Transgenic Herbicide Resistance in Plants

V.S. Rao

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Dedication

This book is dedicated to Sri Rāmachandra

and

To my loving wife Nirmala Devi

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Preface

Currently, the field of weed science is facing a growing challenge in the form of rapid evolution of weeds resistant to a wide spectrum of herbicides whose use has tremendously increased ever since the commercialization of phenoxyacids, beginning with 2,4-D, in 1946. Initially, plant biologists and weed scientists linked the chemistry of the herbicide to the biology of plants and selected suitable newer herbicides to combat global weed problems both in cropped and non-cropped situations. Over the past 45 years, these herbicides have also become the cause and effect of about 240 weed species showing resistance to 155 of them belonging to 22 of the 25 known herbicide sites of action around the world. This trend is not going to abate any time soon. Instead, it is expected to exacerbate.

An altogether different, but not unrelated, development took place in the late 1980s which culminated in the use of genetic engineering to produce crop varieties resistant to herbicides, particularly the nonselective ones whose field use is precluded because of crop toxicity. In this fast-developing area, termed transgenic engineering, two approaches are usually followed to insert herbicide resistance into a crop plant. In one approach, either the plant enzyme or other sensitive biochemical target of herbicide action was made to be insensitive to the herbicide, or the unmodified target protein was induced to overproduce, thus permitting normal plant metabolism to occur. The other approach was the introduction of an enzyme or enzyme system that degrades or detoxifies the compound in the plant before the herbicide reaches the site of action. Plants modified by both approaches were obtained by transferring non-plant (and even from within the plant kingdom) genes that encode herbicide resistance traits into the target plant genome. In doing so, plant scientists linked the biology of plants to the chemistry of the herbicide.

In this process of genetic engineering, scores of herbicide-resistant transgenic varieties, beginning with the bromoxynil-resistant 'BXN' cotton line in 1994, have been developed in several crops. This technology was taken further by applying it to engineer resistance in crops to other biotic stresses like insects, plant pathogens, etc. as well abiotic stresses, besides improving qualitative and quantitative traits of crops. Currently, two transgenic traits—herbicide-resistance and insect-resistance—dominate the crop biotech technology by accounting for about 95 percent of the global area under biotech varieties in maize, soya bean, cotton, and rapeseed (canola).

Despite the obvious pecuniary and non-pecuniary benefits derived by farmers of more than two dozen countries which adopted these transgenic crop varieties over the past two decades, transgenic technology has raised more risks and issues so far than could be resolved both at the farmer level and consumer level. After all, farmers and consumers are the vital components in the success of any agricultural technology. Consequently, development of transgenic crops has been viewed more as a profit-driven rather than need-driven process.

At the present time, few topics in global agriculture are more polarizing and controversial than transgenic engineering.

However, transgenic engineering has the arsenal to address various problems plaguing agriculture in a more effective way than ever and to meet the future food and fiber needs of the rising global population. As in the case of the atom, electronics, computers, and communication, the more we understand the intricacies of this plant-related technology, the better we will be able to utilize it for the betterment of mankind.

This work has been designed to bring out a comprehensive reference-cum-textbook on the basic principles of herbicide resistance and transgenic engineering besides a detailed discussion on the current status of transgenes used to engineer herbicide (also insect) resistance in crops, and development of herbicide-resistant transgenic crop events and stacked varieties and hybrids. The book also deals with the role of transgenic technology in phytoremediation of soils and environment from organic contaminants such as herbicides, insecticides, fungicides, oil spills, explosives, etc. and inorganic pollutants that include natural elements. Additionally, it deals with other chapters that discuss the global adoption and regulation of transgenic crops, while bringing to the fore the benefits transgenic crops were reported to have derived to the global community and discussing the various risks and issues that concern and affect both farmers and consumers. The presentation is based on facts, tinged with impartiality.

As it is impossible to review the entire research done in each of the areas under discussion, I chose to include only the more useful material to bring relevance and objectivity to the subject under discussion. I also chose to exclude some topics because of space constraints. I accept full responsibility for choice of information, presentation, interpretation, and discussion. However, I cannot assume responsibility for the contents of the original references as well as the success or otherwise of a gene expression, gene-sequencing, genotyping, transformation methods/protocols, etc. The reader is advised to refer to the original source for more information. If trade and commercial names are mentioned, it was only as a matter of convenience to the reader but not as an endorsement of a particular product or variety. I generally followed American orthography while writing this book.

During the course of working on this edition, I have been the recipient of constant encouragement given by my loving wife, Nirmala Devi, as well as my beloved daughter, Madhavi Lata Rajavasireddy, and beloved son Srinivas R. Vallurupalli. Not to be outdone by them were my son-in-law Rajiev and daughter-in-law Neelima, my dear grandsons Nikhil and Milind, and granddaughters Ria, Reva and Rayna who have always surrounded me and given me immense joy.

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I have reproduced in this book certain copyrighted material, including some figures and the accompanying discussions and material published in scientific journals, books, and the Internet. I have received help in other ways too to help me prepare this book. I gratefully acknowledge the help extended and permissions granted to the following highly regarded scientists and individuals.

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Acronyms

Genes, Gene Elements

***Aac*C3; *aac*C4 genes**	3-*N*-aminoglycoside acetyl transferases 3 and 4
ACCase	Acetyl-CoA carboxylase enzyme
***aad*1 gene**	Aryloxyalkanoate dihydrogenase gene 1 (from *Sphingobium herbicidovorans*)
addA	Aminoglycoside-3-adenyltransferase gene
adh1	Alcohol dehydrogenase gene 1 (class I)
AHAS	acetohydroxyacid synthase gene
alcA	Alcohol dehydogenease I gene promoter
AlcR	alcohol dehydogenease transactivator protein
als	Acetolactate synthase gene
m*ALS*	mutated Acetolactate synthase gene
***amp*R**	Ampicillin restriction gene
***aph*IV**	Aminoglycoside phosphotransferase type IV gene
APX	Ascorbate peroxidase promoter
Arab-SSUlA/CTPl	**A. thaliana* small subunit 1A ribulose-1,5-bisphosphate carboxylase
ARG7	Arginosuccinate lyase locus 7
AroA	Aaromatic amino acid gene (glyphosate-resistance)
aroA-M1	Aaromatic amino acid gene mutant 1 gene
AtuORF1 3'UTR	**A. tumefaciens* 3′ untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 1
AtuORF23 3'UTR	*A. tumefaciens* 3′ untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 23.
AtUbi10	*A. thaliana* polyubiquitin UBQ10 comprising the promoter 5′ untranslated region and intron
avhppd-03	**A. fatua p*-hydroxyphenylpyruvate dioxygenase
BADH	Betaine aldehyde dehydrogenase gene
bar	Bialaphos resistance gene
bla	β-lactamase gene, codes for antibiotic ampicillin
ble	Bleomycin resistance gene
bxn	Bromoxynil resistance gene from *Klebsiella anthropic* subsp. *ozaenae*
CaMV 35S	Cauliflower mosaic virus of the 35S RNA promoter
CAT	Chloramphenicol acetyltransferase
***Cla*I**	Endonuclease restriction enzyme (cleaves double-stranded DNA) in a site-specific manner: recognition site is: ATCGAT
CMP	Cestrum yellow leaf curling virus

CO1	mitochondrial gene *cytochrome-C oxidase-1*
colE1	Carries a gene for colcin E1 (the *cea* gene), a bacteriocin toxic to bacteria including *E. coli*
cp*4 *epsps	*epsps* gene from *Agrobacterium* spp. Strain CP4
Cre-*lox*	Cyclization recombination locus X (site-specific recombination system of bacteriophage P1)
cry1A.105	Gene from *B. thuringiensis* subsp. *kumamotoensis*, encodes Cry1A.105 protein comprising of the Cry1Ab, Cry1F and Cry1Ac proteins
cry1Ab	Gene from *B. thuringiensis* subsp. *kurstaki* (*B.t.k.*) strain HD-1, encodes Cry1Ab δ-endotoxin
cry1Ac	Gene from *B. thuringiensis* var. *kurstaki* HD73 encodes Cry1Ac δ-endotoxin
cry1F	Gene from *B. thuringiensis* var. *aizawai* strain PS811, encodes Cry1F protoxin
cry1Fa2	Gene from *B. thuringiensis* var. *aizawai*, encodes modified Cry1F δ-endotoxin
cry2Ab	Gene from *B. thuringiensis* strain 14-1, encodes Cry2Ab δ-endotoxin
cry2Ae	Gene from *B. thuringiensis* subsp. *Dakota*, encodes Cry2Ae δ-endotoxin
cry3Bb1	Gene from *B. thuringiensis* subsp. *kumamotoensis* strain EG4691, encodes Cry3Bb1 δ-endotoxin
cry34Ab1	Gene from *B. thuringiensis* strain PS149B1, encodes Cry34Ab1 δ-endotoxin
cry35Ab1	Gene from B. *thuringiensis* strain PS149B1, encodes Cry35Ab1 δ-endotoxin
CS-dmo	Coding sequence of dicamba monooxygenase
csrt-1	Mutated form of acetolactate synthase (contains a single nucleotide change, resulting in a single amino acid substitution in the ALS protein)
CsVMV	Cassava vein mosaic virus promoter
CTP	Chloroplast transit peptide
ctp2	DNA sequence for the N-terminal of chloroplast transit peptide from *A. thaliana* EPSPS gene
dhfr	Dehydropholate reductase gene
dmo	Dicamba monooxygenase gene from *Stenotrophomonas maltophilia*
dms	Dicamba O-demethylase (dicamba) gene
***Eco*RI**	Endonuclease enzyme isolated from strains of *E. coli*. Recognition site: GAATTC
***Eco*RI site**	*E. coli* recognition site of endonuclease: cleaves the phosphodiester bonds of DNA at specific nucleotide sequences
ecry3.1Ab	Synthetic form of *cry3A* and *cry1Ab* genes from *B. thuringiensis* encodes Cry3A-Cry1Ab δ-endotoxin
5'e1	Tapetum specific E1 gene (GE1) of *Oryza sativa*
epsps	5-enolpyruvylshikimate-3-phosphate synthase gene
***epsps* ace5**	Modified *epsps* gene derived from **A. globiformis*
***epsps* grg23ace5**	Modified *epsps*-glyphosate resistance gene 23 from **A. globiformis*
2m-*epsps*	Double mutant *epsps* gene

