resistance as well as genes imparting resistance to different herbicides were stacked. In 2016, globally, 455 million acres were planted with crops that include herbicide tolerance, insect resistance, and stacked gene products. This includes 227.6 million acres under herbicide-tolerant crops only [2]. This broad adoption of HT crops has resulted in increased value to farmers due to reduced weed control costs and increased productivity, cumulatively worth around \$63 billion, over a period of 1996–2014 [94].

Another herbicide target is the enzyme acetohydroxyacid synthase (AHAS), also called acetolactate synthase (ALS). This enzyme is required for the synthesis of branched-chain amino acids and inhibition of its activity results in plant death due to the depletion of essential amino acids [95]. More than 50 ALS inhibitors have been discovered that fall in five chemical classes: sulfonylurea [SU], imidazolinones [IMI], triazolopyrimidines [TP], pyrimidinylthiobenzoates [PTB], and sulfonylamino-carbonyl-triazolinones [96]. Different mutations in the ALS gene are known to impart tolerance to some or all of the above herbicides. These mutated forms of the enzyme have been used to develop crops tolerant to specific herbicide chemical classes. Such crops have been commercialized under the name Clearfield from 1992 onwards [97]. Largely, the Clearfield crops were developed using non-GE plant breeding methods and were nontransgenic in nature. However, recently BASF launched genetically modified soybean with the trade name Cultivance in collaboration with Embrapa in Brazil [98]. Following the introduction of Clearfield corn in 1992 by BASF, a large number of crops including rice, wheat, brassicas, corn, soybean, lettuce, sunflower, etc. have been developed for tolerance to ALS-inhibitory herbicides [97].

Because glyphosate herbicide-tolerant crops have been the most widely adopted weed control system, the extensive use of

glyphosate among the farmers and lack of integrated weed management practices have led to the development of glyphosate resistance in weeds. By mid-2017, around 37 glyphosate-resistant weed species had been reported [93]. Therefore, new strategies have been under development to control emerging glyphosate-resistant weeds. This has stimulated the discovery of new trait genes encoding new modes of action. The most prominent next-generation weed control systems include Enlist and Roundup Ready Xtend in corn, cotton, and soybean [99]. The Enlist technology includes the use of 2,4-dichlorophenoxyacetic acid (2,4-D), which is a broad-spectrum herbicide and controls more than 90 weed species including glyphosate-resistant weeds such as horseweed and water hemp [100]. The trait is encoded by the bacterial aryloxyalkanoate dioxygenase (AAD) gene product, which inactivates 2,4-D and provides excellent tolerance to this chemical in plants [101]. Dow AgroSciences, LLC, has used stacked HT-tolerant gene strategy to transform soybean, corn, and cotton for tolerance to 2,4-D and other herbicides, for effective weed control [102] and to allow implementation of this weed management system. Monsanto has also developed a Roundup Ready Xtend Crop system that stacks dicamba and glyphosate tolerance for weed control [102]. Dicamba tolerance is provided by the bacterial dicamba monooxygenase gene (DMO) that inactivates dicamba when sprayed on crop plants [99]. Another category of herbicides is the 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors. HPPD is a key enzyme required for the production of pigments and for the development of chloroplasts. Inhibition of this enzyme affects chloroplast biosynthesis, which leads to bleaching followed by plant death. The overexpression of the resistant forms of HPPD, that is less sensitive to HPPD inhibitors, is the strategy used to impart tolerance to HPPD-inhibiting herbicides such as Balance Flexx, Callisto, and Lumax [103–105]. Recently Bayer Crop Sciences has developed transgenic soybean, Balance GT, that carries a gene stack of both the glyphosate-tolerance trait combined with tolerance to isoxaflutole-based HPPD-inhibiting herbicide [106]. In general, the stacking of multiple modes of action for herbicide tolerance will be more effective for weed control and is expected to delay the development of resistance in weeds, thus increasing the durability of the HT traits.

Other high-potential herbicides are PPO (protoporphyrinogen oxidase) inhibitors and ACCase (acetyl coenzyme A carboxylase) inhibitors. PPO is a gene product that acts in the chlorophyll/heme biosynthetic pathway. Chlorophyll is an essential light-harvesting pigment and heme is required as a cofactor in many enzymes. Inhibiting the synthesis of this enzyme causes light-dependent membrane damage resulting in burning, desiccation, and growth inhibition of the plants [107]. Overexpression of naturally resistant bacterial PPO genes results in resistance to PPO-inhibiting

herbicides [108, 109]. Monsanto and Sumitomo have partnered to develop the next generation of crops tolerant to PPO-inhibiting herbicides [110]. Similarly, ACCase carries the first step in fatty acid biosynthesis. Inhibiting the activity of ACCase results in cessation of fatty acid biosynthesis that eventually leads to plant death. Point mutations in this gene provide resistance to the application of ACCase-inhibitory chemicals, like aryloxyphenoxypropionate class of herbicides abbreviated as FOP herbicides [111]. Development of herbicide-tolerant crops using these different modes of action will diversify the weed control tool box and provide more effective weed controls for a wide variety of weeds while reducing the probability of developing herbicide resistance. Although multiple and diverse HT modes of action are available for stacking, and despite the challenges with glyphosate weed resistance, glyphosate is still widely used and is a component of new HT stacks. Glyphosate is expected to continue to be used in the near future in commercial HT stack releases [82, 110].

Though multiple modes of action for herbicide tolerance are available today, these are based on the existing herbicide chemistries. The discovery of new herbicides is not being as actively pursued as for insecticides and fungicides where the search for novel chemistries and modes of action are areas of focus. The 1970s was the era when herbicides with new modes of action were discovered every 2–3 years [93]. Today, the availability of a good number of herbicide chemistries bearing different modes of action has reduced the need to further discover and develop new ones [82].

