Compendium of Plant Genomes
Series Editor: Chittaranjan Kole

Jameel M. Al-Khayri
S. Mohan Jain
Dennis V. Johnson *Editors*

The Date Palm Genome, Vol. 2

Omics and Molecular Breeding

Springer

Compendium of Plant Genomes

Series Editor

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Whole-genome sequencing is at the cutting edge of life sciences in the new millennium. Since the first genome sequencing of the model plant Arabidopsis thaliana in 2000, whole genomes of about 100 plant species have been sequenced and genome sequences of several other plants are in the pipeline. Research publications on these genome initiatives are scattered on dedicated web sites and in journals with all too brief descriptions. The individual volumes elucidate the background history of the national and international genome initiatives; public and private partners involved; strategies and genomic resources and tools utilized; enumeration on the sequences and their assembly; repetitive sequences; gene annotation and genome duplication. In addition, synteny with other sequences, comparison of gene families and most importantly potential of the genome sequence information for gene pool characterization and genetic improvement of crop plants are described.

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The Date Palm Genome, Vol. 2

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ISSN 2199-4781 ISSN 2199-479X (electronic)
Compendium of Plant Genomes
ISBN 978-3-030-73749-8 ISBN 978-3-030-73750-4 (eBook)
https://doi.org/10.1007/978-3-030-73750-4

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Small country farm at Bordighera, Italy, 1884, Claude Monet. *Image source* Wikimedia (https://commons.wikimedia.org/wiki/File:Monet_-_Palmen_in_Bordighera.jpg#/media/File:Monet_-_Palmen_in_Bordighera.jpg)

This book series is dedicated to my wife Phullara and our children Sourav and Devleena

Chittaranjan Kole

Preface to the Series

Genome sequencing has emerged as the leading discipline in the plant sciences coinciding with the start of the new century. For much of the twentieth century, plant geneticists were only successful in delineating putative chromosomal location, function, and changes in genes indirectly through the use of a number of "markers" physically linked to them. These included visible or morphological, cytological, protein, and molecular or DNA markers. Among them, the first DNA marker, the RFLPs, introduced a revolutionary change in plant genetics and breeding in the mid-1980s, mainly because of their infinite number and thus potential to cover maximum chromosomal regions, phenotypic neutrality, absence of epistasis, and codominant nature. An array of other hybridization-based markers, PCR-based markers, and markers based on both facilitated construction of genetic linkage maps, mapping of genes controlling simply inherited traits, and even gene clusters (QTLs) controlling polygenic traits in a large number of model and crop plants. During this period, a number of new mapping populations beyond F2 were utilized and a number of computer programs were developed for map construction, mapping of genes, and for mapping of polygenic clusters or QTLs. Molecular markers were also used in the studies of evolution and phylogenetic relationship, genetic diversity, DNA fingerprinting, and map-based cloning. Markers tightly linked to the genes were used in crop improvement employing the so-called marker-assisted selection. These strategies of molecular genetic mapping and molecular breeding made a spectacular impact during the last one and a half decades of the twentieth century. But still they remained "indirect" approaches for elucidation and utilization of plant genomes since much of the chromosomes remained unknown and the complete chemical depiction of them was yet to be unraveled.

Physical mapping of genomes was the obvious consequence that facilitated the development of the "genomic resources" including BAC and YAC libraries to develop physical maps in some plant genomes. Subsequently, integrated genetic–physical maps were also developed in many plants. This led to the concept of structural genomics. Later on, emphasis was laid on EST and transcriptome analysis to decipher the function of the active gene sequences leading to another concept defined as functional genomics. The advent of techniques of bacteriophage gene and DNA sequencing in the 1970s was extended to facilitate sequencing of these genomic resources in the last decade of the twentieth century.

As expected, sequencing of chromosomal regions would have led to too much data to store, characterize, and utilize with the-then available computer software could handle. But the development of information technology made the life of biologists easier by leading to a swift and sweet marriage of biology and informatics, and a new subject was born—bioinformatics.

Thus, the evolution of the concepts, strategies, and tools of sequencing and bioinformatics reinforced the subject of genomics—structural and functional. Today, genome sequencing has traveled much beyond biology and involves biophysics, biochemistry, and bioinformatics!

Thanks to the efforts of both public and private agencies, genome sequencing strategies are evolving very fast, leading to cheaper, quicker, and automated techniques right from clone-by-clone and whole-genome shotgun approaches to a succession of second-generation sequencing methods. The development of software of different generations facilitated this genome sequencing. At the same time, newer concepts and strategies were emerging to handle sequencing of the complex genomes, particularly the polyploids.

It became a reality to chemically—and so directly—define plant genomes, popularly called whole-genome sequencing or simply genome sequencing.

The history of plant genome sequencing will always cite the sequencing of the genome of the model plant *Arabidopsis thaliana* in 2000 that was followed by sequencing the genome of the crop and model plant rice in 2002. Since then, the number of sequenced genomes of higher plants has been increasing exponentially, mainly due to the development of cheaper and quicker genomic techniques and, most importantly, the development of collaborative platforms such as national and international consortia involving partners from public and/or private agencies.

As I write this Preface for the first volume of the new series *Compendium of Plant Genomes*, a net search tells me that complete or nearly complete whole-genome sequencing of 45 crop plants, eight crop and model plants, eight model plants, 15 crop progenitors and relatives, and three basal plants is accomplished, the majority of which are in the public domain. This means that we nowadays know many of our model and crop plants chemically, i.e., directly, and we may depict them and utilize them precisely better than ever. Genome sequencing has covered all groups of crop plants. Hence, information on the precise depiction of plant genomes and the scope of their utilization are growing rapidly every day. However, the information is scattered in research articles and review papers in journals and dedicated Web pages of the consortia and databases. There is no compilation of plant genomes and the opportunity of using the information in sequence-assisted breeding or further genomic studies. This is the underlying rationale for starting this book series, with each volume dedicated to a particular plant.

Plant genome science has emerged as an important subject in academia, and the present compendium of plant genomes will be highly useful to both students and teaching faculties. Most importantly, research scientists involved in genomics research will have access to systematic deliberations on the plant genomes of their interest. Elucidation of plant genomes is of interest not only for the geneticists and breeders, but also for practitioners of an array of plant science disciplines, such as taxonomy, evolution, cytology,

physiology, pathology, entomology, nematology, crop production, biochemistry, and obviously bioinformatics. It must be mentioned that information regarding each plant genome is ever-growing. The contents of the volumes of this compendium are, therefore, focusing on the basic aspects of the genomes and their utility. They include information on the academic and/or economic importance of the plants, description of their genomes from a molecular genetic and cytogenetic point of view, and the genomic resources developed. Detailed deliberations focus on the background history of the national and international genome initiatives, public and private partners involved, strategies and genomic resources and tools utilized, enumeration on the sequences and their assembly, repetitive sequences, gene annotation, and genome duplication. In addition, synteny with other sequences, comparison of gene families, and, most importantly, the potential of the genome sequence information for gene pool characterization through genotyping by sequencing (GBS) and genetic improvement of crop plants have been described. As expected, there is a lot of variation of these topics in the volumes based on the information available on the crop, model, or reference plants.

I must confess that as the series editor, it has been a daunting task for me to work on such a huge and broad knowledge base that spans so many diverse plant species. However, pioneering scientists with lifetime experience and expertise on the particular crops did excellent jobs editing the respective volumes. I myself have been a small science worker on plant genomes since the mid-1980s and that provided me the opportunity to personally know several stalwarts of plant genomics from all over the globe. Most, if not all, of the volume editors are my longtime friends and colleagues. It has been highly comfortable and enriching for me to work with them on this book series. To be honest, while working on this series I have been and will remain a student first, a science worker second, and a series editor last. And I must express my gratitude to the volume editors and the chapter authors for providing me the opportunity to work with them on this compendium.