ept	*Estrogen-induced pituitary tumor 1* gene
E9 3'	3′ non-translated region of the pea RbcS gene E9
EcR	Ecdysone receptors from moths
ER	Human estrogen receptor
Fad	Fatty acid desaturase enzyme
Flp/*frt*	Flp: enzyme flippase; *frt*: flippage recognition target from *Saccharomyces cerevisiae*
FMV 35S	Figwort mosaic virus 35S RNA
FMV/Tsf1	Figwort mosaic virus 35S RNA from the Transferrin 1 gene of *A. thaliana*
gat	Glyphosate *N*-acetyltransferase gene
gat4621	Modified *gat* gene based on the sequences of the three *gat* genes from *Bacillus licheniformis*
GFP	Green fluorescent protein
Gin/*gix*	Gin: G inversion; *gix*: the recombination sites (*Gin-gix* system from bacteriophage Mu)
gm-hra	*Glycine max*-herbicide resistant acetolactate synthase gene
gox	Glyphosate oxidoreductase gene
GT	Glucocorticoid receptor from rat
Gumbi	*Glycine max* ubiquitin
GUS	β-D-glucuoronidase
H4A748	Promoter that codes for the S-phase specific Histone 4
His3	Histidine 3
3′histonAt	The 3′ untranslated region of the histone H4 gene of *A. thaliana*
hpp	Hydroxyphenylpyruvate dioxygenase gene
hppdPfW336	p-hydroxyphenylpyruvate dioxygenase gene of **P. fluorescens* strain A32 modified by replacing amino acid Gly with Trp at position 336
hsp70	Heat shock protein 70 gene
intron1 h3At	First intron of Gene II of the histone H3.III variant of *A. thaliana*
IPT	Isopentenyltransferase gene
***lox*P**	Locus X-over P1
manA	Phosphomannose isomerase gene
matK	Chloroplast gene: maturaseK
mcry3A	Synthetic form of *cry3A* gene from *B. thuringiensis* subsp. *tenebrionis*, encodes mcry3A δ-endotoxin
neo	Neomycin phosphotransferase gene
nos	Napoline synthase gene *A. tumefaciens*
***nos* 3'**	Napoline synthase 3'-polyadenylation signal
3'nos	The 3′ polyadenylation signal from non(un)-translated region of napoline synthase from *A. tumefaciens*
***Not*I**	DNA restriction enzyme from **N. otitids-caviarum*
npt*II/*neo	Neomycin phosphotransferase II from *E. coli*
***npt*III**	Neomycin phosphotransferase III from *Streptococcus faecalis R* plasmid
OCS	Octopine synthase from *A. tumefaciens*
ORF	Open reading frame
ORF25 polyA	3′ polyadenylation signal from ORF25 (*A. tumefaciens*)
ORI ColE1	Origin of replication for colcin E1
Ori-pBR322	Origin of replication from pBR322 for maintenance of plasmid in *E. coli*

ori-V	Origin of replication for *Agrobacterium*-derived from the broad host range of plasmid in RK2
ORIpVS1	Origin of replication from the *Pseudomonas* plasmid pVS1
Ori-322	Origin of replication in *E. coli* plasmid pBR322
OsAct2	*Oryza sativa actin2* gene
OsCc1	*Oryza sativa cytochrome c* gene1
OsDMC1	*Oryza sativa* disrupted meiotic complementary DNA1 gene
OsTubA1	*Oryza sativa* α-tubulin gene which consists of four exons and three introns
OTP	Optimized transit peptide which directs translocation of proteins to chloroplasts. It is derived from plant sequences obtained from maize and sunflower ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCo)
pat	Phosphinothricin acetyl transferase gene **S. viridochromogenes* strain Tu494
pcd	Phenmedipham hydrolase gene derived from *Arthrobacter oxydans* strain P52
PCLSV	Peanut chlorotic streak caulimovirus promoter
P-*e*35S	Cauliflower mosaic virus 35S RNA containing the enhancer region that directs transcription in cells
PG	Polygalacturonase gene
PGD1	Phosphogluconate dehydrogenase 1 promoter
Ph4a748	The promoter region from the gene for histone H4 from *A. thaliana*
Ph4a748At	A sequence including the promoter region of the histone H4 gene derived from *A. thaliana*
PhL	Phosphorylase-L gene
pmi	Phophomannose isomerase (Mannose-6-phospho isomerase) from *E. coli*
***pin*II**	Proteinase inhibitor II from potato (**S. tuberosum*)
pol	Polymerase gene
Ppo	Polyphenol oxidase gene
***Pqr*A**	Paraquat resistance gene
Ps7s7	Duplicated promoter region derived from subterranean clover stunt virus genome segment 7
***psb*A**	Q_B protein (D_1 protein: PS II reaction center) from *Amaranthus hybridus*
P35S3	Promoter region of the CaMV 35S transcript
PSsuAra	A rubisco small subunit gene from *Arabidopsis thaliana*
***pvu*I**	*Proteus vulgaris* restriction enzyme I
rbcl	Chloroplast gene: *ribulose-bisphosphate carboxylase*
rbcS	Ribulose-1,5-bisphosphate carboxylase small subunit
RuBisCo	Ribulose bisphosphate carboxylase oxygenase enzyme
SAG21	Senescence-associated gene 21
SCP1	Super core promoter 1
Sul	Sulfonamide resistance encoding gene
T-35S	CaMV 35S 3′ polyadenylation signal
Tahsp17.3'	**T. aestivum* 3′ untranslated region heat shock protein
tCUP	**T**obacco **c**onstit**u**tive **p**romoter
Tdc	Tryptophan decarboxylase gene
TetR	A tetracycline repressor protein

tetO	Tetracycline operator sequence
TEV	Tobacco etch virus
5'tev	5' end of tobacco etch virus
tfdA	Gene which encodes ferrous iron-dependent dioxygenase that uses α-ketoglutarate as a co-substrate
TpotpC	Coding sequence of an optimized transit peptide, containing sequence of the RuBisCo small subunit genes of **Z. mays* and **H. annuus*
TPotp Y	Optimized transit peptide derivative (position 55 changed to tyrosine) containing sequence of the RuBisCo small subunit genes of *Z. mays* and *H. annuus*
tRA	A tetracycline transactivator fusion protein
TS- CTP2	Targeting sequence from the *Shk*G gene encoding the chloroplast transit peptide region of *A.thaliana* EPSPS
Ubi	*Ubiquitin*
ubi4	Ubiquitin gene 4
ubi7	Ubiquitin gene 7
ubi9	Ubiquitin gene 9
Ubi Zm1	Ubiquitin *Z. mays* promoter, and the first exon and intron
UBQ10	Ubiquitin-10 gene promoter poly (UBQ10) of *A. thaliana*
uidA	Gene which encodes β-D-glucuoronidase (*E. coli* K12)
3'-UTR	3′ untranslated region
3'-UTR of wheat HSP 17.3	3′-UTR of wheat heat shock protein 17.3
vip3Aa20	*Gene from B. thuringiensis* strain AB88, encodes modified Cry3A δ-endotoxin
XhoI	**X. holcicola* type II restriction endonuclease which cleaves DNA to give specific double-stranded fragments with terminal 5′-phosphates
xylA	Xylulose isomerase encoding gene
zm-epsps	*Z. mays*-5-enolpyruvylshikimate-3-phosphate synthase
ubi Zm1	Ubiquitin *Z. mays* promoter, and the first exon and intron
ZFNs	Zinc finger nucleases
Zm-hra	Modified acetolactate synthase (als) from *Zea mays*
ZmPer5 3' UTR	*Zea mays* peroxidase 3′ untranslated region
***Zm ubi1* (*ubi Zm1*)**	*Zea mays* ubiquitin and the first exon and intron

**Agrobacterium tumefaciens*; **Arabidopsis thaliana*; **Arthrobacter globiformis*; **Avena fatua*; **Bacillus thuriginensis; *Escherichia coli*; **Helianthus annuus*; **Nocordia otitids-caviarum*; **Pseudomonas fluorescens*; **Solanum tuberosum*; **Streptomyces viridochromogenes; *Triticum aestivum*; **Xanthomonas holcicola*; **Zea mays*

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The Author

Dr. Vallurupalli Sivaji Rao, native of Gudlavalleru, Krishna District, Andhra Pradesh, India, is an eminent weed scientist with a Bachelor's degree in Agriculture from Karnataka University (1961) and Master's degree in Agronomy from Osmania University (1963), both in India. He followed them up with a Ph.D. degree in weed science from Cornell University, USA, in 1973. He has worked in several universities (including Cornell), governmental research institutions, and international and non-governmental organizations in India and USA for over 50 years, teaching and researching in the field of weed science in crops like rice, tea, sorghum, maize, wheat, alfalfa, and groundnut. He has vast experience of conducting lab research on herbicide action mechanisms as well as field research to develop farmer-oriented weed management programs. He has held the positions of senior scientist, professor, director, advisor, and consultant. Currently, he is an Affiliate Member of Department of Plant Sciences, University of California, Davis, California, USA.

Dr. Rao is credited with scores of research publications in addition to authoring the widely popular reference-cum-textbook "Principles of Weed Science", with its first edition published in 1983 and second edition in 2000. He has served the Indian Society of Weed Science as Executive Vice President and was Editor-in-Chief of Indian Journal of Weed Science during 1982–90. He has also served on several national and state committees in India as a member and chairman between 1980 and 1994. Dr. Rao is the recipient of the Lifetime Achievement Award of Indian Society of Weed Science for 2010. He is a life member (emeritus) of some professional societies in India and USA including the Weed Science Society of America. He and his wife have been living in California, USA since 1995.

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CHAPTER 1

Introduction

Knowledge is both bliss and power. The pursuit of knowledge is a characteristic of the human mind which constantly raises questions only to answer them. This act quietens the mind, but only briefly, before embarking on another quest to find answers for more questions. Thus, the human mind constantly empowers itself. Actually, it is science that truly empowers the human mind to solve problems that plague us. This was what the Greek mathematician, engineer, and astronomer Archimedes experienced 2,200 years ago after discovering the principle of buoyancy while taking a bath, and then getting out of the public bath-tub and running naked through the town of Syracuse, Sicily shouting the Greek words "Eureka! Eureka!" (I have found it! I have found it!).

Today, science faces new challenges, perhaps some of the greatest ever. Prominent among them is the need to feed the world's growing population against the backdrop of shrinking arable land and crop-related resources, constraints that limit the potential of crop yields, and imposition of a greater burden on the planet Earth than any other human activity in the history of mankind. After all, crop plants are the first level of the human food chain.

Mankind is currently tasked with increasing agricultural productivity in a manner that embraces the principles of agricultural sustainability without compromising the ability of future generations to meet their own needs. The challenges to productivity include slower rate of growth due to increased competition from weeds, insects, diseases, etc.; decelerating productive and cultivable agricultural land; rapidly growing demand for food and feed; increasing undernourishment; growing carbon dioxide emissions leading to global warming; depleting water/irrigation availability leading to more frequent droughts than ever; escalating salinity in irrigated regions; shrinking labor force; non-remunerative price for crop produce, etc.

The current global population of 7.3 billion is expected to reach 9.3 billion by 2050, with the Asian continent (5.142 billion), powered by India (1.62 billion) and China (1.47 billion), accounting for 55 percent [United Nations 2012a]. In order to meet the increased demand for food both by the rising population and the expected increase in per capita food consumption in developing and underdeveloped nations, agricultural production needs to increase by 50 percent by 2030 and 70–100 percent by 2050 [Tomlinson 2011]. This is equal to an additional one billion tons of cereals and 200 million tons of livestock products each year. This can be met by an increase in cultivable area and enhanced production level per unit of land. Both, however, are beset by several constraints.

Some 1.6 billion ha of the world's best, most productive lands are currently used to grow crops. Parts of these lands are being degraded through farming practices that result in water and wind erosion, the loss of organic matter, topsoil compaction, salinization and soil pollution, and nutrient loss. FAO reports that 70 percent of the world population will

live in urban areas by 2050, up from 49 percent now. This suggests that there will be fewer people to depend on agriculture for their livelihood. Salinization (caused by high levels of salts in water, greater mobility of salts from ground water, changes in climate favoring salt accumulation, and human activities such as land clearing, etc.) of world's arable land is poised to go up from the present 25–30 percent by 2020 and as much as 50 percent by 2050. Of the current area under irrigation, salinity affects 40 percent of it in various degrees. Crop irrigation increases salinity owing to trace elements in irrigation water [GM Science Update 2014]. Besides, production in over 70 percent of dryland agriculture is also limited by salinity stress worldwide [GM Science Update 2014].