5 Next Generation of Insect Resistance and Herbicide-Tolerant Traits

Site-specific nucleases (SSNs) have emerged as a powerful technology for genome engineering. Of the SSNs, the CRISPR-Cas system is most easy to design, affordable, and versatile. Using SSNs, it is possible to add, delete, and edit genes or modulate their expression [112, 113]. The add and delete functions permit transgene manipulations including molecular stacking at preselected (“preferred”) genomic locations in plants [114, 115]. As a result, the trait introgression process is simplified, as multiple transgenes (traits) can be managed or tracked as a single locus to ensure physical and expression stability of the transgenes and surrounding host genome genes. SSN-based development of molecular stacks is an elegant example of genome engineering that impacts superior organization of transgenes relative to the native genes in the genomes of the major crops.

The “edit” function of SSNs is aimed at introducing gene modifications to create gene knockouts and expression modulations, develop new alleles, or engineer genes that express new

protein versions. Therefore, using the edit function, new alleles can be added to the repertoire of the existing alleles to increase allelic diversity. Learnings from the mutation technologies practiced in the past few decades, e.g., T-DNA insertion [116], transposon tagging [117], ethyl methane sulfonate (EMS)-induced mutations screened with TILLING approach [118, 119], and fast neutron (FN)-induced deletions [120], have illustrated the development of a large number of new crop varieties worldwide by essentially modifying genes or alleles to create new versions with new or additional functions. At least 3270 new varieties were developed from the mutation research alone [121].

6 Trait Reengineering

Another important application of the SSN technology is to modify/reengineer the existing transgene(s) already present in commercial GE crops. This could be conceptually explained as the generation of new allelic variants from the existing transgenes. Many of the insect resistance genes currently present in commercial products, as discussed earlier in this chapter, are continuously challenged by insect pests trying to overcome their susceptibility to IR traits. With the advances in protein engineering, reengineered versions of the existing IR genes may become readily available in the future, which when replacing the older version could quickly reestablish protection in the COI, thus avoiding financial losses to the farmers and the industry in general. This approach would bypass the need for recreating a completely new stack, which is a process that could take more than a decade before commercial release. The same concept could apply to HT genes with the purpose to generate new transgene alleles that provide protection to active ingredient analogs for broader or more effective weed control spectrum. Alternatively, and as mentioned for IR genes, HT trait genes can be removed from commercial events and replaced with new/improved genes in the stack.

There are immense opportunities that CRISPR-CAS, a simple and precise genetic scissor, has to offer in crop improvement and its potential impact on trait development and agriculture in general.

7 Future Perspectives

GE traits have been very impactful in crop production. These traits have made possible the industrialization of several crops, contributing to food security worldwide. Traits for IR and HT are among the most impactful and widely used in agriculture, thus improving land management, reducing operational costs, and decreasing environmental impact by minimizing the number of chemical applications

of insecticides and herbicides, which in turn reduces the use of fossil fuels. GE traits have also reduced the exposure of farmers to chemicals by reducing the application of herbicides by 50%, thus improving the quality of their lives [39]. However, despite all those benefits and the great rate of adoption of these technologies, greater adoption of GE crops is needed worldwide to increase productivity in other parts of the world where these technologies have not yet been adopted.

The development of GE traits is not possible without the advancement and development of enabling technologies that improve the ability to discover and develop new and better input traits. Technologies like crop transformation and genome sequencing (like with next-generation sequencing (NGS)) play a key role in the discovery, testing, and development of IR and HT GE crops. Similarly, analytical technologies for molecular and biochemical characterization make possible the analyses of transgenic events and also allow for the generation of key information to assess their safety and support deregulation of these traits. Last but not least, further development of phenotyping technologies will continue to enhance the ability to make clearer determinations of the efficacy of traits and also help assess agronomic performance.

To date GE IR traits commercially available have been mainly derived from *Bacillus thuringiensis* genes. The development of IR traits is very complex and has to meet the criteria not only for trait efficacy, or insect control, but also for minimal to no impact on nontarget organisms and agronomic performance of COI. Due to these technical challenges, the current toolbox has relatively few insecticidal genes with different modes of action available. To increase the availability of genes encoding for insecticidal activity and with a diversity of modes of action for managing insect resistance and prolonged product life, gene discovery efforts have been focused on non-*Bt* gene sources.

In the case of GE HT, stacked modes of action for HT are becoming available to widen the spectrum of weeds that can be controlled, improve weed control efficacy, and also extend trait durability. In recent years, greater emphasis has been given to the implementation of integrated weed management programs whereby herbicides with different modes of action should be applied in rotation or in combination to slow the development of herbicide-resistant weeds.

To date IR and HT GE crops have been produced using random gene insertions into the plant genome using primarily *Agrobacterium*-based or biolistic-mediated transformation methods. However, technologies using SSNs are now available, which allow delivery of transgenes to precise locations in the plant genome. Furthermore, SSNs can be used to edit native genes and create new and trait-specific allelic variants in the host genome. This technology could also be used to reengineer traits developed by

GE, or to create completely new genes with novel modes of action or functions. Such approaches can play a key role in accelerating the development and availability of IR or HT traits in the near future.

Further increases in crop yields will continue to be delivered through greater adoption of GE crops, discovery and development of new IR and HT trait genes, better integrated pest management systems, improved cultural practices, and use of new and novel technologies to enhance trait performance without negative agro-nomic effects in the elite varieties of target crops. IR and HT crops will continue to be, without a doubt, among the most impactful traits to meet the challenge of feeding the world's growing population.

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