I also wish to mention here my thanks and gratitude to the Springer staff, particularly Dr. Christina Eckey and Dr. Jutta Lindenborn for the earlier set of volumes and presently Ing. Zuzana Bernhart for all their timely help and support.

I always had to set aside additional hours to edit books beside my professional and personal commitments—hours I could and should have given to my wife, Phullara, and our kids, Sourav and Devleena. I must mention that they not only allowed me the freedom to take away those hours from them but also offered their support in the editing job itself. I am really not sure whether my dedication of this compendium to them will suffice to do justice to their sacrifices for the interest of science and the science community.

New Delhi, India Chittaranjan Kole

Preface

The date palm is unquestionably the keystone tree species of agriculture in semiarid and arid lands of the Near East and North Africa, and it is now successfully being grown commercially in South Asia, Southern Africa, Iberia, the Americas and Australia. This important multipurpose palm is an essential local nutritional resource for humans and animals, providing a source of income to farmers through fruit sales, the leaves and wood offering an array of other useful products and the trees themselves delivering ecological services by creating a favorable microenvironment for habitation, cultivation of other agricultural crops and animal husbandry.

In the coming decades, the major challenge for plant breeders is to sustain and improve upon date fruit production, through genetic improvement to counter biotic threats such that presented by the red palm weevil and bayoud disease, as well as abiotic stresses caused by drought, extreme temperatures and high soil or water salinity. Genomic studies are the best way forward to achieve such ends, along with the adoption of best practices for cultivation of the palms and improved postharvest fruit handling and marketing to minimize diminished quality and marketable quantity. A deeper knowledge of the date palm genome also offers a promising opportunity to explore such topics as biofortification to discover new products.

This book is the first comprehensive assemblage of contemporary knowledge and research work relevant to genomics and other omics in date palm. It highlights the recent progress in the development of plant biotechnology, associated molecular tools and their usage in plant breeding.

Two volumes of this book are concurrently published.

Volume 1 subtitled *Phylogeny, Biodiversity and Mapping*, consists of 11 chapters arranged in three parts grouped according to subject. Part I *Biology and Phylogeny*, with three chapters focusing on date palm biology, evolution and origin. Part II *Biodiversity and Molecular Identification*, with four chapters covering conformity of in vitro derived plants, molecular markers, barcoding, pollinizer genetics and gender determination. Part III *Genome Mapping and Bioinformatics*, with three chapters addressing genome mapping of nuclear, chloroplast and mitochondrial DNA, in addition to a chapter on progress made in date palm bioinformatics. This volume represents the efforts of 30 international scientists from 10 countries and contains 78 figures and 30 tables to illustrate presented concepts.

Volume 2 subtitled *Omics and Molecular Breeding* consists of 11 chapters arranged in four parts grouped according to subject. Part I *Nutritional and Pharmaceuticals Properties*, with three chapters on the utilization of date palm as an ingredient of various food products, a source of bioactive compounds, along with a chapter on the production of nanomaterials derived from date palm. Part II *Omics Technologies*, consists of a chapter addressing an overview of omics resources followed with two chapters on proteomics and metabolomics. Part III *Molecular Breeding and Genome Modification*, with three chapters focusing on genetic improvement technologies based on mutagenesis, quantitative traits loci and genome editing. Part IV *Genomics of Abiotic and Biotic Stress*, contains a chapter covering metagenomics of beneficial microbes to enhance tolerance to abiotic stress, and another chapter addressing the various genomics advances as they apply to insect control in date palm. This volume represents the efforts of 34 international scientists from 12 countries and contains 65 figures and 19 tables to illustrate presented concepts.

Participating authors were selected based on their reputable scientific expertise in date palm genomic research. Manuscripts were evaluated through a rigorous review process to assure quality presentation and scientific accuracy and edited for language precision.

Each chapter begins with an introduction covering related background materials and provides in depth discussion of the subject matter supported with high quality color photos, illustrations and relevant tabulated data. The chapter concludes with recommendations for future research directions and a comprehensive list of pertinent references to facilitate further reading.

This book is an excellent reference source for scientists engaged in genetics and modern breeding research based on biotechnology and genomic approaches. It is also a valuable source for advanced undergraduate and postgraduate students specializing in biotechnology and molecular breeding as well as for agricultural industries and policy makers especially those concerned with date palm.

We are greatly appreciative of the stalwart efforts of all chapter authors for their contributions towards the success and quality of this book. We are also grateful to Prof. Chittaranjan Kole, the *Compendium of Plant Genomes* Series editor, for his invitation and supervision and to Springer for the opportunity to publish this book.

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Abbreviations

ABA	Abscisic acid
ABEs	Adenine base editing
AFLP	Amplified fragment length polymorphism
Ag NPs	Silver nanoparticles
Au NPs	Gold nanoparticles
BE	Base editing
Bt	*Bacillus thuringiensis*
CaMV35s	Cauliflower mosaic virus 35s
CBE	Cytidine base editing
Cpf1	CRISPR Prevotella and Francisella 1
CRISPR	Clustered regularly interspaced short palindromic repeats
DPMMD	Date palm molecular marker database
DRDB	Date palm genome database
DSB	Double-stranded break
EMS	Ethyl methanesulfonate
eQTL	Expression quantitative trait loci
EST-SSR	Expressed sequence tag-simple sequence repeat
Fn	Francisella novicida
GC	Gas chromatography
GE	Genome editing
GFP	Green fluorescent protein
GMO	Genetically modified organism
GWAS	Genome-wide association studies
HCA	Hierarchical cluster analysis
HDR	Homology directed repair
KEGG	Kyoto encyclopedia of genes and genomes
LC	Liquid chromatography
MAS	Marker-assisted selection
MS	Mass spectrometry
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NiO NPs	Nickel oxide nanoparticles
NMR	Nuclear magnetic resonance
PAM	Protospacer adjacent motif
Pd NPs	Palladium nanoparticles
PGSB	Plant genome and system biology

Pt NPs	Platinum nanoparticles
qRT-PCR	Quantitative real time PCR
QTL	Quantitative trait loci
RAPD	Rapid amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNAi	RNA interference
RNP	Ribonucleoprotein
RPW	Red palm weevil
sg RNA	Single guide
SNP	Single-nucleotide polymorphism
SPR	Surface plasmon resonance
SSR	Simple sequence repeat
TALEN	Transcription activator-like effector nucleases
TILLING	Targeting induced local lesions in genomes
ZFNs	Zinc finger nucleases
ZnO NPs	Zinc oxide nanoparticles

Part I

Nutritional and Pharmaceuticals Properties

Date Palm: Source of Foods, Sweets and Beverages

1

Ricardo Salomón-Torres, Benjamín Valdez-Salas, and Susana Norzagaray-Plasencia

Abstract

The fruit of the date palm (*Phoenix dactylifera* L.) is grown mainly in the countries of the Middle East and North Africa, where it has great economic and cultural relevance. The date is highly appreciated for its elevated nutritional content, its great distinctive flavor and the great benefits it brings to health. Its pulp consists of approximately 70% carbohydrates, mostly in the form of sugars. They also contain appreciable amounts of dietary fiber and are an acceptable source of minerals and antioxidants. The most common way to consume it is fresh or dried through direct intake. Food scientists and the food industry have developed a wide range of products processed from dates, including energy bars, syrup, paste, juice, sugar, jam, candies, condiments and pickled dates, among others. Some products with added value are dates covered with chocolate, pistachios, peanuts, almonds, grated coconut or red chili powder. Among the by-products of dates are soda, energy beverages, bread, pie, cakes, cookies and butter. Other by-products from fermented dates are wine, beer and liquor, vinegar by oxidation and alcohol by distillation. Finally, oil and a coffee substitute are examples of by-products derived from the date seed. This chapter provides a detailed review about processed products from date fruit, including by-products, dates with value-added, sweets and beverages.