Drought and desertification, which determine water availability and quality as well as biodiversity, cause a loss of 12 million ha each year (23 ha min^{-1}) where 20 million tons of grain could have been produced. Environmental degradation is caused by depletion and changes in the availability and quality of resources such as air, water, and soil. According to the Global Land Assessment of Degradation published by FAO, nearly two billion ha worldwide have been degraded since the 1950s, representing 22 percent of the world's cropland, pastures, forests, and woodlands. In particular, some of the Asian, African, and Latin American countries have the highest proportion of degraded agricultural land as revenue-poor national governments pursue seemingly lucrative policies of deforestation for industrial expansion, urbanization, and population growth. This degradation is likely to increase in the coming decades because of changes in the availability and quality of resources such as air, water, and soil.

Of the water that is available for use, about 70 percent has already been in use for agriculture [Vorosmarty et al. 2000]. Many rivers no longer flow all the way to the sea, while 50 percent of the world's wetlands have disappeared, and major groundwater aquifers are being mined unsustainably, with water tables in parts of India, China, Mexico, and North Africa declining by as much as 1 m yr^{-1} [Somerville and Briscoe 2001].

Compounding the challenges mentioned earlier are the predicted effects of climate change [Lobell et al. 2008]. As the sea level rises and glaciers melt, low-lying croplands will be submerged and river systems will experience shorter and more intense seasonal flows, as well as more flooding [Intergovernmental Panel on Climate Change 2007]. As yields of our most important food, feed, and fiber crops decline precipitously at temperatures greater than 30°C, heat and drought will increasingly limit crop production [Schlenker and Roberts 2009].

Considering that there is little possibility of future increase in cultivable land and the prospect of adverse effects due to climate changes, all of the required increase in agricultural production must largely come from the same land while using less water. However, growth of cereal production has been decelerating over the past 50 years. It came down from 3.2 percent in 1960 to 1.8 percent in 2000 and 1.2 percent in 2010. Currently, the major four crops of wheat, rice, maize, and soya bean, which together provide two-thirds of global agricultural calories, are increasing at the non-compounding rates of 0.9 percent, 1.0 percent, 1.6 percent, and 1.3 percent per annum respectively [Ray et al. 2013]. At these rates, production of these crops are likely to increase at 38 percent, 42 percent, 67 percent, and 55 percent respectively. These rates of increase significantly fall short of the projected demands of 76 percent, 59 percent, 101 percent, and 84 percent for wheat, rice, maize, and soya bean respectively by 2050.

Production of rice, the staple food crop of 55 percent of world population in 2050, must be doubled from the current level. At today's levels of increase of wheat (0.9 percent) and rice (1.0 percent) production, there will be no change in the *per capita* wheat and rice harvests to 2050 [Ray et al. 2013]. A similar scenario will prevail in the case of maize and

soya bean as well. Currently, the top three countries that produce rice and wheat, China, India, and USA, have very low rates of crop yield increase.

Yields are no longer improving on 24–39 percent of world's most important croplands areas [Lin and Huybers 2012]. Many of these areas are in top crop-producing nations, having rising population, increasing affluence, or a combination of these factors [Tilman et al. 2011; United Nations 2012b; Finger 2010; Brisson et al. 2010; Lin and Huybers 2012; Ray et al. 2012]. This may increase the difficulty of meeting future crop production goals.

If the world is required to boost the production in these top four global crops that are now responsible for directly providing ~43 percent of the global dietary energy and ~40 percent of its daily protein supply [FAO 2013] from yield increases alone, it has to necessarily increase the production per unit of land. This can only be achieved by raising yield threshold levels and removing the various crop-production constraints such as biotic and abiotic stresses via biotechnological approaches like conventional breeding, genetic engineering, etc.

The losses caused by these biotic and abiotic stresses, which already result in 30-60 percent yield reductions globally each year, occur after the plants are fully grown: a point at which most or all of the land, water, and funds required to grow a crop has been invested [Dhlamini et al. 2005]. For this reason, a reduction in losses to weeds, insects, and pathogens as also to environmental stresses is equivalent to creating more land and more water. Also, crops and other plants are routinely subjected to a combination of different abiotic [Miller 2006] and biotic stresses. In drought areas, for example, many crops encounter a combination of drought, heat, and salinity stresses, besides infestation by weeds, insects, and pathogens.

Agricultural research, like any field of science, continues to evolve in order to meet the current and future challenges. British physicist and ecologist Lord Robert May had said in 2002 that "we couldn't feed today's world with yesterday's agriculture and we won't be able to feed tomorrow's world with today's… but we can try to do it in a way that is more environmentally sensitive by producing crops that are water tolerant, salt tolerant, and resistant to particular insects without putting chemicals on them that are potentially hazardous to wildlife" [IFR 2002].

Transgenic technology in which desired genes and traits are inserted into the plant genome offers a better chance to raise crop yields from the present near-stagnant levels and mitigate some of the biotic and abiotic stresses the crops are subjected to. It also offers new avenues of plant improvement in a shorter period compared to conventional breeding and the fresh possibility of incorporating new genes with low problem of incompatibility.

Crop Yield

Yields can be intrinsic and operational. Intrinsic yield, the maximum potential that can be achieved, is obtained when crops are grown under ideal conditions. By contrast, operational yield is obtained under field conditions, when biotic and abiotic stresses are considerably less than ideal. Genes that improve operational yield reduce losses caused by these stress factors.

The priority for plant biotechnologists is to raise the intrinsic yields of crops. The major barrier in increasing the yield potential of a crop, however, is the complexity of genes involved in this quantitative trait, requiring several genes to be manipulated. Many of the genes now being considered for increasing yield involve greater genetic, biochemical, and phenotypic complexity than current genes for herbicide tolerance and insect tolerance, and

this complexity will sometimes exacerbate the tendency of transgenic crops to produce side effects, some of which may be unacceptable [Gurian-Sherman 2009]. Yield potential may be raised by improving, for example, by enhancing of efficiency of photosynthesis, protein and lipid metabolism, etc. Although several attempts have been made in the past in this area of biotechnology, one general difficulty is that many improvements have been aimed at aspects of plant physiology that are several steps removed from grain yield [Gurian-Sherman 2009].

Biotic Stress

When a plant is affected by stress, it is subjected to sub- and supra-optimal physiological conditions. Stress results in the formation of a reduction in water potential. Biotic stress on plants is caused by other living organisms, known by a general term 'pest', such as weeds, insects, bacteria, viruses, fungi, nematodes, and parasites. Of these, weeds constitute a major and continuing biotic constraint affecting cropping systems worldwide. Although agricultural crops are damaged by thousands of pest species, less than 10 percent of them are generally considered to cause major problems. It is generally known that yield is progressively reduced with increasing number of these pests. The recognition that pest infestations are a major reason for crop varieties not realizing their yield potential fully had prompted early farmers to select and breed plants that survived the infestation. Overall, weeds cause the highest potential loss (34 percent) followed by insect pests (18 percent), and pathogens (16 percent) [Oerke 2006].

Removal of biotic stress factors do not lead to yield increase *per se*. However, the losses caused by them can be minimized by using pesticides. The global market for different pesticides and agrochemicals which grew at 9.8 percent annually between 2007 and 2013 [World Pesticide and Agrochemical Market 2014] is expected to increase approximately by 8.7 percent from the current $50 billion market. Of these, herbicides account for over 40 percent of the market followed by insecticides and fungicides with 27 percent and 21 percent respectively. Although these agrochemicals protect crops from losses, some 20 to 40 percent of the world's potential crop production is still lost annually. Excessive use and misuse of herbicides, insecticides, and fungicides have their own attendant secondary problems such as weeds developing resistance to herbicides, insects to insecticides, and pathogens to fungicides, aside from the adverse impact pesticide products have on the environment and ecosystem.

It is increasingly difficult to discover a new herbicide, and even more difficult to find one with a novel mode of action. Today, approximately 500,000 compounds must be screened to discover a potential herbicide compared with one per 500 compounds screened in the 1940s. Given the difficulty of discovering new herbicides, as it has been particularly over the past 20 yr, expanding the utility of existing ones that have a broad weed-control spectrum and good environmental profile through genetically enhanced resistance is a useful strategy from the agro-ecological point of view.

Development of resistance in weeds due to the continuous use of same herbicides or the ones that have similar molecules is not a new phenomenon ever since their first commercial introduction, beginning with 2,4-D in 1946. It is, in fact, similar to that exhibited by insects to insecticides since 1914 and plant pathogens to fungicides from 1940. Initially, development of herbicide-resistant weeds was slow, but picked up pace from the early 1980s (vide Chapter 2). At the present time, after nearly six decades of the first reports of biotypes of two weed species becoming resistant to 2,4-D in Hawaii, USA and

Ontario, Canada in 1957, 235 species (138 dicots and 97 monocots) have been identified to develop resistance to scores of herbicides worldwide thus far (vide Chapter 2) [Heap 2014]. A great majority of cases of herbicide resistant weeds have so far been found in developed countries. About one-third of these resistant species have been from the U.S. which uses herbicides most intensively. These numbers do not remain static. They keep increasing as and when more weed species are found resistant in virtually every corner of the globe where herbicides have been in continuous use.

The direct consequences of evolution of herbicide-resistant weeds include reduced crop production, higher weed management costs, and tremendous increase in seed banks leading to greater weed pressure on subsequent crop(s). Furthermore, some herbicides which are effective against a wide range of weed species are either nonselective to crops or selective to only specific crops, and so they have little or limited practical utility in most of the cropping situations. Continuous application of the herbicide(s) in question will only lead to a faster evolution of weed species. Generally, a combination of herbicides with cultural and mechanical methods may only delay but not prevent evolution of herbicide resistant weeds [Green and Owen 2011]. At this juncture, inserting a gene from a plant or non-plant source encoding a protein that confers the herbicide-resistant trait was considered to be an indispensable tool for solving problems associated with development of resistance to herbicides. Currently, several genes derived from non-plant sources (vide Chapter 5) are employed to confer resistance to several crops to some of the herbicides used (vide Chapter 6).

Unlike weeds, insects not only cause direct yield losses by damaging and consuming plants, but also act as vectors for many viral diseases. The damage they inflict often facilitates secondary microbial infections on crops. Herbivorous insects and mites are a major threat to global food and feed production. Larval forms of lepidopteran insects are considered the most destructive pests, with about 40 percent of all insecticides directed against heliothine species [Brooks and Hines 1999]. Many other species within the orders Acrina (ticks and mites), Coleoptera (beetles and weevils), Diptera (flies), Hemiptera (aphids, hoppers, cicadas, and shield bugs), and Thysanoptera (thrips) are also considered agricultural pests with significant economic impact. There are innumerable insect pests that can devastate agricultural production. Of these, the ones most notable for their destructive capacity include the Migratory locust (*Locusta migratoria*), Colorado potato beetle (*Leptinotarsa decemlineata*), Boll weevil (*Anthonomus grandis*), Japanese beetle (*Popillia japonica*), and aphids (of family Aphidoidea) which serve as vectors of plant viruses [Ferry and Gatehouse 2010]. Another destructive insect pest is the Western corn rootworm (*Diabrotica virgifera virgifera*) which is called the billion dollar bug due to its economic impact in USA alone [Ferry and Gatehouse 2010].

Insect pest control is heavily dependent on insecticides which are more expensive and damaging to the environment than herbicides. These chemicals are nonselective, killing harmless and beneficial insect species along with target insects, and eventually accumulating in water and soil. They are also hazardous to human health if overused or misused. In the 1960s, the American biologist and conservationist Rachel Carson brought to the attention of the world, the detrimental environmental and human impacts resulting from overuse or misuse of some insecticides. Constitutive exposure to insecticides can lead to the evolution of resistance in insect populations, leading to reduced insect control. Generally, insecticides are too expensive for farmers in the developing world and in any case are often ineffective against sap-sucking insects including the rice brown plant hopper (*Nilaparvata lugens*) [Christou and Chapel 2009].

