S. M. Paul Khurana
Rajarshi Kumar Gaur *Editors*

Plant Biotechnology: Progress in Genomic Era

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Editors
S. M. Paul Khurana
Amity Institute of Biotechnology
Amity University
Gurgaon, Haryana, India

Rajarshi Kumar Gaur
Department of Biotechnology
Deen Dayal Upadhyaya Gorakhpur
University
Gorakhpur, Uttar Pradesh, India

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Foreword

To address the advances of plant biotechnology, the editors, Prof. S. M. Paul Khurana and Prof. R.K. Gaur, have undertaken the thorny assignment of capturing the status and future trends in the various fields of agriculture and food and nonfood plant production systems. *Plant Biotechnology: Progress in Genomic Era*, delivered by the proponents of agricultural biotechnology, offers a wealth of information about the scientific breakthroughs and discoveries aiming to meet the global challenges of the diminishing amount of arable land as well as energy shortage, malnutrition, and famine. Many eminent and erudite scholars such as Prof. Klaus Ammann, Prof. Ajit Verma, Prof. S. K. Khare, Prof. A. N. Pathak, Dr. Swarup Chakrabarty, Dr. G. P. Singh, Prof. T. Satyanarayana, Prof. Poonam Singh (Nigam), Dr. Kishor Gaikwad, Dr. Senjuti Sinharoy, and Prof. Yuri Dorokov have addressed various aspects of plant biotechnology and provided important contributions for the book. The book consists of four parts, i.e., (i) *Gene and Genome*, (ii) *Biofuel and Bioremediation*, (iii) *Plant as Medicine and GE for (Plant) Stress*, and (iv) *Disease and Crop Management*.

Plant biotechnology comprises a distinct science of deriving valuable products from cells, tissues, and entire plants. The field also involves the exploitation of plants and bioprocess applications in different fields of human activities such as energy production, environmental protection, and industrial use of natural resources. Development of successful biotechnology applications requires thinking across boundaries. The interdisciplinary nature of the field becomes evident in the book, as it covers both traditional and recent developments in the fields of microbiology, mycology, and plant pathology. Plant biotechnology innovations will work in practice only if they can be combined with established strategies and common agricultural practices. If successfully completed, moved through the maze of regulatory safety processes and accepted by consumers, they will continue to shape the future of global agriculture and sustainability of agricultural production.

Various powerful genome sequencing and editing technologies have initiated a new era in plant molecular breeding. It has become increasingly possible to understand the connections between phenotypes and genotypes and to describe gene and protein functions underlying the desired traits. The first part excellently familiarizes the reader with the basic concepts and current technologies of plant genetic engineering. It paves the way for the coming parts dedicated to the introduction of advanced plant biotechnology applications aiming to develop sustainable bioenergy

production, biodegradation, plant-derived health products, and plant disease management. Each of these areas is essential for the short- and long-term success of plant biotechnology and for solving global problems. To exemplify, novel sources of biofuels are urgently required to avoid the use of fossil fuels, the main reason for global increases in atmospheric CO_2 concentrations and climate change.

The book consists of 27 enlightening chapters, which contain numerous beautiful and revealing illustrations helpful for the reader to grasp the essence of the message. Throughout the book, the approaches have been scrutinized with a critical eye as is characteristic for dedicated science professionals. I am confident that this excellent book provides an insightful overview of the prospects and challenges of plant biotechnology both to researchers and to students in this fascinating field. I hope that many readers of the book will become informed advocates of plant biotechnology.

University of Helsinki
Helsinki, Finland

Kristiina Mäkinen

Preface

The present growth of biotechnology in the current research often overshadows the emerging role of plant biotechnology, due to (having) dramatic developments in the last decade. This would certainly overcome and complement the earlier standard procedures. The book *Plant Biotechnology: Progress in Genomic Era* consists of 27 chapters, divided into 4 major parts of modern biotechnology:

(i) Gene and Genome
(ii) Biofuel and Bioremediation
(iii) Plant as Medicine and GE for (Plant) stress
(iv) Disease and Crop Management

All parts share a search into the regulation of genes responsible for specific qualitative or quantitative traits. As with any new revolutionary science, biotechnology needs to be continuously monitored and regulated for the benefit of the humanity. Each chapter has been written by distinguished scientists, having made significant contributions and pioneers/leaders in the field. All the articles present the opinion of the authors and their viewpoints about the powerful tools of the advancement of biotechnology.

Part I, Gene and Genome, provides the background information on the status of studies in this field and on the recent methodological developments in plant genetic engineering. The questions regarding regulatory issues of GM crops, especially those concerning the novel technologies used in plant molecular breeding, are discussed in Chap. 1. With the advent of various genome sequencing technologies, it is now possible to understand the connections between the phenotypes and genotypes and to describe the gene and protein functions in the desired traits. To save time, money, and effort, the subsets of genomes can be prepared for targeted sequencing by enriching the genome area of interest (Chap. 2). Chapter 3 reveals how recent developments in sequencing strategies have made it possible to obtain the full sequence of the complex hexaploid genome of wheat, which is globally the second most important cereal, for its improvement. The accumulating sequence data will advance the possibilities to improve crops also by modeling biological phenomena and studying them at the system(s) level as envisioned in Chap. 4. The contributors of Chap. 5 have also expressed concern whether the full genome

approaches in the development of next-generation crops can be exploited or not due to the strict regulation against GM crops.

For studying different genomes, several editing tools, such as ZFNs, TALENs, and CRISPR systems, and bioinformatics are used to discuss their revolutionary applications in precision molecular breeding and functional genomics research (Chaps. 6, 7, and 8).

In Part II, Biofuel and Bioremediation, many novel approaches of microbial bioremediation, including bioelectric technology, and biosurfactants are discussed. This part deals with the plant oils that can be used for the production of biodiesel and their physical and chemical properties (Chap. 9). Subsequently, various processes of fermenting cellulosic hydrolysates, using microbial strains, are explained along with the properties of biodiesel obtained (Chap. 12). Also, the different wastes that are used for producing bioelectricity (Chap. 11) and actinomycetes for soil remediation (Chap. 13) and the use of nanosystems as a carrier and delivery system of various essential oils on the target pathogen are discussed (Chap. 10).