1.1 Introduction

The fruit of the date palm (*Phoenix dactylifera* L.) is cultivated mainly in arid and semiarid regions of the world. However, favorable areas are located within a geographical belt between 24 and 34° N latitude (Ortiz-Uribe et al. 2019). The date is a berry composed of a fleshy mesocarp, covered by a thin epicarp and an endocarp that covers all of its seed. The seed is usually oblong and ventrally grooved, containing a small embryo and a hard endosperm (Salomón-Torres et al. 2020). Currently, it is estimated that there are approximately 3000 date palm cultivars

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J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_1

around the world and each cultivar is derived from a single seed, cloned and multiplied by offshoots (Johnson 2011).

Due to its bioactive components, this fruit contains a potential value to be a functional food, since its intake provides many health benefits, beyond a simple basic nutrition, such as the possible prevention of some diseases, common cancers and other degenerative diseases (Al-Farsi and Lee 2012). The nutritional properties of this fruit make it an important source of energy and a food with high nutritional value. It contains a large amount of sugars, is low in fat and protein and is a rich source of minerals and antioxidants, in addition to being slightly acidic in nature and being an excellent source of fiber (Guizani and Singh 2012).

The daily consumption of 10–30 dates in Middle Eastern countries is common, because they are part of the regular diet. However, in Western countries it is not very commonly part of the daily diet. This fruit can be consumed as fresh fruit (or dried) or as a processed product. There is a great diversity of processed foods based on the date and its seed (Barreveld 1993). These can be date paste combined in foods based on wheat flour, liquid and powdered sweeteners, fermented and non-fermented beverages, oil, among its main products. Likewise, the date can accompany gourmet dishes, contrasting salty food with its sweet taste.

The aim of this chapter is to provide a broad review of the date palm fruit and seed as a food source, which can include added value, in combination with other food products or to generate by-products. The subjects discussed emphasize its genome, the nutritional content of the fruit, the way to prepare concentrated juices, fermented products and pastes for different uses, date as a sweetener and beverages derived from its pulp and seed.

1.1.1 A Short History of Date Palms

The exact origin of the date palm still remains unknown. Recent research suggests that its first domestication may have been at least 6000 years ago in the Middle East, in the southern part of present-day Iraq, being supported by archaeological evidence from ancient Mesopotamia (Chao and Krueger 2007; Ortiz-Uribe et al. 2019; Zaid and Arias-Jiménez 2002). The Persian Gulf region provides the oldest evidence of date palm cultivation, with seeds found in excavations in the United Arab Emirates, Kuwait and Iraq (Tengberg 2012). Alternate hypotheses suggest their possible domestication in Saudi Arabia, North Africa or India (Chao and Krueger 2007; Hazzouri et al. 2015; Sidhu 2006). Because a wild ancestor has not yet been identified, the origin of his domestication remains unspecified. However, it has been suggested that date palm is the result of the hybridization of two or more wild species of *Phoenix*, because it has been found to contain a large number of private alleles, which are characteristic of the same *Phoenix* species (Tengberg 2012).

From its presumed point of origin, this crop spread by means of seeds to the south to the Arabian Peninsula and to the east, until reaching India. To the west, it began in Egypt until it reached Morocco. Its spread came to Spain through the Arab invasion in the eighth century. Finally, the Spaniards introduced it first to Peru and then to Mexico, through the conquest in the sixteenth century (Johnson et al. 2015; Ortiz-Uribe et al. 2019). Centuries later, it begins its propagation through offshoots by means of its transport over long distances, with the rise of modern date palm plantations with elite cultivars in countries such as the USA, Australia, Israel, Morocco, United Arab Emirates, Oman, Mexico, Jordan and Namibia, among others (Johnson et al. 2015).

1.1.2 Date Palm Cultivation

The fruit of date palm is the main source of basic food and income for many populations in producing countries, playing an important role in their society, environment and the economy of those countries (Abd Rabou and Radwan 2017). Over the centuries, the date palm has achieved great spiritual significance in three of the world's

major religions (Zaid and Arias-Jiménez 2002). It is closely linked to the history, culture and diet of Islamic peoples. In the tradition of the Judaism and Christianity, the palm tree is accepted as a symbol of a long and healthy life (Schorr et al. 2018).

Of the three propagation methods that exist for this crop (Chao and Krueger 2007; Rajmohan 2011), the most common is its vegetative propagation through its offshoots, which produces the palm in its juvenile stage. Its separation is made from the base of the palm trunk, when they are 3–5 years old and weighing 10–15 kg. Another method of propagation is from its seed, with the disadvantage that the seedlings can be male with a 50% probability. This method usually causes late maturity and lower fruit quality, compared to the common method (Zaid and Arias-Jiménez 2002). The method of propagation through tissue culture has been the most recent scientific effort to achieve a faster spread of this crop. Two techniques are used for tissue culture: The first is embryogenesis by producing embryos from cells, and the second technique is organogenesis, which uses meristematic cells (Jain et al. 2011).

The best yield in this crop is the result of a high fruit set percentage. The success of this percentage depends on the combination of several factors such as pollen source quality, pollination efficiency, pollination period, male–female compatibility and environmental components such as temperature, irrigation, soil and fertilization (Salomón-Torres et al. 2017b). The pollination of the date palm is carried out naturally by the action of wind. However, for its commercial production, it is necessary to carry it out artificially (García-González et al. 2019). Pollen extracted from 1 male palm is enough to pollinate up to 50 female palms. Pollen sources have an effect on both the chemical properties and the quality characteristics of the fruit and seed of the date (Salomón-Torres et al. 2018), so the proper selection of pollen is important. According to the cultivar and agricultural practices, a single bunch can produce up to 1000 dates, weighing more than 8 kg. Likewise, the date palm begins to bear fruit at 4–5 years of age, reaching its maximum production between the ages of 10–15, producing 40–80 kg of fruit for each palm (Sidhu 2006). Bunch thinning is a practice that was developed to increase the size of the fruit. This practice is common in the Medjool cultivar, since the quality and size of this cultivar generate a higher price in the market (Cohen and Glasner 2015).

According to FAO data, in 2017 there were 8,165,897 mt of dates produced in the world, of which 19.47% were produced by Egypt, being the largest producer of this fruit. The producing countries of Asia report 58.92%, Africa 40.32, America 0.58 and Europe with 0.18% of date production, respectively (FAO 2019).

1.1.3 Date Palm Genome

The first efforts to learn more about the genetic diversity of date palm were through the use of molecular markers. Due to their abundance in the entire genome, molecular markers were used to describe genetic diversity, sex determination and gene characterization in date palm (Salomón-Torres et al. 2017a). Random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR) and simple sequence repeat (SSR) are fingerprinting techniques from DNA-based molecular markers that have been used to perform ancestry, cultivar and relationship to related plants, morphology, disease and pest resistance, drought tolerance and soil adaptability analyses (Johnson 2011). Likewise, the efficiency of start codon targeted (SCoT) molecular markers in date palm genetic diversity was recently evaluated, with satisfactory results (Saboori et al. 2020). With these types of molecular markers, it was possible to observe that the palm employs a XX/XY system for sex determination (Al-Mahmoud et al. 2012). Advantages and disadvantages have been judged based on the level of polymorphism they capture, the quality of template DNA needed, the development cost, the reliability and the level of skill required (Jain et al. 2011).