Therefore, insect pests became the second most important target for transgenic technology. The transgenic engineering of plants to express insect-resistance genes offers the potential to overcome the shortcomings of continued heavy use of insecticides. In doing so, genes that are specific towards a particular insect species are isolated from bacteria and other non-plant sources. Furthermore, the proteins encoded by these transgenes within plants allow effective control of insects that feed or shelter within the plant. Several of these genes have been sourced from bacterial toxins, lectins, and protease inhibitors. The foremost of the toxins have been derived from the spore-forming bacterium *Bacillus thuringiensis* (*Bt*). *Bt* toxins are known as crystal (Cry) proteins or δ-endotoxins (vide Chapters 5 and 6).

Crop plants have also evolved resistance mechanisms that protect them from pathogen species. One of the most prevalent and problematic bacterial diseases in food crops is bacterial blight of rice, causing losses in excess of US$250 million every year in Asia alone. The gene, *Xa21*, isolated from rice-related wild species *Oryza longistaminata* was shown to confer resistance to all known isolates of the blight pathogen *Xanthomonas oryzae* pv. *oryzae* in India and the Philippines [Khush et al. 1990]. This gene encodes a receptor tyrosine kinase [Song et al. 1995]. The transfer of this plant gene to susceptible rice varieties resulted in plants showing strong resistance to a range of isolates of the pathogen [Wang et al. 1996; Tu et al. 1998; Zhang et al. 1998].

Viruses, which also cause significant crop losses, are another major target of transgenic technology. Virus resistance can be achieved by introducing into the crop plant one or more genes from the virus itself. One way to achieve pathogen-derived virus resistance is to express a coat protein gene which can block virus replication. This strategy has been demonstrated in rice, the host to more than 10 disease-causing viruses. Tungro virus is the most damaging viral disease in rice in South Asia and Southeast Asia. This is caused by a combination of two viruses: rice tungro bacillus virus (RTBV) and rice tungro spherical virus (RTSV). Rice hoja blanca virus (RHBV) causes 100 percent yield losses in rice in Central and South America. Pathogen-derived resistance to these diseases has been achieved in experimental plants by exposing coat protein genes from RTBV, RTSV, and RHBV [Kloti et al. 1996; Lentini et al. 1996; Sivamani et al. 1999].

Another virus-resistant transgenic crop is papaya which is seriously affected by papaya ringspot virus (PRSV) in Hawaiian Islands. PRSV is a potyvirus with single-stranded RNA. The inserted transgene was designed with a premature stop codon in the PRSV coat protein sequence to prevent expression of a functional coat protein because, at the time of engineering, it was thought that the protein itself was an important factor in resistance. RNA analysis revealed that the plants with the best resistance exhibited the least detectable message, suggesting the involvement of an RNA silencing mechanism [Tripathi et al. 2006].

Abiotic Stress

Abiotic stress, a natural part of every ecosystem, causes changes in soil-plant-atmosphere continuum and metabolic activities within the plant, leading to reduced crop production. Abiotic stress is an integral part of "climate change," a complex phenomenon with a wide range of unpredictable impacts on the environment. Prolonged exposure to abiotic stress factors such as temperature (heat, chilling, freezing) drought, cold, ozone, salinity, flooding, intense light, and nutrient imbalance (mineral toxicity and deficiency) leads to an altered metabolism and damage to biomolecules. These responses cause deterioration and destruction of crop plants, resulting in low productivity.

In the case of mitigation of salinity, two major genes Na(+) exclusion in durum wheat, *Nax1* and *Nax2*, that were previously identified as the Na(+) transporters TmHKT1;4-A2 and TmHKT1;5-A, have been transferred into bread wheat in order to increase its capacity to restrict the accumulation of Na(+) in leaves [James et al. 2011]. The recent introgression of an ancestral form of the *HKT1;5* gene from the more Na^+-tolerant wheat relative *Triticum monococcum* into a commercial durum wheat species (*Triticum turgidum* ssp. *durum*), which is susceptible to salinity, has increased grain yields on saline soil by 25 percent [Schroeder et al. 2013]. These results indicate that both *Nax* genes have the potential to improve the salt tolerance of bread wheat and even other crops. Besides, these genes have the potential to confer an extra advantage under a combination of waterlogged and saline conditions [James et al. 2011]. Combining HKT transporter traits with vacuolar Na^+ sequestration mechanisms provides a potentially powerful approach to improve the salinity tolerance of crops [Schroeder et al. 2013]. In rice, the most aluminum tolerant of the cereal crops, *NRAT1* gene, which encodes a protein that confers further tolerance to this metal, has been identified by Cornell scientists [ISAAA 2014].

In the short and medium term, as more genes are identified that confer salinity-tolerant traits, their introduction by transgenic methods, alone or in combination, should elevate salinity tolerance in other crop species in future.

The presence of abiotic stress can also have an effect of reducing or enhancing susceptibility to a biotic stress like weed competition, insect infestation, pathogen (fungi, bacteria, viruses, etc.) infection, etc. This interaction between biotic and abiotic stresses is orchestrated by hormone (e.g., abscisic acid) signaling pathways that may induce or antagonize one another. Specificity in multiple stress responses is further controlled by a range of molecular mechanisms that act together in a complex regulatory network. Therefore, the subject of abiotic stress is gaining considerable significance in genetic engineering.

Gene Manipulation

One branch of science that humans have been pursuing from pre-historic times is agriculture. It began when man struggled to survive. Initially, he grew crops in quantities sufficient to support his family. Later, he needed to support others not engaged in agriculture and the growing population. In this pursuit, he had to grow more crops and reap more yields from the same land year after year. For this, he devised and followed best production practices to increase crop yields and feed others. In order to increase yields, he resorted to domestication of wild crop species followed by manipulation of plant genes through selection and much later, by crossing to evolve more useful and productive cultivars. In the process, he altered the genomes of plant species for thousands of years, by choice or otherwise, to derive cultivars with improved quantitative and qualitative traits. This became the forerunner of the birth of a new branch of science called 'genetics' following the path-breaking findings of the Austrian monk, Gregor Mendel, on heredity and segregation of heritable traits after crossing many generations of garden pea between 1856 and 1863.

A flurry of research activities followed on both sides of the Atlantic over the next several decades to give birth to the field of modern or conventional plant breeding. This resulted in an enormous number of improved varieties in economic crops, including the two vital food crops wheat and rice, thus ushering in 'Green Revolution' in the last century. Spectacular advances developed in molecular biology, biotechnology, and genetic engineering (vide Chapters 3 and 4). The speed at which this progress has been achieved

in these fields is probably unparalleled in the history of science, barring inventions of the atom, computer, and communication.

Both conventional breeding and genetic engineering are used to improve the genetic traits of plants for human use. Their main goal is to develop crop varieties that express good agronomic characters as well as to enable crop plants withstand biotic and abiotic stresses.

All plant traits are encoded by genes. A plant has 10,000–50,000 genes, depending on the species. Each of the genes is associated with specific traits. Many genes encode enzymes that catalyze specific biochemical reactions. Before the advent of genetic engineering, plant breeders used the genes of a plant to select specific desirable traits. In conventional plant breeding, the genetic composition of plants is modified by making crosses and selecting new superior genotype combinations. However, conventional breeding has limitations. First, crop improvement depends solely on the desirable genes available naturally, created by induced mutations, or their shuffling for desired recombinations. Second, breeding can only be done between plants that can sexually mate with each other. This limits the new traits that can be added to those that already exist in that species. Third, when plants are crossed, many traits are transferred along with the trait(s) of interest. These include those with undesirable effects on yield potential. Fourth, there is no guarantee of obtaining a particular gene combination from the millions of crosses generated. Undesirable genes can be transferred along with desirable genes, or while one desirable gene is gained, another is lost because the genes of both parents are mixed together and re-assorted more or less randomly in the offspring [ISAAA 2012]. These problems limit the improvements that plant breeders can achieve. It was here that genetic engineering found its niche and utility over three decades ago.

Three key elements have essentially transformed biotechnology into genetic engineering. These are: identification of DNA as the carrier of genetic information by Avery Colin McLeod and MacLyn McCarty in 1944, discovery of the structure of DNA by James Watson and Francis Crick in 1953, and discovery of a recombinant technique by which a section of DNA is cut from the plasmid of an *E. coli* bacterium for transfer into the DNA of another by Stanley Cohen and Herbert Boyer in 1973.

The first genetically engineered plant was tobacco when Fraley et al. [1983] produced in 1982, an antibiotic-resistant tobacco plant by using the soil bacterium *Agrobacterium tumefaciens* to insert a small segment of DNA (T-DNA), from a Ti plasmid, into the plant cell. This was followed by the herbicide-tolerant tobacco in 1986, the frost-resistant strawberry and potato in 1987, and the virus-resistant tobacco in 1992 (vide Chapter 4). The year 1994 saw the first transgenically engineered, but short-lived, whole food tomato line 'Flavr Savr' developed by the Davis, California-based Calgene and the first herbicide-resistant crop tobacco which was engineered to become resistant to bromoxynil developed by the European Union. These path-breaking developments laid the groundwork for all transgenic crops developed over the next two decades (vide Chapters 4 and 6). Transgenic plants have been developed in scores of species to be useful for agriculture and forestry. Some of these include maize, soya bean, cotton, canola (rapeseed), sugar beet, rice, wheat, potato, tobacco, papaya, lucerne (alfalfa), linseed (flax), pea, tomato, squash (zucchini: *Cucurbita pepo*), sugarcane, sorghum, sweet pepper (*Capsicum annuum* var. *annuum*), brinjal (eggplant: *Solanum melongena*), banana and plantain, creeping bentgrass (*Agrostis stolonifera*), chicory (*Cichorium intybus* var. *foliosum*), pine (*Pinus* spp.), poplar (*Populus* spp.), Jatropha (*Jatropha curcas*), petunia, etc.

The traits inserted in these crops include herbicide resistance; insect resistance; virus resistance; resistance against fungal and bacterial infections; abiotic stress tolerance;

delayed fruit ripening; male sterility; production and quality of biofuels (e.g., Jatropha, high starch-to-sugar maize, low-lignin poplar, etc.); pharmaceutical products of therapeutic value; phytoremediation of the soil contaminated by explosives (TNT, RDX, etc.), toxic elements (mercury, selenium, etc.) and organic pollutants (polychlorinated biphenyls); production of drugs (e.g., Elelyso: taliglucerase alfa from carrot cells for the treatment of Gaucher's disease, a rare genetic disorder); production of bioplastics, detergents, substitute fuels, and petrochemicals; etc. As weeds and insect pests are the primary targets for transgenic technology, a vast majority of commercially grown transgenic plants are modified for herbicide resistance, insect resistance, and both [James 2006].

Initially, genetic engineering was done to carry genes that deliver single traits. Later, plants have been transformed to carry two or more genes that code for proteins having different modes of action and enzymes. As multi-trait stacks are tightly linked, they exhibit an extremely low rate of segregation, essentially behaving as a single gene. Biotech stacks provide better chances of overcoming the myriad of problems in the field such as weeds, insect pests, diseases, and environmental stresses, low yields and nutritional quality, etc. simultaneously.