In Part III, Plant as Medicine and GE for (Plant) stress, Chap. 14 summarizes current research dealing with medicinal properties and health benefits of *Withania somnifera* with a focus on antioxidant, anticancer, and antimicrobial properties, while Chap. 20 describes defense mechanism and diverse actions of fungal biocontrol agents against plant biotic stresses. Besides, clinical trials and action mechanism of potent compounds extracted from lichens are also described (Chap. 15). It is apparent that nanotechnology offers a wide range of applications and is a highly promising technology for revolutionizing (modern) agriculture (Chap. 17). Chapter 16 describes the aloe vera plantlets under controlled experimental conditions in order to analyze its potential on morphogenesis and secondary metabolites from the test plant. The contributors of Chap. 18 introduce model of legume species that have been used to expand our understanding of the traits associated with root nodule symbiosis (RNS) in plants. Chapter 19 presents an overview of recent advances on the development and application of CRISPR/Cas9 system in plants.

In Part IV, Disease and Crop Management, Chap. 21 summarizes future research on ROS through classical as well as advanced biotechnological methods for a better understanding of plant biology. Chapter 24 describes at length on methods and the prospects of P-TMAs which are especially important for individualized cancer therapy, as well as cases of bioterrorism and pandemics. Furthermore, Chap. 26 provides a comprehensive account on the various diagnostic techniques available for citrus greening/HLB and also discusses the recent advancements in its detection. Various plant viruses have also been described as used for gene silencing vectors and the next-generation vectors (Chap. 22) and RNA gene silencing which focus on the perspectives for utilizing this mechanism as a tool for control of viruses in plants (Chap. 23). Chapter 25 discusses ddPCR applications of plant pathogens using citrus pathogens in duplex and triplex assays. Precise information on chloroplast-virus interaction as developed for disease control strategies and genetically engineered plants with better photosynthetic efficiency and yields is given in Chap. 27.

We wish to express our deep gratitude to all the contributing authors, including many eminent scientists worldwide, who are pioneers in plant biotechnology. While

every effort was made to avoid ambiguity and to maintain uniformity and/or consistency in style, the presented results, ideas, and organizational details of the chapters still reflect personal opinion and preference of the respective authors.

We are highly grateful to the many reviewers, colleagues, and friends, involved in the venture, for their help, advice, and cooperation as well as to the Springer Nature for their kind assistance and ungrudging patience.

Finally, with love and affection, we are also deeply indebted to our families for their patience and understanding.

Gurgaon, India — S. M. Paul Khurana
Gorakhpur, India — Rajarshi Kumar Gaur

Contents

Editors and Contributors

Editors

Prof. (Dr.) S. M. Paul Khurana had his PhD in 1969 on Papaya Viruses and his 2-year postdoctoral research (1970–1972) on Advanced Plant Virology at Kyushu University, Fukuoka, Japan, with Prof. Zyun Hidaka as JSPS Fellow. He also availed GOI DBT Overseas Fellowship for 1 year at the University of Minnesota, St Paul (USA), with Prof. EE Banttari and specialized in Immunodiagnostics (March 1987–April 1988). Moreover, he worked at the Central Potato Research Institute (CPRI), Shimla, since 1973, as Scientist/Sr. Scientist; Principal Scientist and Head, Virus/Seed Pathology (1976–1982/1988), Principal Scientist and Head, Plant Pathology Division (1988–1993); Project Coordinator, AICRP-Potato (1994–2004); Director CPRI, Shimla (2002–2004); and Vice Chancellor, Rani Durgavati University, Jabalpur (2004–2009). He also served as Visiting Consultant for CIP/FAO (1992, 1996, 1997) and then as the Director of Amity Institute of Biotechnology, AUUP, Noida (2009–2010), and moved to Amity University Haryana at Gurgaon, where he served as Dean, Science, Engineering, and Technology (2013–2016), & Professor of Biotechnology since 2016 to present, in August 2010 for establishing the Institute of Biotechnology (2010–2015). In addition, he is an internationally recognized Plant Virologist/Pathologist and Biotechnologist having 53 years of experience, 230 research papers, and 100+ reviews, guided 16 PhDs, edited 18 books, etc.

Dr. Rajarshi Kumar Gaur earned his PhD in 2005, now Professor, Department of Biotechnology, Deen Dayal Upadhyaya Gorakhur University, Gorakhpur, Uttar Pradesh, India. His PhD was on molecular characterization of sugarcane viruses, viz., mosaic, streak mosaic, and yellow luteovirus. He received MASHAV Fellowship in 2004 from the Israel Government for his postdoctoral studies and joined The Volcani Centre, Israel, and BenGurion University, Negev, Israel. In 2007, he received the Visiting Scientist Fellowship from Swedish Institute Fellowship, Sweden, for 1 year to work in the Umeå University, Umeå, Sweden. He has made significant contributions on sugarcane viruses and published 130 national/international papers and presented nearly about 50 papers in the national and international conferences. He was awarded as Fellow of Linnean Society, London. Currently, he is handling many national and international grants and international collaborative projects on plant viruses and disease management.