The information provided by molecular markers was limited. It was known that the date palm genome was composed of 18 chromosome pairs (2n = 36) (Zhang et al. 2011). A study estimated the genome size of the date palm at 1.29 Mbp, by flow cytometry using the fluorochrome propidium iodide (Zonneveld et al. 2005). A group of researchers from Qatar announced in 2011 the release of the first draft of the genome sequence of the date palm for the Khalas cultivar. It suggests a size ~658 Mbp, with a GC content of 37% (Al-Dous et al. 2011), where male genomes share the same heterozygous genotypes and female genomes the same homozygous genotypes. This was the first publicly available resource of the date palm genome. A second group of researchers from Saudi Arabia reported in 2013 the genome assembly of the same palm variety, with a size of 605.4 Mbp, covering 90% of the genome (~671 Mbp) and 96% of its genes (~41,660 genes) (Al-Mssallem et al. 2013). In 2014, a study announced the first genetic map of the date palm, in which an analysis of its karyotype with 64,783 single-nucleotide polymorphisms (SNPs) was made, where structural information of its genome is provided (Mathew et al. 2014). Likewise, it was observed that the markers linked to the palm genus are in the lower half of chromosome 12, suggesting that this may be the chromosome that defines the sex of the palm.

Whole genome re-sequence of 62 date palm varieties, originally from 12 countries in the Middle East and North Africa, was recently published. This re-sequencing revealed the diversification existing in this crop, providing essential information about the population structure and evolutionary history. Likewise, genetic markers were defined for identification of varieties and genome-wide association study (GWAS) approaches for mapping genes of agricultural importance (Hazzouri et al. 2015).

In a next step, GWAS will be useful for date palm researchers to identify critical genetic mechanisms to improve yields, disease resistance and fruit enhancement, and will open doors so that in the near future they can implement genetic improvement programs in the cultivation of date palm, through genomic selection.

There is still much to do with the genome of the date palm. It is necessary to create linkage disequilibrium maps, quantitative trait loci (QTL), copy number variation (CNV), selective sweep, haplotype blocks, etc., in order to enrich existing genetic resources. With a broader knowledge about gene expression in date palm, new transgenic palms may be developed, for example, resistant to red palm weevil (*Rhynchophorus ferrugineus* Olivier) and the fungal causative agent of the *Fusarium oxysporum* f. sp. *albedinis* W.L. Gordon. Likewise, it will be possible to have access to seed products of backcrosses that allow the development of improved date palms.

1.2 Source of Foods

1.2.1 Quality Characteristics and Nutritional Contents

Date palm can be classified by its cultivars, according to the characteristics of its fruit. It is estimated that worldwide there are approximately 3000 date palm cultivars. However, some cultivar names may be synonyms, since the name given to a cultivar in one country could be known in another with a different name (Johnson 2011). Each cultivar is derived from a unique single seed, cloned and multiplied by offshoots. Date palm cultivars have specific quality characteristics: color, size, sweetness, texture, maturity, stalk size, tolerance to tree moisture, medicinal properties and quality in general (Jaradat 2015).

Fruits can also be classified based on their moisture content (Jayasuriya 2012; Khairi 2015), same that generates a texture as follows:

Dry Date Cultivars They are characterized by being grown in regions with a moisture <20%, very dry and hot climate, since the crop requires 2093–2316 °C heat units. This category includes cvs, Abattamoda, Bartamoda, Deglet Beida, Gondaila, Horra, Kulma, Ruthana, Segao, Sakoty and Thoory, among others.

Semidry Date Cultivars These cultivars require a moisture between 20 and 30%, a total of 1400–1500 °C heat units and a moderately dry climate. In this classification are cultivars: Ajwa, Amry, Barni, Barhee, Dayry, Deglet Noor, Medjool, Mishrig Wad Laggai, Sewy and Zahidi, among others.

Soft Date Cultivars They require a moisture >30% and a total of between 1100 and 1150 °C heat units. The cultivars in this classification are: Abada, Amhat, Bentaisha, Hallawy, Hayany, Honey, Khadrawy and Khalas, among others.

Depending on the cultivar, the quality traits in the fruit show a wide range of variation according to their stage of maturity (Sidhu 2006). Within its physical changes in its growth curve, the fruit of the date also shows great changes in its chemical composition, mainly in sugars and tannins (Aleid 2012). Figure 1.1 shows the characterization of the growth curve of the Medjool cultivar grown in Mexico, for the 2018 and 2019 growing seasons, at 165 days after its pollination. Regarding commercial fruit quality in Mexico, skin separation and length of the fruit define the quality of the Medjool date. Length classification parameters are as follows: choice (<3.81 cm), fancy (3.81–4.44 cm), extra fancy (4.44–5.08 cm), large (5.08–5.71 cm), jumbo (5.71–6.35) and superjumbo (>6.35 cm). Skin separation of 0–10% in any of these classifications is acceptable (Ortiz-Uribe et al. 2019).

The growth of the fruit presents a progressive level of maturity in four great stages, which are known by their Arabic names: kimri, khalal, rutab and tamar. Hababauk is the term used for the female flower and the period just after pollination when the young fruit is still creamy white before gradually turning green at the kimri stage. The duration of each of the maturation stages is observed in days after pollination or elapsed weeks. These may vary according to the cultivar and cultivation practices (Al-Alawi et al. 2017; Aleid 2012; Alqarni and Bazzi 2019). At its first stage of growth (kimri), the fruit is small and green, with a hard texture, characterized by rapid growth. In its second stage (khalal), the fruit reached its maximum size and begins to change its color green to yellow or red. The third stage (rutab) is characterized by the loss of weight and moisture, turning the fruit into brown color. In its last stage (tamar), the fruit is ripe, has lost most of its moisture, is brown in color and is ready to be harvested (Aleid 2012).

Carbohydrates are the most abundant fruit component and are in the form of reducing sugars (fructose, glucose, mannose and maltose) and non-reducing sugars (sucrose), with small amounts of polysaccharides, such as cellulose and starch (Al-Shahib and Marshall 2003). The date is an excellent source of energy, due to its high sugar content, providing 330 kcal/100 g (Salomón-Torres et al. 2019). The most abundant mineral elements found in the date are: potassium

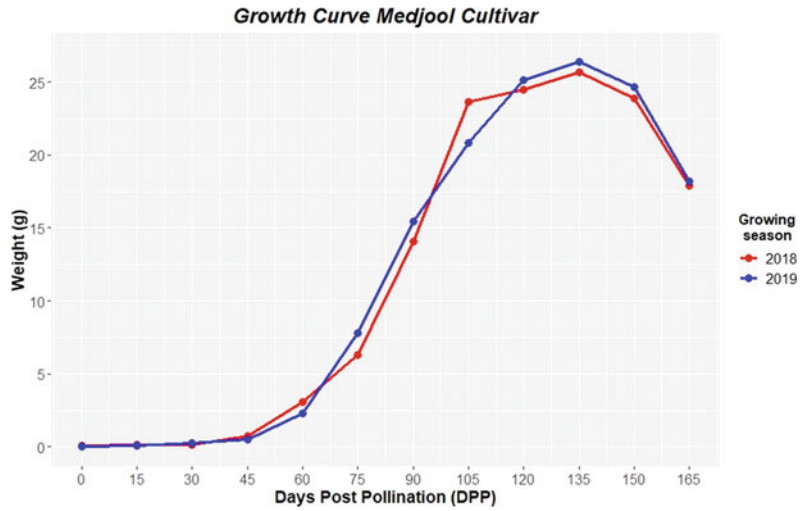

Fig. 1.1 Growth curve of the Medjool cultivar grown under the agroclimatic conditions of northwestern Mexico (figure constructed by Ricardo Salomón-Torres)

(345–1285), magnesium (31–150), phosphorus (35–74), calcium (5–206), sodium (1–261), copper (0.001–0.8), iron (0.1–1.5), manganese (0.01–0.5) and zinc (0.02–0.6), all in mg/100 g (Al-Farsi and Lee 2008).