In genetic engineering, a genetic material is inserted followed by selection. Insertion is done by a vector-mediated transformation or one of the vector-less direct methods into the host plant cell and then, with the help of genetic elements in the construct, the genetic material inserts itself into the chromosomes of the host plant. Genetic engineers must also insert a 'promoter' gene from a virus as a part of the package, to make the inserted gene express itself. This process is profoundly different from conventional breeding even if the primary goal is only to insert genetic material from the same species.

However, the technique of genetic engineering offers a new type of genetic modification. It enables direct and purposeful transfer of one or just a few genes of interest from species, families, and even kingdoms which could not previously be sources of genetic material for a particular species, and even to insert custom-designed genes that do not exist in nature. Thus genetic engineering allows movement of genetic material from any organism to any other organism. It also offers the ability of creating a new genetic material and expression of products like never before.

The plant genome is a complex entity made up, in part, of genes and genetic elements that interact in complex regulatory pathways to create and maintain the organism. The new genetic material that enters the genome of the host plant must fit into this total complex or it may end up destabilizing it. Genome is like a complex computer program or an ecological community. When a new sub-program is introduced within the larger complex computer program, it may fit in well or can create unpredictable effects and may ultimately cause the whole program to crash. Similarly, in a complex ecological community, the introduction of a new species may survive or cause a catastrophic effect on the ecosystem. Unlike in a computer program, the changes that a new genetic material or species may bring about cannot be predicted or be evident in a short time span.

The gene that is transferred, called transgene, holds information that will give the host plant a trait. However, it cannot control the location where the trait is inserted into the genome with any precision or with a guarantee of stable expression. Regardless of the method of transformation, the site of insertion of the transgene is fairly random. As the effect of a gene on the host plant is governed by its location, the lack of control over location is the cause of unexpected effects. This is not altogether an unexpected phenomenon because transgenic engineering, unlike conventional breeding, involves organisms with desperate

evolutionary backgrounds. Thus transgenic engineering is more a random process than conventional breeding.

Transgenic engineering, however, is not bound by the limitations of traditional plant breeding. It physically removes the DNA from one organism and transfers the gene(s) for one or a few traits into another. Since crossing is not necessary initially, the 'sexual' barrier between species is overcome. Therefore, traits from any living organism can be transferred into a plant.

Although there are many diverse and complex techniques involved in transgenic engineering, its basic principles are reasonably simple [ISAAA 2012]. There are five major steps in the development of a genetically engineered crop. For every step, it is very important to know the biochemical and physiological mechanisms of action, regulation of gene expression, safety of the gene, and the gene product to be utilized. Even before a genetically engineered crop is made available for commercial use, it has to pass through rigorous safety and risk assessment procedures before being approved for commercialization.

The length of time in developing a transgenic plant depends upon the gene, crop species, available resources, and regulatory approval. It may take 6–15 yr before a transgenic line is ready for commercial release [ISAAA 2012]. This transgenic technology has been used over the past two decades to develop scores of transgenic crop lines incorporated with various qualitative and quantitative traits in several crops around the world.

As transgenic technology in the West was driven predominantly by the potential commercial gain, research has so far focused mainly on the weed (and insect) problems farmers of industrialized nations faced. There has been little interest and attempt in producing crops with resistance to the weed species that plague subsistence farmers in the developing world, even though this would have an immediate impact on global food security [Christou and Chapel 2009].

Currently, close to 180 million ha are under transgenic crops globally, and this area may rise to 400–500 million ha by 2030 at a time when over 120 crops are expected to be transgenically engineered with desired traits and adopted worldwide. This, however, is dependent on the extent of adoption by developing nations, particularly the growth engines China, India, Brazil, Argentina, and South Africa. Further expansion is also dependent on global regulatory procedures and how best the risks and issues (vide Chapter 9) associated with them are answered to the satisfaction of farmers and consumers.

Once a transgenic crop line is developed, it needs to go through regulatory system before it is commercialized. However, there is no uniform global regulatory framework in place. Each country has its own regulatory framework (vide Chapter 8). Even within a country, there is a wide variation in review and assessment because the biotech variety intended for food use undergoes through a different perspective from the one used for non-food or feed purpose. Many a time, assessment, approval, and regulation are based not entirely from technology standpoint.

Although genetic engineering provided a significant breakthrough in terms of substituting land scarcity for agriculture and enhancing the production efficiency of certain edible crops, few topics in agriculture are more polarizing and controversial than this growing field of biotechnology. This is because the proponents vehemently praise the virtues of the technology and the progress made thus far in offering farmers alternatives to herbicides and insecticides while the opponents zealously point out the perils it has brought upon the farmers, agro-ecology, and soil ecosystem aside from its potential impact on health of consumers. However, what has been found thus far is that the benefits derived from biotech crops by farmers varied with the crop, the traits it carried, farm size, and

the country that adopted them. The success achieved by one farmer and one country is no guarantee that other farmers and countries will also taste them. In reality, every technology has its benefits and risks. Transgenic engineering is no exception.

Future of Transgenic Engineering

The transgenically engineered crop varieties developed over the past 20 yr have certainly offered a means to enhance global agricultural sustainability. This technology holds promise to meet the agricultural needs of the 21st century, particularly in regard to increasing plant productivity, both directly and indirectly, while enhancing quality of the produce. It also aids in reducing the footprint of pesticide chemicals (herbicides, insecticides, fungicides, etc.) and carbon on the environment. Transgenic engineering has the required arsenal to address various problems plaguing agriculture in a more effective way than ever. As in the case of atom, electronics, computer, and communication, the more we understand the intricacies of this plant-related technology, the better we will be able to utilize it for the betterment of mankind.

Currently, there are scores of useful genetically engineered traits in the pipeline. Future transgenic technology (second and third generations) is expected in the following:

1) Enhancing the photosynthetic efficiency of crop plants as well as protein and lipid metabolism;
2) Increased nitrogen use efficiency and reducing the detrimental environmental impacts such as water eutrophication caused by nitrogen compounds in fertilizers and greenhouse gas emissions emanating from their synthesis;
3) Improved phosphorus efficiency and availability;
4) Enhanced nutritional quality in staple food crops such as rice, wheat, maize, and sorghum (β-carotene, iron, protein, etc.) besides other nutritious legume crops chick pea, pigeonpea, groundnut (peanut), etc.;
5) Better drought tolerance in most of the crops in the light of global warming and shrinking water resources;
6) Greater tolerance of crops to frost, salinity, and flooding;
7) Longer shelf life in tomato and major fruit crops like banana, mango, apple, etc.;
8) Higher levels of health-promoting antioxidants like flavonols and flavonoids in fruits and juice;
9) Silencing of polyphenol oxidase to avoid bruising and browning of potatoes and apples;
10) Greater tolerance of plants to arsenic;
11) Restoration of fertility;
12) Enhancing plant characteristics (panicle size, seed quantity per panicle, etc.) for higher yields;
13) Lowering of seed-shattering habit of crops, particularly food crops;
14) Apomixis in fruit crops;
15) Male sterility and self-incompatibility;
16) Lower lignin content in tree crops for paper making;
17) Providing renewable alternatives to fossil fuels, such as feedstocks for biofuels, e.g., sugar (sugar beet, sugarcane), starch (maize, wheat), oil (rapeseed), and woody ligno-cellulose (poplar, willow, Miscanthus, etc.);
18) Developing oilseed crops that accumulate omega-3-long-chain polyunsaturated fatty acids (LC-PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid

(EPA) representing a potential sustainable terrestrial source of fish oils which can reduce the risk of cardiovascular disease;

19) Biofortification (nutritional enhancement) of staple crops, fruit crops, vegetable crops, and animal products with higher vitamin A, iron, zinc, etc.;
20) Phytoremediation of organic contaminants (herbicides, insecticides, oil spills, explosives, industrial chemicals, etc.) and inorganic pollutants (natural elements: cadmium, cobalt, iron, lead, mercury, selenium, tungsten, etc.); and
21) Biopharming which includes production of vaccines, therapeutics, antibodies, and enzymes from crops like rice, tobacco, potato, maize, lucerne (alfalfa), barley, etc.

With regard to herbicide-resistant crops, transgenic research may be directed more towards developing crop varieties resistant to herbicides other than glyphosate and glufosinate besides multi-herbicide tolerant stacks. This helps in controlling weeds resistant to these nonselective herbicides to which several crops have become or are becoming tolerant in the recent past. Furthermore, herbicide-resistant traits may be combined with traits other than those with insect resistance.

Any new plant variety, developed through transgenic engineering, carries the risk of unintended consequences. This is because transgenic plants contain desirable traits which offer a range of benefits above and beyond those that emerged from innovations in traditional agricultural biotechnology. However, this technology is complex because it deals with genes, the actions of genes, and interactions with other genes, more often derived from non-plant sources.

Although transgenic engineering has proved its utility in successfully transforming many crops with desired traits, agro-ecological and human safety concerns remain a contentious issue. This calls for using **intragenic** engineering technology, which involves insertion of DNA fragments from the same plant species or cross-compatible species in a sense or antisense orientation (vide Chapter 4), as an alternative. In this method, the undesirable genes are silenced and desirable genes are enhanced by linking with beneficial genes by using tissue-specific or near-constitutive promoters. Attempts have been made to successfully use this technology (vide Chapter 4) in tomato to redesign and improve the quality of Calgene's Flavr Savr tomato; to eliminate discoloration of a high-yielding potato variety; and to enhance oleic acid and oil content and shattering resistance in rapeseed (canola). This suggests that intragenic engineering can be used to insert traits such as resistance to herbicides, diseases, salinity, drought, frost, flooding, etc.

Another alternative to transgenic engineering with regard to herbicides is employing non-transgenic or partial-transgenic technology. The former was used to develop imidazolinone herbicide-resistant lines in maize, rapeseed (canola), rice, wheat, sunflower, and lentil, while the latter was used to produce β-carotene-rich 'Golden' rice variety by inserting two genes, one derived from a plant (daffodil) and the other from a soil bacterium.

One promising area of future genetic engineering is the artificial chromosome technology involving mini-chromosomes. The current technology is beset with certain limitations. One of them is that stacking of multiple genes in one germplasm takes many years, but still faces the possibility of segregation of transgenes in later generations [Yu and Birchler 2007; Yu et al. 2007; Xu et al. 2012]. Another limitation is linkage drag, which refers to the reduction in fitness of a cultivar due to deleterious genes introduced along with the beneficial gene during backcrossing [Xu et al. 2012; Yu and Birchler 2007]. A mini-chromosome is an extremely small version of a chromosome, the threadlike linear strand of DNA and associated proteins that carry genes and functions in the transmission of hereditary information. A normal chromosome is made of both centromeres and telomeres

with much intervening DNA while a mini-chromosome contains only centromeres and telomeres, the end section of a chromosome, with little else. Mini-chromosome technology enables circumventing the conventional problems of genetic engineering. It opens up new possibilities for the development of crops carrying multiple genes that confer resistance to herbicides, insects, viruses, fungi, and bacteria as also for the development of proteins and metabolites that can be used to treat human illnesses.

One of the most important bio-technological developments over the last 10 years, together with advances in bioinformatics, is the availability of high throughput DNA sequencing methods at very affordable prices. These techniques allow the production of Gigabases of DNA sequences for around US$1000, and as a consequence, the amount of information present in DNA databases has doubled every 18 months. As a result of these high throughput methods, the genome sequences of the main plant species are now known. The resulting datasets include the genomes of model species, such as *Arabidopsis thaliana*, those of the main crops, rice, maize, and soya bean, and other important species such as poplar, cotton, grapevine, apple, cassava, and sorghum [GM Science Update 2014].