Contributors

Mostafa Abdelrahman Molecular Breeding Laboratory, Arid Land Research Center, Tottori University, Tottori, Japan
Botany Department Faculty of Science, Aswan University, Aswan, Egypt

Razi Ahmad Enzyme and Microbial Biochemistry Laboratory, Chemistry Department, IIT Delhi, New Delhi, India

Rabia Amir Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan

Klaus Ammann University of Bern, Bern, Switzerland

Sandeep Arora Department of Molecular Biology and Genetic Engineering, (CBSH), G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

Kaustav Bandyopadhyay Amity Institute of Biotechnology, Amity University, Gurgaon, Haryana, India

Vagner A. Benedito Division of Plant and Soil Sciences, West Virginia University, Morgantown, WV, USA

Swarup Kumar Chakrabarti ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh, India

Sarika Chaturvedi Amity Institute of Biotechnology, Amity University, Gurgaon, Haryana, India

Cristina Cruz Faculty of Science, Centre of Biology, University of Lisboa, Campo Grande, Portugal

Anica Dadwal Division of Biological Sciences & Engineering, Netaji Subhas University of Technology, New Delhi, India

Ashis K. Das Plant Pathology Lab, ICAR-Central Citrus Research Institute, Nagpur, Maharashtra, India

Sunny Dhir Amity Institute of Virology & Immunology, Amity University, Noida, Uttar Pradesh, India

Yuri L. Dorokhov N. I. Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, Russia
A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia

Kishor Gaikwad ICAR-National Institute for Plant Biotechnology, New Delhi, India

R. K. Gaur Department of Biotechnology, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur, Uttar Pradesh, India

Kumar Gaurav Amity Institute of Biotechnology, Amity University, Gurgaon, Haryana, India

K. Gopalareddy Division of Crop Improvement, ICAR-Indian Institute of Wheat and Barley Research, Karnal, India

Om Prakash Gupta Division of Quality and Basic Sciences, ICAR-Indian Institute of Wheat and Barley Research, Karnal, India

Subhas Hajeri Citrus Pest Detection Program, Central California Tristeza Eradication Agency, Tulare, CA, USA

Adil Hussain Department of Agriculture, Abdul Wali Khan University, Mardan, Pakistan

Sidra Hussain Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan

Sudisha Jogaiah Plant Healthcare and Diagnostic Center, Department of Studies in Biotechnology and Microbiology, Karnatak University, Dharwad, India

Yun Kang Noble Research Institute, Ardmore, Oklahoma, USA

Hemant Kardile ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh, India

Parampreet Kaur ICAR-National Institute for Plant Biotechnology, New Delhi, India
Punjab Agricultural University, Ludhiana, Punjab, India

Sunil Kumar Khare Enzyme and Microbial Biochemistry Laboratory, Chemistry Department, IIT Delhi, New Delhi, India

Amit Kharkwal Amity Institute of Microbial Technology, Amity University, Noida, Uttar Pradesh, India

S. M. Paul Khurana Amity Institute of Biotechnology, Amity University, Gurgaon, Haryana, India

Tatiana V. Komarova N. I. Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, Russia
A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russia

Igor Kryvoruchko Bogazici University, İstanbul, Turkey

Arindam Kuila Department of Bioscience & Biotechnology, Banasthali Vidyapith, Banasthali, Rajasthan, India

Narendra Kumar Amity Institute of Biotechnology, Amity University, Gurgaon, Haryana, India

Rajesh Kumar Department of Biosciences, School of Sciences, Mody University of Science and Technology, Sikar, Rajasthan, India

Yogita Maheshwari USDA-ARS, San Joaquin Valley Agricultural Sciences Center, Parlier, CA, USA

Neha Malviya Department of Biotechnology, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur, Uttar Pradesh, India

Avinash Marwal Department of Biotechnology, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

Avinash Mishra Division of Biotechnology and Phycology, CSIR-Central Salt and Marine Chemicals Research Institute, Bhavnagar, Gujarat, India
Academy of Scientific and Innovative Research, Council of Scientific and Industrial Research, Ghaziabad, India

Megha Mishra Department of Biosciences, School of Sciences, Mody University of Science and Technology, Sikar, Rajasthan, India

Poonam Singh Nigam School of Biomedical Sciences, University of Ulster, Coleraine, UK

Hafiza Noor-ul-Ain Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan

Richard Owusu-Apenten Department of Clinical Sciences & Nutrition, University of Chester, Chester, UK

Anjali Pande Department of Molecular Biology and Genetic Engineering, (CBSH), G. B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

Vanita Pandey Division of Quality and Basic Sciences, ICAR-Indian Institute of Wheat and Barley Research, Karnal, India

Jaykumar Patel Division of Biotechnology and Phycology, CSIR-Central Salt and Marine Chemicals Research Institute, Bhavnagar, Gujarat, India
Academy of Scientific and Innovative Research, Council of Scientific and Industrial Research, Ghaziabad, India

A. N. Pathak Centre Research for Development, Parul University, Vadodara, Gujrat, India

Nikolay M. Petrov New Bulgarian University, Sofia, Bulgaria
Institute of Soil Science, Agrotechnologies and Plant Protection "N. Pushkarov", Sofia, Bulgaria

Kumar Pranaw Enzyme and Microbial Biochemistry Laboratory, Chemistry Department, IIT Delhi, New Delhi, India

Bikash Raul National Institute of Plant Genome Research, New Delhi, India

Tulasi Satyanarayana Division of Biological Sciences & Engineering, Netaji Subhas University of Technology, New Delhi, India

Vijayanandraj Selvaraj USDA-ARS, San Joaquin Valley Agricultural Sciences Center, Parlier, CA, USA

Pradeep Sharma Division of Crop Improvement, ICAR-Indian Institute of Wheat and Barley Research, Karnal, India

Priyanka Sharma Amity Institute of Microbial Technology, Amity University, Noida, Uttar Pradesh, India

Shilpa Sharma Division of Biological Sciences & Engineering, Netaji Subhas University of Technology, New Delhi, India

Shivani Sharma Department of Bioscience & Biotechnology, Banasthali Vidyapith, Banasthali, Rajasthan, India

Susheel K. Sharma ICAR Research Complex for NEH Region, Manipur Centre, Imphal, Manipur, India

Vinay Sharma Amity University, Jaipur, Rajasthan, India

Ekaterina V. Sheshukova N. I. Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow, Russia

Gyanendra Pratap Singh ICAR-Indian Institute of Wheat and Barley Research, Karnal, India