The most abundant element in the date pulp is sugar, with content that can vary between 70 and 80% (g/100 g), followed by fiber content (6.40–11.50%), protein (2.30–5.60%), lipids (0.20–0.50%) and minerals (4.9–1088) all in mg/100 g (Salomón-Torres et al. 2019). Dates are a good source of phenols, carotenoids and flavonoids. They also contain high levels of essential amino acids and vitamins such as A, B1, B2, B3 and C, as well as strong antioxidant, anticancer and antiviral activities (Assirey 2015). Purportedly, date fruit not only provides these health benefits, but also has various medicinal values that include gastroprotective, hepatoprotective, antihyperlipidemic and nephroprotective activities (Tang et al. 2013). These characteristics of the date make this fruit as a great food for either derivative products or by-products of date.

1.2.2 Date Foods

Although it is not a staple food by definition, such as wheat, corn, rice or potatoes, the date has played this role, due to the absence of other commodities. Derived from the health benefits granted by this fruit, there is great interest in the date as a source of food, this as the main ingredient in the preparation of various foodstuffs (Barreveld 1993). Currently, health professionals suggest the consumption of functional foods such as date, since they have a high potential to prevent chronic diseases (Maqsood et al. 2020). A study conducted in Saudi Arabia observed that Arab workers who lived on a diet based on dates, rice and fish maintained better health than Indian workers who did not eat dates (Ahmed et al. 1995). Likewise, a survey was carried out in the desert of Saudi Arabia of almost 4000 regular consumers of dates, where no cases of deficiency disease were found among them (Ahmed et al. 1995), showing the great contribution of the date fruit to nutrition and human health.

The date as food can be consumed in two ways: as fresh fruit (or dried) and as a processed date product. The most common way of eating it is out of hand, or it can also be chopped and filled, or chopped and used in various products such as granola bars, cereals, bread, cookies, cakes, among others. Commonly, low-quality dates are processed to convert them mainly into paste, powder (date sugar), jams, syrups, juices, wine and vinegar (Aleid 2014).

Since the date is rich in sugars, but low in protein and fat, its consumption has extended to its combination with other foods, which seek to complement this nutritional deficiency, making a mixture of flavors that are exquisite on the palate. This variant of intake has been exploited mainly in countries where there is a low tradition of fresh consumption, such as the European countries, the USA and Mexico, where gourmet dishes have been developed as shown in Fig. 1.2a–d. The gourmet dishes based on dates are commonly filled with cream cheese, goat cheese or other types of cheese, combined with protein of animal origin such as bacon, serrano ham and Spanish chorizo sausage, among others. Dates can be part of salads (Fig. 1.2e–f) or can accompany a traditional dish like pizza, hamburger, risotto, pasta or sandwich (Fig. 1.2g–j), providing the sweet contrast to the savory or sour taste of the dish. Within Mexican gastronomy, there is the date tamale, which consists of the mixture of corn dough with date paste in an envelope of corn husk, which is cooked by steaming (Ortiz-Uribe et al. 2019).

Date confectionery products are another form of added value to encourage consumption. These are found in various presentations such as: whole dates covered with chocolate (including white), which can contain a layer of grated coconut, pieces of almonds, pistachios or peanuts. Dates are filled with peanut butter, combined with whole seeds or in pieces (Fig. 1.3). A very popular form of consumption in Mexico is pieces of date with red chili powder (Ortiz-Uribe et al. 2019) (Fig. 1.3g).

An alternative to ingesting this fruit directly is the handmade preparation of energy bars and balls based on dates (Fig. 1.4a–b). This bar or ball can be fortified with multiple nutritional

Fig. 1.2 Gourmet dishes cooked with dates. **a** Baked date wrapped with bacon and cheese, **b** date skewers stuffed with cheese and vegetables, **c** Medjool date tapas stuffed with goat cheese, Spanish chorizo and some vegetables, **d** stuffed date with nuts and cheese, **e**, **f** salads with dates, nuts, purple onion, pomegranate and other vegetables, **g** date and ham on a bagel, **h** chicken breast hamburger with dates, **i** risotto with dates, a typical dish of Italian gastronomy, **j** pasta with dates. *Source* www.facebook.com