At the same time, the genomic variation within a species has become accessible due to resequencing of different breeding lines (cultivars). In the case of *Arabidopsis* and rice, more than 1000 sequences from different cultivars have been obtained and published [1001 Genomes Project n.d.; Huang et al. 2012]. This genome sequence data is helping to identify the genetic basis of domestication of the main crop species, and the many major genes affecting the performance of crops, including yield and disease resistance. Progress is also being made towards the identification of minor genes affecting quantitative traits that would have been more difficult to identify using classical molecular biology and genetics.

Over the past 5 years, newer methods to produce genetically modified plants are being increasingly developed. These enable mutations at very specific locations or to target gene sequences at specific sites. Classical methods of genetic engineering cannot predict the location of the new gene in the plant genome, and the insertion may cause the new gene to have low levels of expression or to insert into another useful gene. In contrast, the targeted transgenic methods enable the new gene in a specific location to avoid unforeseen effects.

These new methods, based on the use of site-directed nucleases (SDN) [Podevin et al. 2013; Goldstein et al. 2012; EFSA GMO Panel 2012], prepare the target site DNA for modification or insertion of a new sequence. Two examples of SDNs are Zinc-finger nucleases and TAL-nucleases in which a hybrid protein comprises a nuclease domain from a bacterium to cleave DNA and a sequence-specific DNA binding domain with a motif from a plant pathogen Xanthomonas. The SDN is expressed in cells of the crop to be engineered by a break at a defined location in the genome, where a mutation or foreign DNA can be introduced. Methods using SDNs are likely to become the normal method in transgenic engineering in future [GMO Science Update 2014].

Missing Links

Despite the significant advances made thus far, there are several missing links in today's transgenic technology. One of them is the country-specific and region-specific technology. Not all countries are alike when it comes to agriculture. Farmers of developing countries, with smaller farm size, poor capital, expensive inputs, and inadequate techniques stand to lose more than their counterparts in developed countries. Many of them do not even have access to good seeds and the available technology at affordable prices. If they had all of them, they would have achieved much better yields as has been proved in certain parts

of the developing world. Any new farming technology will be successful only when it is oriented to the needs of farmers. They will then use it voluntarily without the need for it to be forced upon them.

Another missing link is the relatively weaker participation of public research organizations in developing of biotech crops. In fact, many of the early discoveries have emanated from universities and public research institutions. Despite the fact that several of them are also involved in this field, their research agenda is being increasingly influenced and taken over by the private sector in ways never seen in the past [Altieri 1998]. The public sector, aided by government funding, will be able to serve farming communities better if they could ensure the availability of ecologically sound aspects of biotechnology and making such knowledge available in the public domain for the benefit of society [Altieri 1998].

Yet another missing link—the vital one—is the consumer. The success of any technology is dependent on consumer acceptance. Consumers always exercise choice when they buy any product, be it agricultural or non-agricultural. Denial of this basic right will eventually lead to failure of technology as has been the case in the past. Transgenic technology will be no exception if they (and their animals) are denied the choice of what they consume. Consumers are not yet fully aware of the potential risks—both short term and long term—foods derived from transgenic crops have on human and animal health. In order to make these food products acceptable to consumers all over the world, biotech industry, food companies, and farmer markets are required to answer and allay their concerns satisfactorily by resorting to strict product traceability and labeling standards, besides printing of nutritional facts (including genetic information) on the labels.

This means that transgenic engineering has a long way to go before being fully accepted by both farmers and consumers. The American poet Robert Frost wrote: "The woods are lovely, dark and deep. But I have promises to keep, and miles to go before I sleep, and miles to go before I sleep." This is most relevant in the case of the fast growing field of plant biotechnology which still has a long way to go before the global agricultural needs of the next four decades are met, free of risks and issues to farmers and consumers.

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CHAPTER 2

Herbicide Resistance

Ever since the widespread usage of phenoxy herbicides beginning 1946, weed scientists began pondering over the possibility of development of herbicide-resistant weed populations similar to those exhibited by insects against an inorganic insecticide in 1908 but documented in 1914, and plant pathogens against fungicides since 1940. In 1950, Blackman [1950] warned "… repeated spraying with one type of herbicide will sort out resistant strains within the weed population." In 1954, McCall [1954] wondered whether weeds were becoming more resistant to herbicides. The same year saw a report from the U.K. suggesting that continuous application of 2,4-D has led to resistance of weed species normally susceptible to it. This was followed by two other reports against 2,4-D in 1957, one from Hawaii where biotypes of *Commelina diffusa* (spreading dayflower) in sugarcane fields [Hilton 1957], and another from Ontario, Canada, where biotypes of *Daucus carota* (wild carrot) in sections of highway weeds [Switzer 1957] exhibited resistance.

These and a few other warnings were largely ignored until the first confirmed report of herbicide resistance against simazine and atrazine which failed to control *Senicio vulgaris* in 1968 in a Washington nursery, where they had been used since 1958 [Ryan 1970]. Since then, herbicide resistance problems have been accelerating. Consequently, management of weeds have become increasingly more difficult and complex.

At the present time, 238 weed species (138 dicots and 100 monocots) infest 84 crops and non-cropping areas in 65 countries have been identified to develop resistance to 155 different herbicides belonging to 22 of the 25 herbicide families with as many sites of action [Heap 2014]. As many species showed resistance to herbicides of multiple sites of action, the number of unique resistant cases are much higher (Fig. 2.1; Appendix: Table 1). For example, *Lolium rigidum* is resistant to herbicides of 11 sites of action.

The most wide-spread herbicide-resistant weed species in the world, with four and more sites of action other than *Lolium rigidum* include *Echinochloa crus-galli* var. *crus-galli* (10), *Poa annua* (9), *Eleusine indica* (7), *Alopecurus myosuroides* (6), *Amaranthus tuberculatus* (=*A. rudis*) (6), *Echinocloa colona* (6), *Lolium perenne* ssp. *multiflorum* (6), *Amaranthus palmeri* (5), *Ambrosia artemisiifolia* (5), *Avena fatua* (5), *Conyza canadensis* (5), *Raphanus raphanistrum* (5), *Amaranthus retroflexus* (4), *Bromus tectorum* (4), *Chenopodium album* (4), *Conyza bonariensis* (4), *Ischaemum rugosum* (4), *Kochia scoparia* (4), *Setaria viridis* (4), *Sisymbrium orientale* (4), and *Sorghum halepense* (4) [Heap 2014]. 27 showed resistance to three (3) sites of action and 41 to two (2) sites of action, while the remaining species exhibiting action at no more than one site (vide Appendix: Table 1) [Heap 2014].

Among weed families, Poaceae contributed the most number of resistance species of 75 followed by Asteraceae (37), Brassicaceae (21), Amaranthaceae (12), Cyperaceae

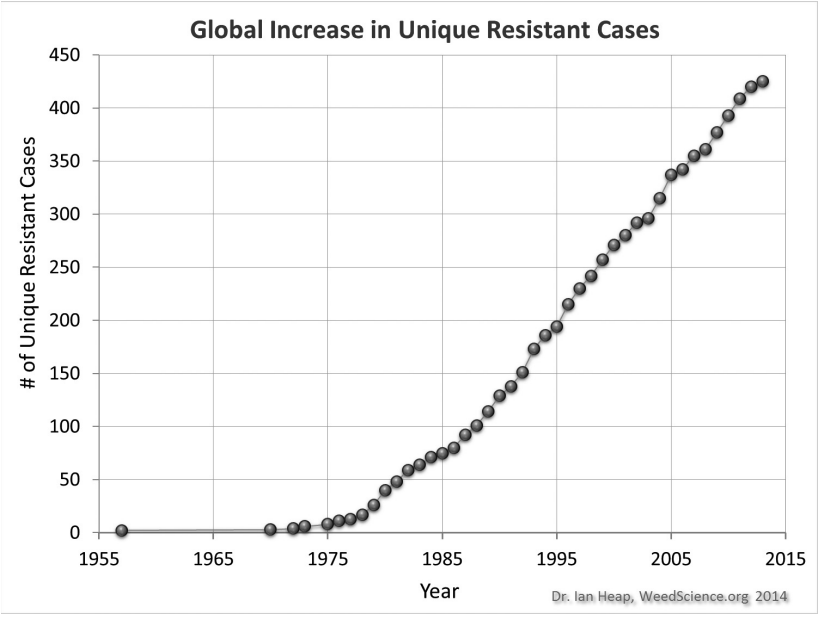

Fig. 2.1. Global Increase in Herbicide-Resistant Weeds (Unique Cases) from 1957 [Heap 2014].

(10), Scorphulaceae (9), and Chenopodiaceae (8), Alismataceae (6), Polygonaceae (6), and Caryophyllaceae (5) [Heap 2014].

Of the various crops in which herbicide-resistant weed species were found so far, wheat tops with 65 followed by maize/corn (58), rice (50), and soya bean/soybean (46), rapeseed/canola (20), and cotton (17) [Heap 2014].

Generally, '**resistance**' is defined as the **inherited ability** of a plant species/biotype to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type [WSSA 1998]. This is the dose normally used for satisfactory weed control. In both crop plants and weeds, herbicide resistance may be naturally occurring or induced by genetic engineering or selection of variants produced by tissue culture or mutagenesis [WSSA 1998]. Generally, this heritable resistance trait is found in crop plants, as against weeds, thus forming the basis for herbicide selectivity. Resistant weeds are those plant species that express the genetic variation required to evolve mechanisms to escape control. Like other crop pests, herbicide-resistant weeds are the result of intensive selection pressure in weed populations. When a herbicide causes selection pressure, susceptible plants are killed while the resistant plants survive to reproduce without confronting any competition from susceptible plants.

The term **herbicide-resistance** is normally used while referring to a) evolution of resistance to herbicides over a period of time and b) the resistance trait introduced in a plant transgenically. This is also the term used in this book in both instances. However, the term herbicide-tolerance is also used intermittently, particularly when the source of published information cited in the book has used it.

On the other hand, '**tolerance**' is the **inherent ability** of a species to survive and reproduce after herbicide treatment [WSSA 1998]. This implies that there is no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant. Tolerance may

also be considered the natural or normal variability of response to herbicides that exists within a species and can easily and quickly evolve.

Evolution, Spread, and Types of Herbicide Resistance

Herbicides do not induce resistance. Instead, they select for resistant individuals that naturally occur within the weed population. The more a herbicide is used, the greater the likelihood of encountering a resistant individual in a field. Once a resistant plant is selected, repeated use of a herbicide over multiple generations allows the resistant plants to proliferate as susceptible plants are eliminated. Once a resistance gene has occurred within a population, failure of the herbicide can be rapid.

There are two pre-requisites for the evolution of herbicide resistance in plant populations: a) the occurrence of heritable variation in genetic composition for herbicide resistance and b) natural selection for increased resistance to herbicides.

In response to repeated treatment with a particular herbicide or class (family) of herbicides, weed populations change in genetic composition such that the frequency of resistance alleles and resistant individuals increase [Jasieniuk et al. 1996]. In this way, weed populations become adapted to the intense selection pressure imposed by herbicides. The evolution of resistance under continuous application of a herbicide may be considered as an example of recurrent selection in which there is a progressive and, sometimes, rapid shift in average fitness of populations of weeds exposed to it. This shift in fitness, a genetic trait, is directly related to an increase in frequency of the resistance trait (phenotype) in the population. The selection pressure for herbicide resistance is contributed by: a) efficiency of the herbicide, b) frequency of herbicide use, c) duration of herbicide effect, d) method of herbicide use, e) selection pressure that is characteristic of the herbicide, and f) resistance mechanism in weed species.