Senjuti Sinharoy National Institute of Plant Genome Research, New Delhi, India

Ashish Srivastava Amity Institute of Virology & Immunology, Amity University, Noida, Uttar Pradesh, India

Vivek Srivastava International Pannacea Limited, Gurgaon, Haryana, India

Mariya I. Stoyanova Institute of Soil Science, Agrotechnologies and Plant Protection "N. Pushkarov", Sofia, Bulgaria

Anita Talan Institut National de la Recherche Scientifique, Quebec city, Canada

Neha Thakre School of Plant Sciences, University of Arizona, Tucson, AZ, USA

Jagesh Kumar Tiwari ICAR-Central Potato Research Institute, Shimla, Himachal Pradesh, India

Monika Tyagi Amity Institute of Microbial Technology, Amity University, Noida, Uttar Pradesh, India

Ajit Varma Amity Institute of Microbial Technology, Amity University, Noida, Uttar Pradesh, India

Jerome Verdier Research Institute of horticulture and seeds (IRHS), INRA, Agrocampus-Ouest, Universite d'Angers, Angers, France

Rakesh Kumar Verma Department of Biosciences, School of Sciences, Mody University of Science and Technology, Sikar, Rajasthan, India

Dinesh Yadav Department of Biotechnology, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur, Uttar Pradesh, India

Dinesh Kumar Yadav Department of Botany, University of Allahabad, Allahabad, Uttar Pradesh, India

Neelam Yadav Department of Botany, University of Allahabad, Allahabad, Uttar Pradesh, India

Sangeeta Yadav Department of Biotechnology, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur, Uttar Pradesh, India

Sarika Yadav Department of Biochemistry, Sri Venkateshawara College, University of Delhi, New Delhi, India

Raymond Yokomi USDA-ARS, San Joaquin Valley Agricultural Sciences Center, Parlier, CA, USA

Nobiyuki Yoshikawa Faculty of Agriculture Department of Plant-bioscience, Iwate University, Morioka, Japan

Byung-Wook Yun School of Applied Bioscience, College of Agriculture & Life Sciences, Kyungpook National University, Daegu, South Korea

Part I

Gene and Genome

Selected Innovative Solutions for the Regulation of GM Crops in Times of Gene Editing

1

Klaus Ammann

Abstract

The analysis of the structure of the regulatory discourse needs a look behind the curtain about reasons for the dissent, and we need to acknowledge the 'Genomic Misconception': Transfer of transgenes similar to natural mutation. The conclusion is to have a critical look at the present-day regulation and shift to a professional discursive structure of the regulatory rules. The unfortunate decision of the European Court to include all Gene Editing products hinders progress but encourages also to aim at a basic restructuring of the present-day regulation laws instead of only minor corrections. One of the important discursive elements is to include cultural responsibilities of modern agriculture in its broadest aspects.

Keywords

GM crops · Gene editing · Regulatory discourse · Genomic misconception

1.1 Introduction

If we want to escape years-long fruitless debates on biotechnology and biodiversity, we have to do more than just to deplore the debate full of artificial (or imagined) contrasts, the main arguments are summarized below. The debate needs a professional *discursive structure* and we must embrace different kinds of knowledge, and new solutions should not be excluded, on the contrary: in new regulatory structures surprising new discoveries of better crops and in the science of GM safety also have to be anticipated. Basically, a mutual understanding of the different views on

K. Ammann (✉)
University of Bern, Bern, Switzerland
e-mail: klaus.ammann@ips.unibe.ch

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agricultural strategies (from organic farming to the application of new breeds may stimulate the debate and lead to innovative solutions.

In their publication of Zetterberg and Edvardsson Björnberg (2017), the authors come up with a programmatic summary, which can well serve here as a motivation to go ahead with a courageous proposal for a regulatory change of GM crops:

> *In recent years, the EU legislation on genetically modified (GM) crops has come under severe criticism. Among the arguments are that the present legislation is inconsistent, disproportionate, obsolete from a scientific point of view, and vague in terms of its scope. In this paper, the EU GM legislation (mainly the "Release Directive", 2001/18/EC) is analyzed based on five proposed criteria: legal certainty, non-discrimination, proportionality, scientific adaptability, and inclusion of non-safety considerations. It is argued that the European regulatory framework does not at present satisfy the criteria of legal certainty, non-discrimination, and scientific adaptability. Two ways of reforming the present legislation toward greater accommodation of the values expressed through the proposed criteria are briefly introduced and discussed.* From Zetterberg and Edvardsson Björnberg (2017)

With the necessary courage and organized workforce those plans can be realized in a few months of intensive work, and it should also be possible to push the solutions through in the complex system of European and international regulation.

1.2 The Structure of a Regulatory Discourse

1.2.1 First: Look Behind the Curtain

We need to see behind the curtain and focus on the main driver elements behind the debate. The industry, together with important farmer organizations, wants to see better results of the new breeds in the field for commercial marketing. The scientists focus on facts, strive for innovation and progress in agricultural breeding, and they believe in new solutions to fight the hunger in the world still existing Council for Agricultural Science and Technology (CAST) et al. (2017). Decisive opposition comes from professional NGOs like Greenpeace and Friends of the Earth, most often with arguments which are not supported by science. Both opponent sides build on heavy financial support and are reluctant to lose their expensive structural organization. Scientists often do not understand that a discourse on modern breeding including the public institutions is an absolute necessity. NGOs also fear that public support will faint, a support which is still of very important dimensions: Bouillon (2014). Recently, part of the GM opposition deplores to lose the debate related to the more precise methods of Gene Editing which might be more acceptable to the public and politics. GM-Opponents still consider the modern breeds full of risks but are unable to present convincing facts Steinbrecher and Paul (2017). The debate is often carried in a merciless way, major players risk major loss of income by losing the debate Miller et al. (2008). The main driver behind opponent campaigns is often *diffuse fear*, built on questionable interpretations of substantial equivalence and sustainability and full of additional false arguments constructing negative but unsubstantiated effects of modern breeding. But such negative contributions counting on the natural fear mongering for the public, are contradicted heavily by breeding

optimism, here one example dealing only with the great genomic potential of wild relatives: Wettberg et al. (2018): The conclusions:

> *Collections of wild relatives of crops will be most useful to breeding programs if they reflect the breadth of adaptations present in natural populations, which we argue is best accomplished when collections span the full geographic and environmental range of the species. Our collection expands both the genomic diversity and environmental range of the two closest wild relatives of chickpea, increasing the size of the collection by over an order of magnitude. The variation in substrate, elevation, and climatic range encompassed by the collection increases the likelihood that the assembled germplasm contains variation in phenology, drought, heat and cold stress. Indeed, we observe phenotypes that are correlated with environmental variation in the form of seed color crypsis and responsiveness to drought, and we have identified variation in seed nutrient density, phenology, resistance to pod borer, heat tolerance, and water deficit response. We are also actively exploring segregating variation in Fusarium wilt and Ascochyta blight resistance, nitrogen fixation and plant architecture, each of which represent traits that are of great interest for chickpea crop improvement. Our collection also highlights the need for conservation of CWRs. Rapid development in southeastern Anatolia is accompanied by the fragmentation and loss of native landscapes. Two of the populations reported here were lost or fragmented in subsequent years (2014, 2015), while other populations are threatened by human activities.* ***These facts underscore the urgency of the need to collect, characterize, and preserve both in situ and ex situ wild relatives of crops as essential components of humankind's agricultural heritage and future*** *1 McCouch and Crop Wild Relative (2013), 36, Tanksley and McCouch (1997) and 37 Maxted et al. (2012). Comments from Wettberg et al. (2018).*

It will be important to abstain from unilateral thoughts and try to integrate various methods and approaches for a healthy and future-minded agriculture: Ammann (2012a), Dollacker (2018), Van Wensem et al. (2017), and Ricroch et al. (2016a).

1.2.2 Second: The 'Genomic Misconception' of Existing GM Regulations

Not surprisingly, molecular science and unbiased views on agricultural history should be able to ease down the contrasts in this debate, here two of many arguments:

(a) The process of gene transfer is identical, whether done in natural mutation or modern biotechnology, a view supported in the past many times by Nobel Prize Winner Werner Arber Arber (2010), summarized with details of the regulatory history in the *Genomic Misconception*, a review published 2014 by Ammann (2014).

According to latest papers of Werner Arber, Genetic Engineering represents a safe approach for innovations improving nutritional contents of major food crops Arber (2017a, b).

(b) It is on the other side clear that the *application* of the huge potential of the new methods including *Gene-Editing*, will have important consequences in the future of agriculture. There is a plethora of new crop trait possibilities which are already tested or need to be tested, whether involving "foreign DNA" or not, since all new traits done with molecular methods *embrace a certain procedural*

novelty. The present-day politics of many scientists aims at the full exclusion of those very precise Oligo-Mutations which end up without "foreign" DNA in the product, should be fully excluded from regulation Breyer et al. (2009b). This sounds convincing, but a closer look at the methods of Gene Editing will lead to more precautionary conclusions, as shown below. Some insight in the present day debate on regulation of GM crops can be read in a selection of publications – it is nearly impossible to distill out of the considerable variety of regulatory thoughts into a clear, simple concept (see Chap. 4): Zetterberg and Edvardsson Björnberg (2017), Eriksson (2018a, b, Eriksson et al. (2018), Davison and Ammann (2017), Eriksson and Ammann (2017), Ricroch et al. (2016b, Tagliabue et al. (2016), and Tagliabue et al. (2017).

1.2.3 Third: Organo-transgenic Thoughts

The consequences from the Genomic Misconception analysis are the following:

It is fact, after Wood et al. Wood and Lenne (2001), that our main world crops (Rice, Wheat and Sorghum) have been chosen by our ancestors because they already lived in *large monodominant stands*, an important precondition of efficient food production. The often-heard argument that huge monocultures are directly and negatively related to modern breeding has no logic or historic background. On the contrary, modern breeding can be key to conceive a more ecological methodology in agriculture Ammann (2007b, 2012b). In consequence, we need proposals to merge organic farming with its good sides in biodiversity management, but unfortunately having a strict focus on anti-biotechnology and hostility towards industrial farming with its uncritical perspective on production alone – a critical view which in the latest years received a lot of correction also in conventional agriculture: Consequently, it is better to think the unthinkable such as *Organo-Transgenics*: Indeed, organic farming and biotech farming could actually go together under well-defined circumstances – across ideological and commercial barriers. Ammann (2008, 2009), Ammann and van Montagu (2009), about cisgenic potatoes see Gheysen and Custers (2017).

But the regulatory conclusions from 2.1 to 2.3 are, despite numerous proposals published, somehow complex.

1.3 Conclusion: Regulatory Proposals: The Idea of a Dynamically Scalable Regulation

The regulatory views should in consequence not be black and white for part or the whole modern and traditional breeding: A *dynamically scalable regulatory modus* should be more realistic and more acceptable to friends and foes, see Wolt et al. (2010, 2015) and Podevin et al. (2012a) and Wolt (2017). The Gene Editing

methods which finally do not contain any foreign DNA should still be regulated in a modest way for a few years, then released swiftly after and almost certain positive outcome to the world agriculture applications. More details about the Dynamically Scalable Regulation can be checked out in the citations above. One illustration from Jeffrey Wolt explains concisely the strategy.

The anticipated scrutiny of the various regulatory methods is well summarized in Wolt et al. (2015), specifically in its Fig. 1.1 below.