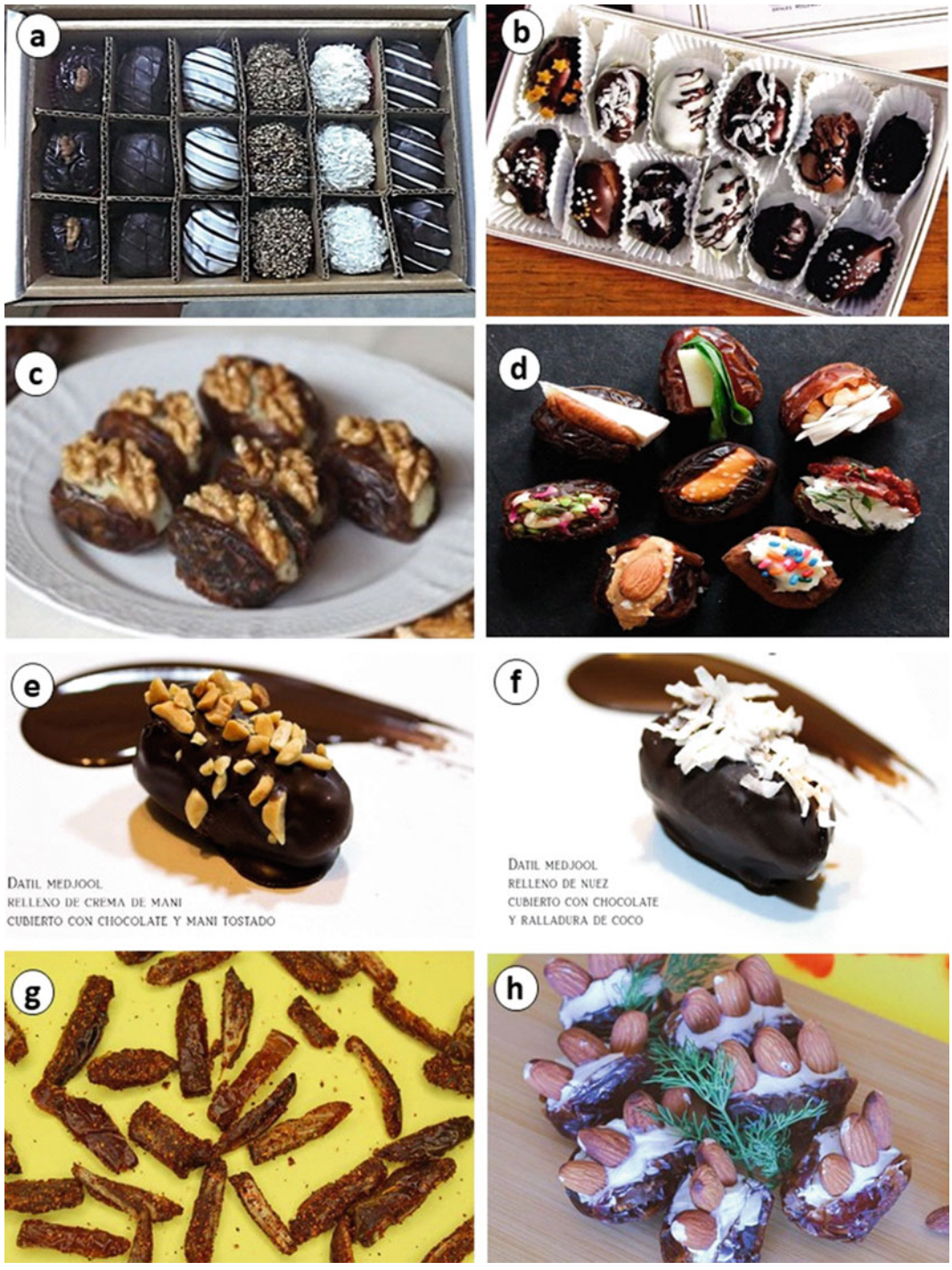

Fig. 1.3 Dates confectionery products. **a**, **b** Various presentations of date covered with chocolate, **c** date stuffed with cheese and nuts, **d** date filled with various options, **e** whole date stuffed with peanut butter covered with chocolate and roasted peanut pieces, **f** whole date stuffed with nuts, covered with chocolate and grated coconut, **g** date pieces with chili powder, **h** date stuffed with a sweet paste and whole almonds. *Source* www.facebook.com

options, for example, hazelnut, oatmeal, squash, sunflower, almond, peanut, pistachio, grated coconut, cranberry, raisin, fig and apple. Its elaboration process consists of washing the fruit in its tamar stage, removing the seed and macerating the pulp to obtain a paste. Once the paste is obtained, it is mixed in a homogeneous mass with the seeds or nuts to taste. The mixture is bonded to the desired thickness with a roller, and the bars are cut to the desired size. Finally, they are wrapped in cellophane and stored in airtight plastic containers (Vijayanand and Kulkarni 2012). It is also possible to find a vast market of date bars and rolls, covered with different types of seeds, nuts and other added ingredients (Fig. 1.4c, d).

Various products that belong to foods designed for breakfast have been identified. These include dates and, with a simple homemade preparation with milk, are ready to eat. These product variants have been developed mainly in the USA, and these commonly consist of cereals, which combine dates with other nuts and seeds (Barreveld 1993).

Another popular representation in date consumption is its combination in foods derived from wheat flour (Fig. 1.5). It is common to find bakery products such as pie, bread, cake, pancake, cookies and shortbreads, in which date paste is included in the bakery product or containing pieces of date inside the food or on the outside as decoration, and can be combined with seeds or nuts. For its preparation, one can combine different proportions of wheat flour dough with date paste (Aleid 2014). The development of this type of food is more homemade or handmade than industrial production (Abd Rabou and Radwan 2017). One form of cookie filling is the date empanada (Fig. 1.5f), which is a typical food in Mexico, where bread is filled

Fig. 1.4 Fortified date bars. **a** Date bars with different seeds, **b** date balls seeds and grated coconut, **c**, **d** commercial alternatives of date bars. *Source* www.Facebook.com

Fig. 1.5 Date products with wheat flour. **a** Dates with nuts, cream and toasted bread, **b** date pie with walnuts, **c** date bread, **d** date cake, **e** date pancake, **f** date empanadas, **g** bread with dates, **h** date shortbread. *Source* www.facebook.com

with date paste (the issue of date paste is discussed in the sweets section).

Studies have been carried out on the use of date paste in bread and cookies, where wheat flour has been replaced with date paste in various proportions (Mathew et al. 2014). The results showed that the bread had a greater volume with the addition of 8% date paste, with 12% the dough becomes sticky distorting the characteristics of a bread, and the sensory test revealed a greater taste for control (i.e., with 0% of date paste) (Barreveld 1993). The case of cookies, a 20% rate (the highest proportion) of date paste, gave the best result, showing a lower cracking tendency in cookies with increasing paste content. Another study replaced sucrose with date paste in breads and cookies, improving their mineral and vitamin content (Mikki et al. 1983).

In Mexico, date production is aimed at the export market as fresh fruits; the processing industry in this fruit is not yet established. However, there is an active development of handmade products that can be found in limited quantities in agricultural markets, agri-food fairs (Fig. 1.6) and high-end stores.

Fig. 1.6 Exhibition of products at a date fair in Mexicali, Mexico. **a** Tasting of date fresh, date bread, date with chili powder and handmade date liquor, **b** fresh date and with various added values (photographs by Ricardo Salomón-Torres)

Also, a variety of dishes and recipes using dates, as well as baked foods, dairy products, cereals or confections, can be found on the following Web sites: https://datesaregreat.com/date-recipe-center/#recipe-grid+p:2 http://www.goldenpalmdates.com/recetas.php.

1.2.3 Date Seeds

According to the variety and the agroclimatic conditions of its cultivation, the date seed can be 6–18% of the total weight of a mature date (Abdul Afiq et al. 2013; Platat et al. 2014; Salomón-Torres et al. 2020). These provide high fiber content, low amounts of sugars and fatty acids, as well as appreciable amounts of minerals (Al-Farsi and Lee 2011; Salomón-Torres et al. 2020). There have been developed from date seeds coffee-like beverages (Ghnimi and Almansoori 2015) (the issue of a coffee-like beverage is discussed in the beverages section); also, thanks to its high fiber content, its use has been suggested in certain processed foods (Sirisena et al. 2015). Date seed is considered an agricultural waste after the consumption of its fruit and an industrial waste after the technological (Al-Hooti et al. 1997) and biological transformation of its fruit (Nancib et al. 1999). However, thanks to its high nutritional content, currently date seed powder is marketed in Saudi Arabia, as a unique powerhouse of minerals, energy and fiber that may help with heart health, blood pressure and digestion (Fig. 1.7a). It has also been used as feed for cattle, horses, goats, sheep, camels and poultry (Adeosun et al. 2016).

The extraction of its oil has become one of the main alternative uses of the date seed. Its lipid content is commonly less than 13%, and its oil is of the oleic–lauric type, since these fatty acids are the most abundant (Devshony et al. 1992; García-González et al. 2019). Due its high resistance and stability to thermal treatment, its use as edible cooking oil (Fig. 1.7b) and for the production of margarine has been suggested (Abdul Afiq et al. 2013). This oil is characterized by having a more color yellow than other vegetable oils because it could be easily preserved because of its high oxidative stability (Basuny and Al-Marzooq 2011).

One possible use of date seed oil is in food products that require a substantial amount of oil, as in the production of mayonnaise. Basuny and Al-Marzooq (2011) evaluated the sensory quality

Fig. 1.7 Date seed products. **a** Date seed powder as powerhouse of minerals and **b** organic date seed oil. *Source* www.facebook.com

of mayonnaise made with date seed oil, against commercial mayonnaise based on corn oil. The parameters evaluated were taste, flavor, color and texture, which were superior to those compared to the control samples. Other potential uses of date seed oil are considered in the cosmetic, pharmaceutical and to a lesser extent food industries (Devshony et al. 1992). Options have been explored for its use in the production of biofuels, particularly for the formulation of biodiesel (Elnajjar et al. 2018).

1.3 Sweets

Although date fruit has not been transformed into products with added value on a large commercial scale, as it has been done with other fruits, it has generated its own industry where a wide range of processed products or by-products has been developed (Siddiq and Greiby 2014). Date pulp is used to prepare concentrated juices, fermented products and pastes for different product uses (Chandrasekaran and Bahkali 2013). Commonly for the elaboration of these products, cull or second-grade dates are used, which are of low quality, too hard, too small, not fully developed or with an unappealing appearance (Aleid 2014; Najib and Al-Yousef 2014). This section describes most of the processed date products, relating their processing, composition, uses and other considerations.