The intensity of selection in response to herbicide application is a measure of the relative mortality in target weed populations and/or the relative reduction in seed production of survivors; this will be proportional, in some manner, to herbicide dose [Maxwell et al. 1990]. The duration of selection is a measure of the period of time over which phytotoxicity is imposed by herbicide. Both intensity and duration will interact to give seasonal variation in the process of selection which will, in turn, depend upon the phenology and growth of a weed species. For example, in the case of a preemergence herbicide that inhibits seedling emergence over a time period, the intensity of selection may be much higher on weed seedlings emerging early in the life of a crop than those emerging later. The occurrence and speed of evolution of herbicide resistance are determined by: a) number of alleles involved in the expression of functional resistance, b) frequency of resistance alleles in natural (unselected) populations of weed species, c) mode of inheritance of the resistant alleles, d) reproductive and breeding characters of the weed species, e) longevity of weed seeds in the soil, f) intensity of selection which differentiates resistant biotypes from susceptible ones, and g) absolute fitness of resistance and susceptible genotypes.

Factors Leading to the Evolution of Herbicide Resistance

Factors that lead to, or stimulate and accelerate, the evolution of herbicide resistance are manifold. These include biological characteristics of the weed species, characteristics and application of the herbicide, and cultural practices adopted for weed control.

Weed Characteristics

The most likely weed characteristics that favor increase in resistance against a particular herbicide are: a) annual growth habit, b) high seed production, c) relatively rapid turnover of the seed-bank due to high percentage of seed germination each year, d) several reproductive generations per growing season, e) extreme susceptibility to a particular herbicide, and f) frequency of resistant gene(s) among weeds.

Weed species less likely to develop resistance generally have a) a slower generation time, b) incomplete selection pressure for most herbicides, c) ability to adapt to changing environment, d) lower fitness for resistant biotypes, and e) extended seed dormancy in the soil. These factors increase the number of susceptible biotypes in the population.

Herbicide Characteristics

The following properties of herbicide molecule build resistance in weeds: a) mechanism of action, b) a single site of action, c) frequency of herbicide use, d) broad spectrum control, and e) longer residual activity in the soil.

Cultural Practices

Complete reliance on herbicides for weed control can greatly enhance the occurrence of herbicide resistant weeds. Cultural practices can also increase the selective pressure (discussed latter in the Chapter) for the development of herbicide resistant biotypes. These are: a) shift away from multi-crop rotations towards mono-cropping, b) little or no cultivation or tillage for weed control or no elimination of weeds that escape herbicide control, c) continuous or repeated use of a single herbicide or several herbicides that have the same mode of action, d) higher herbicide use rate relative to the amount needed for weed control, and e) weeds in orchard and vineyard systems as well as roadsides.

The level of herbicide resistance in weeds varies with weed biology and resistance mechanism. In some cases, resistance occurs when the species survives a labeled rate of application, while in other cases the species can survive up to 1,000 times the labeled rate. There are two levels of herbicide resistance characteristics: low-level and high-level. Low-level resistance includes, a) continuum of plant responses from slightly injured to nearly dead, b) display of an immediate response by majority of plants, and c) presence of susceptible plants in the population, especially when herbicide resistance is determined early. In high-level resistance a) plants are slightly injured to uninjured, b) few plants have intermediate responses, and c) susceptible plants can be present in the population.

Genetic Variation and Mutation

Evolution of herbicide resistance is dependent on the extent of genetic variation and frequency of occurrence of mutation. In a susceptible population, genetic variation must be present for occurrence of the evolution of herbicide resistance. Most weed species contain adequate genetic variations that allow them to survive under a variety of environmental stresses. Genetic variation, which has a direct relationship with natural selection, may be measured by such quantitative measures as plant height, time to flowering, and total biomass.

Genetic variation can be preexisting or arise *de novo* by mutation (or recombination) following herbicide application. We can thus distinguish two situations as regards genetic variation for herbicide resistance in non-selected populations: a) factors affecting the acquisition of resistance by novel mutation and b) factors affecting the probability of preexisting variation for resistance.

Given the existence of genetic variation, the rate of evolution will be determined by the mode of inheritance of resistance traits and intensity of selection. The evolution of resistance under persistent applications of herbicides may be considered as an example of recurrent selection in which there is a progressive, and sometimes rapid shift in average fitness of weed populations exposed to herbicide. Once established, gene flow via seed and propagule distribution contributes to the spread of resistant weeds. A major determinant in the selection of herbicide-resistant biotypes is the effective selection intensity that differentiates resistant individuals (more fit) from susceptible ones (less fit) in the face of selection (to the application of herbicide).

There are two ways in which resistance traits may arise within a weed population. A major resistant gene(s) may be present at low frequency so that selection acts to change a population, which is initially susceptible [Maxwell and Mortimer 1994]. Alternately, recurrent selection may occur continuously to achieve a progressive increase in average resistance from one generation to the other, with changes in gene frequency at many loci conferring resistance. Thus, herbicide resistance is developed by gene mutations or conferred by preexisting genes.

In general, gene mutations conferring resistance to a herbicide class are not induced by application of the herbicide, but rather, occur spontaneously. Spontaneous mutations at gene loci recur with characteristic frequency such that new mutations are continuously generated in natural populations of weeds. Mutations at some loci, particularly those encoding a specific herbicide site of action, may confer resistance. Typical spontaneous mutation rates in biological organisms are often cited as 1×10^{-5} or 1×10^{-6} gametes per locus per generation [Merrell 1981].

These values are for single, nuclear gene inheritance of evolution of herbicide resistance evolution. The rate of mutation to a single, dominant resistant allele of 1×10^{-6}, the probability of occurrence of at least one resistant plant in a 30 ha field with a weed density equal to, or exceeding, five plants m^{-1} is greater than 0.95 for a random mating species [Jasieniuk et al. 1996]. Thus, at least one resistant plant is almost certain to occur in a weed population of this size despite the low rate of mutation to resistance. Factors such as seed non-viability and seedling mortality may cause the plant's death prior to reproduction. However, should it survive and reproduce and if the corresponding herbicide or herbicide class is applied for several generations, the single resistant plant could give rise to a predominantly resistant population of weeds.

If the mutation rates are lower than 1×10^{-6}, i.e., 1×10^{-8} to 1×10^{-10}, the probability of occurrence of a resistant mutant is markedly reduced. It requires densities greater than 50 plants per m^2 for a resistant mutant to occur if the mutation rate is 1×10^{-8} [Jasieniuk et al. 1996]. The positive correlation between the size of a susceptible weed population and probability of occurrence of resistant mutant plants may partly explain why herbicide resistance has evolved in some species but not in others. The probability of herbicide-resistant plants occurring in a population is greater for weeds with high densities than for those which occur at low densities.

The frequency of mutation may be influenced by environment and dosage of herbicide. For example, atrazine applied at sub-lethal doses to certain susceptible genotypes of

Chenopodium album resulted in a progeny with triazine-resistant characteristics similar to highly resistant plants [Bettini et al. 1987]. This indicates that herbicide resistance could be induced by low doses of the chemical in certain genotypes. Most herbicides are extensively screened for efficacy in the field before release for commercial use. Herbicides are not accepted for market without at least 85–90 percent control of the target weeds. Therefore, it is very likely that resistance traits are present, if undetectable, in weed populations before large-scale selection with herbicides.

Mechanisms of Herbicide Resistance

The most common and important mechanisms of herbicide resistance are those which interrupt the transport of herbicides to biochemical sites of action, reduce the sensitivity of target sites, and detoxify the chemical or enhance repair can potentially confer resistance. These include the following:

1. Sequestration or Compartmentalization of the Herbicide in the Apoplast: Some plants restrict the movement of herbicides within the cells or tissues and prevent them from causing harmful effects. In this case, the herbicide may be inactivated either through binding (often to sugar moiety) or removed from metabolically active regions of the cell to inactive regions where it exerts no effect.
2. Altered Target Site: The herbicide has a specific site of action where it acts to disrupt a particular plant process or function. If the target site is altered, it no longer binds to the site and is unable to exert its phytotoxic effect. This is the most common herbicide resistance mechanism.
3. Differential Uptake and Translocation: In resistant biotypes, the herbicides are not taken up readily due to abnormal production of waxes, reduced leaf area, etc. Similarly, in resistant biotypes the apoplastic and symplastic transport of herbicide is reduced due to differential modifications.
4. Enhanced Metabolism: Weeds that have the ability to quickly degrade a herbicide may potentially inactivate it before it reaches its site of action within the plant, thus enhancing metabolism.
5. Over-expression of the Target Protein: If the target protein on which the herbicide acts is produced in large quantities by the plant, then the effect of herbicide becomes insignificant.
6. Enhanced Production of the Target Site: When production of the target site is enhanced, the herbicide will be unable to inactivate the enzyme. Thus, the enzyme spared by the herbicide will carry on the normal plant metabolic activities.
7. Modification of cell membrane function and structure.
8. Altered sensitivity of the key target enzyme caused by mutation(s).
9. Enhanced metabolic breakdown and conjugation of the herbicide.
10. Enhanced degradation of herbicide-generated toxic products.

These mechanisms, and consequently the expression of resistance, are controlled by genetic loci.

Inheritance of Herbicide Resistance

There are three modes of inheritance of herbicide resistance: Nuclear inheritance, Cytoplasmic inheritance, and Quantitative inheritance.

Nuclear Inheritance

Resistance to most classes of herbicides is caused by nuclear inheritance. These include auxinic herbicides, aryloxyphenoxypropionics, benzoics, bipyridiliums, dinitroanilines, sulfonylureas, substituted ureas, glycines, etc. In nuclear inheritance, the resistance-conferring alleles are transmitted through pollen and ovules. Adaptive evolution is achieved by the selection of phenotypes encoded by many genes (i.e., polygenes), each with a small additive effect. Generally, herbicide resistance is conferred by major genes present in weeds. In the majority of cases in which the number of genes has been determined, resistance is controlled by a single, major gene [Jasieniuk et al. 1995]. The predominance of major gene inheritance is attributed to the two following factors.

1. The most recently developed herbicides are highly target site-specific and interfere with single enzymes in major metabolic pathways. Mutation of the gene encoding for the enzyme may alter a plant's sensitivity to the herbicide and cause resistance.
2. Repeated application of these herbicides imposes strong selection, often causing 95–99 percent mortality, against susceptible phenotypes in weed populations.

Adaptation to herbicide is possible only if resistant genes are present in a population and have a significantly large phenotypic effect to allow the survival of a few individuals in a single generation [Mazur and Falco 1989]. With polygenic inheritance, recombination among individuals for many generations is required to bring together a sufficient number of favorable alleles to produce a highly resistant phenotype. Polygenic inheritance of resistance is thus more likely under conditions of weak selection as would occur with sublethal herbicide application [Jasieniuk et al. 1996].

Cytoplasmic Inheritance

Cytoplasmic inheritance of resistance occurs with triazine herbicides in several weed species. The gene conferring triazine resistance is located in the chloroplast genome [Hirschberg and McIntosh 1983]. Transmission of the chloroplast resistant gene mostly occurs by pollen, the paternal parent. For example, the mutation that confers maternally inherited triazine resistance involves a single base substitution in the *psbA* chloroplast gene which codes for a photosystem II (PS II) membrane protein to which triazine herbicides bind. The expected frequency with which a mutation occurs in the chloroplast genome and gives rise to gamete-transmissible triazine resistance is very low. The probability ranges from 1×10^{-9} to 1×10^{-12} mutations per gene locus.