The full text of interpretation in Wolt is given here with some editing of the author for the reason of a precise argumentation for his dynamically scalable regulation scheme in Fig. 1.1, including the citations with some added items:

Regulatory discussion of a wide range of new breeding techniques applied to crop development was initiated in 2011 with an EU-convened international workshop that considered the techniques then available for site-directed genome editing Lusser and Davies (2013b), and Lusser et al. (2011, 2012. Based on the categorizations identified by this group, its elaboration by Podevin et al. (2012b) see also Devos et al. (2014)—and accounting for the emergence of new techniques in the interim—a schema for regulatory characterization specific to genome editing techniques can be described (Fig. 1.1). This schema considers the approach to DSB repairs that are achieved by NHEJ (SDN1), homologous recombination (SDN2) or transgene insertion (SDN3) and whether the technique for introduction of the GEEN is transient *(Category 1),* introduces rDNA within the plant genome with subsequent removal *(Category 2)* or entails stable plant genome integration of rDNA *(Category 3).* The OMM approach produces DSB repaired by NHEJ and therefore is analogous to SDN1 in terms of its regulatory characterization to the extent the

Category / Method	Category 1 Transient expression resulting in site-specific DSB and repair	Category 2 Stable genomic introduction of rDNA with intermediate steps to generate transgene-free null segregants	Category 3 Stable genomic integration of recombinant DNA
SDN1* Site-directed random mutation involving NHEJ	Low	• Low for deletions • Case-by-case for addition • Higher as size of insertion increases	N/A
SDN2 site-directed homologous repair involving one or very few nucleotides	Case-by-case	Case-by-case	N/A
SDN3 site-directed transgene insertion	N/A	N/A	High, moderated for well characterized insertion sites

Fig. 1.1 Relationship of site-directed genome approach to the anticipated degree of regulatory scrutiny of the plant phenotype obtained. ∗Current uses of OMM are analogous to SDN1 in terms of regulatory scrutiny. (From Wolt et al. 2015)

changes are viewed as point mutations and not template insertions: Hartung and Schiemann (2014); Lusser and Davies (2013b). It is somehow plausible to exclude Oligo-Mutations from the usual regulatory scheme, as many US and EU authors conclude. But apart from this solution, indeed rather simple and tempting from the regulatory point of view, this exclusion will meet decisive opposition from many GM critiques such as Steinbrecher and Paul (2017), we propose a differentiated solution, by taking up the views of Jeffrey Wolt et al. within the following three categories.

1.3.1 Category 1 of a New Dynamic Regulation

Techniques involve transient introduction of recombinant DNA using in vitro synthesized nucleic acids and DNA delivery methods that *do not integrate* into the host genome Pauwels et al. (2014). These techniques, therefore, resemble transgenic processes but produce phenotypes that are indistinguishable from plants obtained through conventional plant breeding. The techniques would include site-specific point mutations with oligonucleotides (OMM), site-specific random mutations by NHEJ (SDN1) and site-specific mutations with DNA repair via homologous recombination (SDN2). Novel techniques avoiding the use of rDNA through direct introduction of the nuclease or mRNA encoding the nuclease Baltes et al. (2014), Baltes and Voytas (2015), and Martin-Ortigosa et al. (2014) to catalyze similar mutation events would also fall into this category.

1.3.2 Category 2 of a New Dynamic Regulation

Consists of stable introduction of rDNA into the host genome and an intermediate step involving expression of SDN1 or SDN2 to effect DSBs and repairs. Subsequent breeding selection for null segregants results in phenotypes that are indistinguishable from phenotypes obtained through conventional plant breeding. Therefore, evidence will generally be lacking in the product to indicate a transgenic process was involved in the intermediate step. Plant phenotypes developed by SDN1 methods as described in either of the forgoing categories represent simple point mutations and with few exceptions (Canada) regulators do not consider crops developed by mutagenesis in the same context as GM crops. The regulatory opinions regarding plant phenotypes developed by SDN2 methods are not as clear, as the nature and extent of the edits used to effect the desired change in the phenotype obtained by the technique would influence opinions as to whether the phenotype represented a GM product. For instance, deletions are viewed as less consequential than are additions. And in the case of additions, the greater the number of bases added, the greater the level of regulatory concern. Important in this context is the determination as to whether the NHEJ accomplished by the technique is viewed as a template insertion into the genome Lusser and Davies (2013a).

1.3.3 Category 3 of a New Dynamic Regulation

Category 3 finally involves techniques which result in stable integration of rDNA where 'Genome editing with engineered nucleases' (GEEN) is used to specifically target delivery of a transgene or multiple transgenes through insertion by homologous recombination (SDN3). Current examples of this technique involve the site-directed stacking of transgenes D'Halluin and Ruiter (2013). Thus, they simply represent a refined technique to accomplish transgenesis and would be considered no differently than GM products by regulators. The European Food Safety Authority (EFSA) Panel on Genetically Modified Organisms—an expert panel providing independent scientific advice to EFSA on GMOs—has developed the regulatory opinion that existing EFSA guidance documents apply to the SDN3 technique EFSA GMO Panel (2012), see also other important EFSA-publications: EFSA Gmo Panel Working Group on Animal Feeding Trials (2008), EFSA Guidance (2011), EFSA Guidelines and Renn Ortwin (2012), EFSA Independence (2012), EFSA letter and Paoletti Claudine (2015), and EFSA Opinion (2015). But because the technique can specifically target transgene delivery into the genome, it has the potential to minimize potential hazards associated with gene disruption or regulatory elements in the recipient genome.