Date Paste One form of preservation of this fruit is its transformation to paste (Fig. 1.8c), which can later be used by the bakery industry, as an ingredient in other products or to produce powder, syrup, candy and other products. It has been suggested that the use of 4–8% of date paste in bread making generates a significant improvement in the rheological properties of the product (Tang et al. 2013). The transformation process consists of using macerated dates (pitted), mixed with predetermined amounts of hot water (95 °C) for 5–15 s or steamed for 3 min, until the pulp of the fruit is converted into a semisolid mass form, which will then be stored (Aleid 2014; Siddiq and Greiby 2014). A great challenge for the producers of date paste is to ensure that the quality of the product is maintained when it is stored, since a common problem with date paste is the degradation of its color, loss of moisture and the increase of its hardness. To maintain a desirable color and prolong the shelf life of the date paste, ascorbic acid or in combination with 0.2% citric acid is generally used (Tang et al. 2013). This will minimize the

Fig. 1.8 Date sweet products in Mexico. **a** Date sugar, **b** date honey (syrup), **c** homemade date paste, **d** date butter (photographs by Ricardo Salomón-Torres)

pH changes of the date paste for 8 and 16 weeks of storage at temperatures of 25 and 5 °C, respectively (Yousif et al. 1991). A moisture content of 20–23% and a water activity < 0.6 are considered as the minimum safety threshold to prevent deterioration by microorganisms, enzymes or non-enzymatic reactions (Aleid 2014). The nutritional content of date paste has been characterized for some cultivars. The Medjool cultivar contains protein (2.12%), fat (1.35%), moisture (34.73%), total sugars (53%), dietary fiber (7%) and total phenolic content (225 mg GAE/100 g FW) (Sánchez-Zapata et al. 2011), while for the Deglet Noor date paste, moisture (5.3%), pH (4.4), acidity (2.93%) and total sugars (61.99%) are reported (Mrabet et al. 2008) and also for Khalas cultivar, moisture (11.2%), total soluble solids (83.2%), protein (2.13%), pH (5.05), acidity (0.38%), total sugars (80%) and crude fiber (2.2%), respectively (Aleid 2014).

Date Syrup, also known in Arabic as *rub al tamr* or *dibis*, is a viscous dark brown liquid, resulting from the interaction between protein and reducing sugars with the high temperature of its elaboration (Vijayanand and Kulkarni 2012). Commonly, low-quality dates are selected for the production of concentrated date syrup, providing

good amounts of fructose and glucose in almost equal amounts and to a lesser extent sucrose (Aleid 2012). Date syrup is the extract of ripe fruits (tamar stage) and is generated for human consumption in baked goods, ice cream and for the production of vinegar, ethanol, single-cell protein and caramel coloring (Roukas and Kotzekidou 1997). Given its high sugar content, it is also used as a sweetening agent, as a substitute for honey (Fig. 1.8b), malt syrups, glucose, maple syrup, molasses and high fructose, as well as in all crystalline forms of sugar (Vijayanand and Kulkarni 2012). The most common process for the production of date syrup is through hot water, which consists of mixing the pulp or paste of the date, with certain proportions of water (commonly pulp-to-water ratio 1:3), adjusting the temperature of the mixture for the required period (Hamza et al. 2015). The juice produced (sugar solution) is passed through a filter to separate solid materials and obtain a clear solution. The solution obtained is heated under vacuum to concentrate it at 70 ºBrix, to prolong its shelf time (Aleid et al. 2015). Citric acid can also be added at a concentration of 0.5% (Vijayanand and Kulkarni 2012). Finally, the viscosity of the syrup can vary at different temperatures, the origin of the cultivar and the concentration of the syrup. Other date syrup production options have been explored with the use of enzyme treatments. Abbès et al. (2011) analyzed the effect of hydrolytic enzymes (pectinase/cellulase) on the physical–chemical characteristics and sensory properties of date syrup. Their results report an increase in reducing sugars, which helps to avoid the phenomenon of crystallization in date syrup. Also, the syrup obtained after the extraction presents a lighter color, being evaluated in the sensory tests. For the traditional syrup extraction in the Deglet Noor, Allig and Kentichi cultivars, there is reported a content of soluble sugars 70, 69 and 62%; reducing sugars 27, 66 and 24%; protein 1.24, 1.31 and 0.97%; total phenols 461, 356 and 400 mg GAE/100 g; pH 4.87, 4.48 and 4.82; acidity 0.27, 0.18 and 0.20; and water activity 0.458, 0.474 and 0.466, respectively (Abbès et al. 2011).

Press Cake A by-product derived from the extraction process of date syrup, which depends on the method of extraction, is made up of approximately 30% of the remaining material, consisting of pulp, with or without seeds (Barreveld 1993). Press cake is used for animal feed and for microbial conversions (Hajian and Hamidi-Esfahani 2015).

Fructose A by-product derived from date syrups is the separation of their sugars, particularly from fructose, because it is the sweetest natural sugar and the ideal dietary sugar, it can be used in high-quality products (Aleid 2006; Siddiq and Greiby 2014).

Date Jam Preserves are products derived from the transformation of a fresh food, and in order to prolong their storage life, natural elements such as sugar, salt and organic acids are used (Barreveld 1993). Date jam is a common form of preserves in this fruit. Its elaboration process consists of using the date pulp of the rutab or tamar stages, mixing it with the same amount of water, adding sugar (sugar-to-date pulp ratio of 55:45) and boiling them for 15–45 min until obtaining a total soluble solid (TSS) density from 60 to 70 ºBrix. Pectin is added 1% concentration to ensure gelation, and the pH is adjusted from 3.0 to 3.5 with citric or tartaric acid (Barreveld 1993; Sidhu 2006; Vijayanand and Kulkarni 2012). The chemical characteristics of date marmalade from Allig, Deglet Noor and Kentichi cultivars were characterized by Besbes et al. (2009), reporting a total sugar content 90, 91 and 82%; reducing sugars 86, 56 and 35%; protein 1.29, 1.39 and 0.84%; total phenols 308, 493 and 190 mg GAE/100 g; pH 4.02, 4.10 and 4.07; dietary fiber 5.90, 4.85 and 13.75%; and water activity 0.734, 0.690 and 0.689, respectively.

Date Jelly Fruit jellies are semisolid, coming from the mixture of fruit juice, pulp and sugar. The preparation of date jelly was reported by Yousif et al. (1990) and consists of using the pulp of this fruit soaked in water with 1:2 ratio at 25 °C for a period of 5 min, and then drained and ground until a paste is produced. Date juice is prepared by adding water to the date paste in a 3:1 (w/w) radius. The mixture is boiled with

gentle stirring for 5 min, after a filtering process to remove the fiber and other impurities. The date juice obtained is boiled with sugar at a 50/50 ratio, until reaching TSS of 65 ºBrix. Ascorbic acid (0.1%) and benzoic acid are added separately and dissolved in small amounts of hot syrup and then added to the boiling mixture. Yousif and Alghamdi (1999) studied the suitability of nine cultivars produced in Saudi Arabia for the production and storage of date jelly. The results show that 50/50 date juice/sugar ratio produces the best jelly. The Sefri and Barni cultivars were identified as the most suitable for the production of date jelly. They also reported that their storage at a temperature of 25 °C for up to 32 weeks did not affect the quality of the product, with the exception of a noticeable change in its sugar composition and a slight change in color. Likewise, Yousif and Alghamdi (1999) reported the chemical properties of date jelly from Barni and Sefri cultivars, with a moisture content of 31.5 and 31.7%; pH 3.11 and 3.1; protein 0.32 and 0.26%; total sugars 58.2 and 53.1%; and ºBrix 66.9 and 64.8, respectively.