Quantitative Inheritance

Quantitative patterns of inheritance of a phenotypic characteristic (trait) are controlled by polygenes. In this, the additive action of numerous genes, perhaps minor, results in a trait (e.g., height, seed production, etc.) showing continuous variability. The different minor genes that affect several processes will rapidly add up to a high level of resistance [Neve and Powels 2005a]. For instance, one gene may limit translocation of the herbicide, another may cause rapid metabolism, and yet another may affect the target site slightly [Gressel 2009]. Generally, differential resistance is quantitatively inherited.

Unlike monogenic traits, polygenic traits do not follow the Mendelian inheritance (separated traits). Genes, with each one causing a small increase in fitness under

herbicide selection, may systematically promote increased fitness in genotypes as genetic recombination occurs over successive generations. Implicitly, the rate of evolution is likely to be slower than for single nuclear-encoded genes.

Awareness of the possibility of quantitative resistance has led to recommendations to apply labeled herbicide rates to weeds of the size recommended by the registration.

Resistance to chlorsulfuron by *Lolium perenne* is conferred by additive minor genes inherited in a quantitative manner [Mackenzie et al. 1995]. Resistance of *L. rigidum* to diclofop-methyl is controlled by at least three resistant genes and low herbicide dose can rapidly evolve polygenic broad-spectrum herbicide resistance by quantitative accumulation of additive genes of small effect [Busi et al. 2012].

Imposition of Selection Pressure by Herbicides

Selection pressure is an interaction between natural variation in a species and factors in its environment that cause a certain plant to have an advantage over the others. It pushes the evolution of a particular species toward a greater prevalence of this variation. Its effectiveness is measured in terms of differential survival and reproduction, and consequently in change in the frequency of alleles in a population.

In the case of weed resistance, herbicide application exerts a powerful selection pressure on huge populations of weed species in both herbicide-resistant transgenic crops and conventional crops, exposing individuals with a genetic ability to survive herbicide treatment. While the population as a whole suffers high mortality, the herbicide is effectively selecting individual plants that possess any of the genetically endowed traits (resistant genes) which enable them to survive the herbicide dosage used. These survivors produce seed and contribute to the gene pool of subsequent generations, enriching the population with resistance genes. Thus, herbicide resistance results from selection pressure working on genetic diversity. Selection by herbicides changes the population over time. If, in the first year, one plant in a million plants of a weed species treated with a herbicide exhibits resistance, in the second year it will multiply into more resistant plants, and the process repeats in the following years to end up in the evolution of even more resistant plants.

This evolution of herbicide resistance in weeds is dependent on the intensity of selection imposed by herbicides. Most herbicides are applied at rates that result in the mortality of 90–99 percent of the susceptible weeds. If genetic variation for resistance is present due to mutation or gene flow, even at very low frequencies, repeated herbicide application will normally result in a rapid increase in the frequency of resistant individuals until they dominate the population [Jasieniuk et al. 1996].

Selection pressure imposed by a herbicide is a primary factor that determines the rate of enrichment of herbicide resistance in a weed population. In general, selection pressure is a measure of the ability of a herbicide to differentiate between susceptible and resistant plants. The higher the intensity of selection imposed by a herbicide against susceptible species, the faster the expected rate of evolution and spread of resistance. Several herbicide characteristics and patterns of use result in higher mortality than others do and thus impose a more intense selection pressure for the development of resistance. These include a single target site and highly specific mechanism of action, long-term soil residual activity, and frequent applications [LeBaron and McFarland 1990].

Seed production is an essential component to assess selection pressure. Effective kill by a herbicide is measured as the percentage reduction in seed yield at the end of the growing season. Values obtained for both resistant and susceptible plants are then used

to estimate the selection pressure. The selection pressure of a herbicide is calculated as the ratio of the fraction of resistant plants that survive its application to the corresponding fraction of susceptible plants [Gressel and Segel 1990].

Measurement of selection intensity exerted by herbicides is done theoretically and phenotypically. Population geneticists measure selection as the differential survival of alleles or change in gene frequency after the action of the selection agent. However, weed scientists measure selection frequency at the phenotype level. Selection coefficients may be variously defined at the genetic (gene) or zygotic (genotypic) level. The coefficient of selection, '***S***', may be defined as the proportionate reduction in the contribution of a particular genotype (usually the most favored), whose contribution is usually taken to be unity [Maxwell and Mortimer 1994].

It is very important to take into account that the intensity of selection pressure depends on the type of treatment and/or herbicide, its formulation, frequency of application, and the biological characteristics of the weed and the crop. Herbicide selection pressure should be seen in the group of actions carried out in the field: tillage, crop rotation, use of other control methods and cropping. Thus, a herbicide with selection pressure, used sporadically and alternating with other non-chemical control methods, will have a low risk of causing problems of resistance. Some herbicide groups have a higher selection pressure than others. For example, selection pressure is high (within 4–8 yr of continuous application) with ACCase-(acetyl CoA carboxylase) and ALS-(acetolactate synthase) inhibiting herbicides, while it is medium (within 10–15 yr of application) with PS II-, PS I-, carotenoid biosynthesis-inhibiting, and auxin-inhibiting herbicides. For other herbicides the selection pressure is on the low, with resistance occurring only after continuous application for 15 years and more.

Alteration of Selection Pressure

When resistance is determined by major genes, a lowering of the selection pressure may delay the onset of resistance. This can be achieved [Mortimer 1993] by:

a. Reducing the rate of application of the herbicide selected.
b. Invoking mixtures of herbicides. In this, co-evolution of resistance to two different herbicide chemistries would be slow because the frequency of dual-resistant plant would be the compounded frequency of the two herbicides [Gressel and Segel 1990].
c. Adopting rotation of herbicides with non-chemical methods of control.
d. Using herbicides which have fundamentally different modes of action.

Reducing the selection pressure may delay the evolution of resistance for the following reasons:

a. Plants that are susceptible to selective herbicides may contribute progeny to the next generation and hence lower the frequency of resistant alleles in the total population.
b. Where a weed species has a persistent seed-bank, only a fraction of that weed population will be exposed to selection in each cropping season. Hence populations in successive seasons will include susceptible individuals recruited from the seed-bank, and the survival of these individuals may again result in a lowering of the frequency of resistant alleles.
c. Plants that escape mortality may be the recipients of immigrant pollen (from external sources), thereby enabling an influx of susceptible alleles and hence leading to lowering of resistance gene frequency. The effectiveness of gene flow will be

determined by the mode of inheritance of resistance and the frequency of resistant alleles at the time when gene flow occurs. The influence of such immigration will be noticeable if resistance is conferred as a recessive allele that is at low frequency in the population. When resistance is controlled by a dominant allele, the effect is likely to be small, and if maternally inherited, zero.

The reverse situation in which resistance alleles emigrate into the surrounding populations of susceptible genotypes is significant for the management of existing herbicide resistant populations. Where selection is more relaxed due to change in management, the frequency of resistance alleles in the susceptible population will reduce more slowly than the absence of pollen flow.

Effect of Polygenic Resistance on Selection Pressure

The response to selection based on polygenes depends on genetic recombination causing several or many genes (each contributing in a minor way) to 'coalesce' in a single genotype [Mortimer 1993]. Relatively rapid response to selection will occur if low selection pressures are applied since this will strongly select for genotypes showing elevated resistance as individual genes become combined within a genotype. Application of increasingly strong doses of herbicide will intensify the response to selection. If selection pressure is high initially, then genotypes with small enhancement of resistance will be lost from the population and the frequency of recombinations of polygenes or multiple gene amplifications will be greatly reduced.

Fitness

Fitness is the ability of the organism to survive and produce offspring in a given environment. It is the central idea in evolutionary theory. If differences between alleles of a given gene affect fitness, then the frequencies of the alleles will change over generations. In the theoretical plant population model constructed to predict herbicide resistance, two sets of biological processes serve as major factors. These are ecological fitness and gene flow. Knowledge about both factors is necessary to develop effective strategies for management of herbicide resistant weeds.

An individual plant is a unique genotype with variation at many loci affecting fitness. The fitness of a group of plants having a certain genotype is assessed in relation to the fitness of other genotypes lacking key traits of interest. Fitness is a measure of survival and ability of a given genotype (e.g., herbicide-resistant biotypes) to produce viable offspring in competition with the wild type (e.g., herbicide-susceptible biotypes) [Gressel 2002]. It describes the evolutionary advantage of a phenotype, based on its survival and reproductive success. Under conditions of natural selection, genotypes with greater fitness produce, on average, more offspring than less fit genotypes. It is measured over the whole life cycle of a plant, encompassing the effects of selection on mortality and seed production of survivors.

In a single interbreeding population of plants in a homogeneous environment, the genotypic response due to allelic changes at a single locus is considered to occur against a constant environmental and genetic background and the expression 'genotypic fitness' is used. With this approach, it is possible to measure and calculate 'genotypic fitness' in the field and laboratory for a given genotype (homozygote and heterozygote) [White and White 1981].

Ecological fitness of a given biotype indicates that it will leave a greater proportion of its genes in the future gene pool of the population. The most fit plant will leave the greatest number of offspring. Differences in ecological fitness between resistant-(R) and susceptible-(S) biotypes will influence the rate at which herbicide resistance appears, as well as development of resistant populations when not treated with the herbicide. Although herbicide-R biotypes should be less fit than S biotypes, the fitness of resistant populations may vary depending on the mechanism of resistance and the environmental conditions. For example, under agricultural field conditions, triazine-R biotypes than their normal-R counterparts have been shown to be less fit than the S biotypes. However, many studies have not been able to detect fitness penalty in biotypes resistant to ALS inhibitors (e.g., imazethapyr) under those conditions [Ashigh and Tardif 2009]. The existence of fitness penalty under field conditions could be exploited for management of those resistant biotypes affected by it.

For an annual species, the seed produced by a genotype per generation constitutes a fitness estimate for a given environment only at one point in the evolutionary time. While determining the fitness of a weed with a persistent seed-bank, the rate of loss of seed from the soil needs to be measured. Seed carryover from previous generations plus the seed produced in the current generation contribute to total seed production. In order to understand the rate of evolution of resistance or management of resistance, measurements of fitness need to be conducted only under field conditions with the crop, and with and without herbicide application. For plant species reproducing vegetatively, measuring fitness is intrinsically more difficult and may require measurement of biomass or plant parts over a time period appropriate to the species in question.

Fitness is expressed in relative terms whereby genotypes are compared amongst themselves relative to the most successful one but it is important to recognize the following:

a. Absolute fitness contributes to the rate of evolution in its own right. When all other factors are equal, evolutionary rate is proportional to per capita rates of increase of the weed population.
b. Fitness is a measure of genotypic performance in a particular environment. Early studies on resistant biotypes of weeds pointed to the fact that there may be a 'cost' to resistance reflected in traits such as growth and competitiveness. Thus susceptible genotypes have superior fitness to resistant ones in the absence of selection.

Gene Flow and Spread of Herbicide Resistance

Gene flow, also known as gene migration, is the transfer of genetic material or alleles from one plant to another and from one site to another. It results in a change in gene frequency in one population due to movement of gametes, individuals, or groups of individuals from one population to another [Slatkin 1987] and occurs both spatially and temporally [Mallory-Smith and Olguin 2011]. It is a natural process to which all genes are subject and this contributes to evolution of species. Gene flow is of two types: horizontal and vertical. Horizontal gene flow is the movement of genes between disparate, unrelated species as in the case of plants and microbes. On the other hand, vertical gene flow, which is of greater importance in evolution of species, is the exchange of genes between closely related species. It occurs in only one generation between varieties or types of plants within the same species, and sometimes even between species. Thus vertical gene flow is a natural process that occurs incessantly and permanently between biologically compatible organisms and to which all genes are subject.