*Thus, plants developed using SDN1 methods may require less data for risk characterization than more conventional approaches to transgenesis: s*ummary with edits of the author from Wolt et al. (2015)

> *Summary: Genome editing with engineered nucleases (GEEN) represents a highly specific and efficient tool for crop improvement with the potential to rapidly generate useful novel phenotypes/traits. Genome editing techniques initiate specifically targeted double strand breaks facilitating DNA repair pathways that lead to base additions or deletions by non-homologous end joining as well as targeted gene replacements or transgene insertions involving homology-directed repair mechanisms. Many of these techniques and the ancillary processes they employ generate phenotypic variation that is indistinguishable from that obtained through natural means or conventional mutagenesis; and therefore, they do not readily fit current definitions of genetically engineered or genetically modified used within most regulatory regimes.* ***Addressing ambiguities regarding the regulatory status of genome editing techniques is critical to their application for development of economically useful crop traits. Continued regulatory focus on the process used, rather than the nature of the novel phenotype developed, results in confusion on the part of regulators, product developers, and the public alike and creates uncertainty as of the use of genome engineering tools for crop improvement.*** *From Wolt et al.* (2016)

And from the paragraph of the same text of Wolt et al. 2016 "Needs within the regulated community"

> *The need to rapidly innovate to introduce novel traits in crops is heightened by increased world food demand and increasing use of crops as sources of renewable energy (Edgerton 2009). The opportunity for transgenic crop innovation is limited by regulatory hurdles and continued public unease Pew Initiative et al.* (2015) *and Smyth et al.* (2015). *Transgenic technologies continue to elicit considerable public misunderstanding and mistrust despite 19 years of commercial use and over 181.5 million hectares in production globally in 2014 James (*2014). *Largely in response to effective pressure on the part of a broad spectrum of NGO and activist groups Paarlberg* (2014) *and the continuing public pressure it has engendered, the*

regulatory processes for transgenic GE crops (the so-called GMOs) are largely broken in many parts of the world. Implementation of national biosafety laws is encumbered in the developing world Bayer et al. (2010) *and Okeno et al.* (2013) *and long delays in cultivation approvals are reducing the value of innovation in many regulatory domains Smyth and Phillips* (2014)*. New breeding technologies, especially site-directed genome editing, are viable alternatives to transgenic crop production that provide new opportunities for innovation and which in many cases clearly involve a reduced degree of regulatory oversight. Success in advancing GEEN and related technologies for crop improvement will be limited if public views and regulatory response continues to be captured within the overriding theme of GMOs. The continued reliance on process-based definitions as a guide to regulatory oversight—and the adoption of process-focused language in public discourse—detracts from appropriately gauged approaches toward the regulation of genome-edited crops. Thus, the focus on the nature of the novel plant phenotype/trait is lost as the appropriate paradigm for the safety assessment, which encumbers regulatory approvals for crops derived from both established and emerging plant breeding techniques. Lacking a fuller emphasis on this point means that the public may largely misunderstand genome editing and regulators will be faced with pressure to evaluate these products within existing biosafety frameworks. Fortunately, progress is being made by regulators in shaping sensible and pragmatic approaches toward the application of genome editing for crop improvement but at some point to new product-based paradigms for regulation of new breeding technologies must emerge. From Wolt et al*. (2016)

See also the new table from Wolt (2017).

	Category 1	**Category 2**	**Category 3**
SONI	NHEJ or nucleotide replacement with transient introduction of reagent	NHEJ with stable integration of nuclease-encoding rONA and subsequent NS selection	NHEJ with stable integration of nuclease-encoding rONA
SDN2	HDR with transient introduction of reagent	HOR with stable integration of nuclease-encoding rDNA and subsequent NSselection	HOR with stable integration of nuclease-encoding rONA
SDN3	Transient introduction of reagent with site-directed transgene insertion	Site-directed transgene insertion with stable integration of nuclease-encoding rONA and NS selection	Sit e-directed transgene **insertion with stable** integration of nuclease-encoding **rDNA**

See the explanation from the first paper of Wolt et al. (2016), table from Wolt (2017)

Again, in his latest publication Wolt (2017) Jeffrey Wolt insists (as does the author) on a ***Dynamically Scalable Regulatory Modus*** (Box 1.1).

Genome editing with engineered nucleases (GEEN) is increasingly used as a tool for gene discovery and trait development in crops through generation of targeted changes in endogenous genes. The development of the CRISPR- Cas9 system clustered regularly interspaced short palindromic repeats with associated Cas9 protein), in particular, has enabled widespread use of genome editing. Research to date has not comprehensively addressed genome-editing specificity and off-target mismatches that may result in unintended changes within plant genomes or the potential for gene drive initiation.Governance and regulatory considerations for bioengineered crops derived from using GEEN will require greater clarity as to target specificity, the potential for mismatched edits, unanticipated downstream effects of off-target mutations, and assurance that genome reagents do not occur in finished products.

Box 1.1 Genome-Editing Approaches and Categories from Wolt (2017)

In case-by-case assessments of biotechnology-derived plant products, regulators initially seek understanding as to the egree the methodology employed is familiar, as this helps to inform assessment of risks and uncertainties that may be associated with a given trait within the crop of interest. In terms of genome-edited crops, regardless of the reagent system used, an understanding of potential downstream outcomes for the plant product can be ascertained through consideration of the editing approach and the introduction and fate of the reagent. Wolt et al. (2016)

Accomplishing a genome edit uses a site-directed nuclease (SDN) to cause DSBs. Repair of the DSBs occurs by various mechanisms: NHEJ which randomly insert/deletes one to several bases to cause point mutation (SDNl) and homology-directed repair (HDR) involving native or synthetic template insertion (SDN2) or transgene insertion (SDN3). These approaches represent, respectively, insertion of progressively consequential nucleotide base sequences at a targeted DSB. Approaches involving OMM involve simple nucleotide replacements and are analogous to SDN 1 to the degree the edits are viewed as point mutations and not template insertions. Lusser and Davies (2013a)

Additionally, in accomplishing the genome edit, the technique for introduction of the GEEN may be transient in the form of the protein, protein/RNA complex (e.g., Cas9/gRNA ribonucleoprotein complex), mRNA, or DNA that does not integrate into the host genome (category 1); may introduce nuclease-encoding rDNA in the genome that is subsequently removed through NS selection (category 2); or may involve stable genome integration of nuclease-encoding rDNA (category 3). These categories represent a progression of increasingly consequential procedures ranging from transient insertion of short-lived ribonucleoprotein complexes into the cytoplasm of cells to the introduction of nuclease-encoding rDNA into the host genome.

Together, the approach to gene editing used and the category describing introduction and fate of the nuclease roughly reflect the degree of regulatory uncertainty regarding the derived phenotype. The topology of current regulatory views concerning a given outcome of genome editing in terms of these factors are shown below (as adapted from Wolt and colleagues Wolt et al. 2016*). An additional layer of regulatory consideration with respect to category 2 and category 3 will be whether the design of the reagent has the potential to enable a gene drive.*

Since governance and regulatory decision making involves robust standards of evidence extending from the laboratory to the post-commercial marketplace, developers of genome-edited crops must anticipate significant engagement and investment to address questions of regulators and civil society*. From Wolt* (2017)

1.3.4 Summary: Clearly, There Is Still an Ongoing Regulatory Dispute Among Scientists

Most scientists and an important number of European scientists insist in a non-regulatory status of Oligo-Mutations not containing "foreign DNA", a position which can be seen with some scientific merits: It sounds somehow logic that mutational breeding not containing foreign DNA should not fall under the present day tedious and cumbersome regulation.

But still, the argument cannot be dismissed that after all, the new Oligo-mutation breeds are made with an *over-all new methodology*, which needs a minimum of regulatory proofing. Indeed, research has not addressed comprehensively the potential for mismatched edits and the results are contradictory: Considerable amount of mismatched edits: Fu et al. (2013), Sander and Joung (2014), Schaefer et al. (2017), and Zischewski et al. (2017) low incidence of such edits: Veres et al. (2014) unanticipated downstream effects of off-target mutations, and assurance that genome reagents do not occur in finished products. We should agree therefore to the *Dynamically Scalable Regulatory Modus* as described above. Unfortunately, the European Court has recently taken regulatory decisions which are clearly outside proper scientific thought, details below in Chap. 6.

1.4 Outlook

Recent papers demonstrate with more detailed conclusions and comments about the Regulation of GM crops within Europe, that the scene of new ideas is volatile: Ammann Klaus (2017), Davison John and Ammann Klaus (2017), Eriksson and Ammann (2017), Ricroch et al. (2016b), Tagliabue et al. (2016, 2017), and Tagliabue and Ammann (2018). More interesting ideas and concepts on modern regulation have been published mainly by German and US authors: Hokanson et al. (2018), Raybould et al. (2012), and Roberts et al. (2014). And the question is, why the European Union needs a national GMO opt-in mechanism: Eriksson et al. (2018). In Europe, it is still the question, whether regulatory hurdles for genome editing should be process- or product-based approaches in different regulatory contexts: Sprink et al. (2016), although most authors lean towards a product-view. In the eyes of the author this question depends on the perspective of the treated crop regulation. The divide among scientists often focuses to the regulation of Oligo-Mutations which lack in the final product "foreign" DNA, many follow the US decisions of not regulating those breeds, some, like the author, would like to see instead of such a rather theoretical molecular regulatory divide a *dynamically scalable regulation* as defined above, which includes also the Oligo-Mutations without foreign DNA, but with a minimum stretch of only 2–3 years of regulatory scrutiny – as described above. A very detailed and important debate about the regulation of Gene Editing is given by Jeffrey Wolt 2017: Wolt (2017), see above and remarks below:

Deciding on regulatory needs for 'Genome editing with engineered nucleases' (GEEN) it is not done with the simplistic distinction between products with or

without foreign DNA, the questions on safety situation are more complex, the author agrees fully with Jeffrey Wolt, here his more detailed comments from 2017 (p.220ff).

One concern are the off-target effects, which are still not studied enough: Jacobs et al. (2015), Jeffrey Wolt's comments Wolt (2017).

> *For example, increased editing efficiency is observed over time for genome modifications targeted using CRISPR- Cas9 in cultured soybean embryos. This may indicate continued expression of Cas9. during embryo and plant development and the potential for dose-dependent (concentration x time) effects that may increase off-target mutations. Addressing this will require further consideration off genome analysis of CRISPR-edited plant lines as well as improvements in computational tools, reagent design, and experimental methodology; collectively, these can lead to further insurances regarding the limited potential for unanticipated effects at likely mismatch sites within the genome. From Wolt* (2017)

The other, lesser concerns are the possibilities of Gene Drive events, Wolt's own comments:

> *The creation of gene drives in crop plants or livestock is of lesser concern than applications where gene drives are expressed in wild organisms under open release conditions – as may be the case if gene drives are used to eliminate invasive plant species or overcome pesticide resistance in weedy species. For domesticated plants and animals, there is limited opportunity for uncontrolled gene drive escape and dissemination because of long generation times and control of breeding lines which, respectively, reduce gene drive efficiency and provide a means to observe and remove undesired phenotypes which may be inadvertently developed. Addition,1lly, since domesticated crops and food animals are not competitive with sexually incompatible wild species, the probability is low for environmental establishment of gene drive-bearing crops or livestock. See the important citation: National Academies of Sciences* (2016)*, from Wolt* (2017)

In this complex situation of a multifaceted dispute, it is important to conduct future discourses under the auspices of a *modern discourse strategy*, as already promoted by Churchman (1979, 1984) and Rittel and Webber (1973, 2005).

> *"The search for scientific bases for confronting problems of social policy is bound to fail, because of the nature of these problems. They are "wicked" problems, whereas science has developed to deal with "tame" problems. Policy problems cannot be definitively described.* ***Moreover, in a pluralistic society there is nothing like the undisputable public good; there is no objective definition of equity; policies that respond to social problems cannot be meaningfully correct or false; and it makes no sense to talk about "optimal solutions" to social problems unless severe qualifications are imposed first.*** From Rittel and Webber (1973, 2005)

1.5 The Unfortunate Decision of the European Curia

The decision surprised many:

Hopes were real that the European Court would take a decision along those lines of analysis, as one of the closest experts like *Advocate General Michal Bobek* still in January 2018: (cited in the GAIN Report) GAIN Report et al. (2018)