Date Butter Date fruits in the tamar stage are also used for the preparation of date butter (Fig. 1.8d), which is similar to peanut butter in its use (Hajian and Hamidi-Esfahani 2015). Its preparation is very similar to the preparation of date jam, with the difference being that the pH is adjusted to 4.5–4.7, the TSS of 74–75 ºBrix, a sugar-to-date pulp ratio of 40:60 and with an activity of water from 0.61 to 0.66 (Vijayanand and Kulkarni 2012).

Date Sugar/Powder This can be produced from date paste or dehydrated dates (Krueger 2015; Sablani et al. 2008). The paste, once diluted with water, dries until it has a moisture content of less than 5%, and then ground to resemble granulated sugar (Hajian and Hamidi-Esfahani 2015). Dried pitted dates are ground directly until a coarse powder is obtained. Date sugar has a sweet caramel flavor much more nuanced than brown sugar, although with a similar appearance (Fig. 1.8a). The high sugar content in the date suggests that it could be developed as a potential source of refined sugar (Samarawira 1983). The presence of fiber and other unsweetened compounds could be present in date sugar up to 50%, which will allow the presence of small grains of sugar that will not dissolve in baked goods or hot liquids, so their use could be limited for specific applications, where this small inconvenience is unimportant (Manickavasagan 2012). Date sugar can be used as a one-to-one replacement for granulated or refined sugar in baking recipes, in confectionery and in certain foods as a sweetening agent. Date sugar is marketed in the USA, and in Mexico it is possible to find it in some markets as an organic product (Ortiz-Uribe et al. 2019). In the Gaza Strip and Palestine, the use of date powder is reported for nutritional, medicinal and hair painting purposes by some women, when mixed with oil (Abd Rabou and Radwan 2017).

Date Candy Candies are preparations of sugar, honey and other natural or artificial sweeteners, which are combined with fruits, seeds, chocolate and other ingredients or flavorings, in different presentations or forms. With the purpose of improving a traditional *arkouy* recipe (Radia et al. 2018), they developed a process to produce natural dietary sweet from date, with 75% date paste, 20% carob powder and 5% olive paste, which could be ingested as a nutritional supplement for any consumer. Their results showed that date candy in a bar shape is viable as a natural dietary sweet of date, having a high acceptance in sensory tests. Also, another process for the generation of date candy has been developed from immature dates, which have a highly astringent property (Vijayanand and Kulkarni 2012).

Date Chutney Chutney is a generic term for a seasoning consisting of a sweet source (fruits), an acid ingredient (such as lemon or vinegar), vegetables and spices (Barreveld 1993). The preparation of date chutney is to boil the chopped fruit for 15 min, then add sugar and seasonings and keep boiling for another 20 min. Another way to prepare it is reported by Al-Hooti et al. (1996), where fruits of the Bushibal, Gash Gaafar, Gash Habash, Lulu and Shahla cultivars, in the kimri and khalal stages, are peeled and sliced, mixed with spices and fried for 25–30 min with mustard

oil. Once the fruit has softened, vinegar is added over medium heat for another 10 min, and then sugar is added, keeping the temperature at 106 °C for a specified time. Finally, potassium sorbate dissolved in water is added to the chutney preparation. In Mexico, it is common to find this product at fairs and exhibitions about dates (Ortiz-Uribe et al. 2019), highlighting the spicy flavor and diversity of chilies such as habanero (*Capsicum chinense* Jacq.) and jalapeno (*C. annuum* L.).

Date Relish This can be produced with dates in tamar stage, where they are cooked with an equal amount of water for 45 min, and in rutab stage, the dates are cut in circular rings. Small pieces of carrots, onions, ginger and dates are cooked over a low flame. Sugar, salt and other seasonings are added and cooked for 20 min. Subsequently added to the mixture are skim milk powder, pectin and acetic acid, cooking at a final temperature of 106 °C, and finally add potassium sorbate, dissolved in a small amount of water, mixing it in the cooked dough (Al-Hooti et al. 1996), being ready for consumption the next day.

Date Pickles In the Arabian Peninsula, pickles are used as an appetizer, they are served with all meals and these can be pickles-in-oil or pickles-in-vinegar. The elaboration process is very similar to that used in other fruits and vegetables. Khatchadourian et al. (1987) reported the processing of date pickles from three cultivars (Nabbut Ghrein, Miskani and Hulwa) in kimri stage, which were washed, peeled, removed seed and steam blanched for 2 min, to be packaged in glass jars. Sugar 20%, initial acetic acid (vinegar 5% acid strength) of 2.0% v/v, was cooked for 15 min at 71 °C. Other components such as table salt (3.0%), mixed spices (1.5% including paprika, cayenne pepper, coriander, ginger, pepper and allspice) and sodium benzoate (0.05%) were added. Finally, it is stored at room temperature for up to 6 months. The green color, size and shape of the kimri stage make pickled dates look similar to olives (Sidhu 2006).

Dates-in-Syrup Fruits in syrup are an essential ingredient in bakery, as well as in the most modern proposals in confectionery. For its elaboration, the dates are peeled and seeded to be packed in glass jars with 50 ºBrix sugar syrup. The pH of the syrup is adjusted to 2.8 with citric acid, and the hot syrup is added to the fruit jars, for sealing and storage (Vijayanand and Kulkarni 2012).

1.4 Beverages

Beverages are an important part in social, political, economic and religious functions of our modern world, as well as of ancient cultures. As food, beverages not only nourish, but are also a means to preserve food, and they can contain medications, induce the alteration of a state of consciousness or be part of some ritual or religious activity (Biwer 2018). Various beverages have been developed taking into account the sugar content in the date as the main nutrient, as well as the aroma, taste, color and quality in general that this fruit gives to the product developed (Barreveld 1993). This section includes the description of most beverages produced from the date fruit.

1.4.1 Fermented

Date Wine Among the fermented products using dates are wine, alcohol, organic acids, baker's yeast, antibiotics and single-cell protein (Ashraf and Hamidi-Esfahani 2011). Wine is a beverage derived from fruits (also some vegetables) where its sugar is transformed into alcohol, in a content that normally is between 11 and 13% (Sivakumar 2002). Wine is best known and associated with grapes, but you can also use other fruits, including dates. In the same way as grapes, with wild yeast on the skin of dates, the fermentation process is promoted, turning the sugars of ripe dates into ethanol:

$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$. (Schorr et al. 2018).

Essentially, the winemaking process is very simple, but for the production of a wine with high quality, requires a good selection of raw material, selection of yeast strains, achieving ideal

conditions for fermentation, considering the effect of added nutrients, as well as a thorough knowledge of each of the processes (Barreveld 1993). For the fermentation process, yeast strains used include *Saccharomyces uvarum*, *S. cerevisiae*, *S. carlsbergensis* and *S. ellipsoideus* (Sivakumar 2002). The strain *S. cerevisiae* var. *ellipsoideus* has been widely used to produce quality wine, since it generates 12% of ethanol, 0.35–0.54% of acidity and pH of 4.0–4.2 (Ashraf and Hamidi-Esfahani 2011).

Bhusari et al. (2013) developed a process to make date wine using S. *cerevisiae* NCIM 3495. Ripe dates were cut into small pieces, adding 20 g of them to 100 ml of distilled water, and after homogenizing the mixture, ammonium nitrate (0.5%) was added. The must was filtered, and the pasteurization process was carried out at 110 °C for 15 min, rapidly cooling to 5 °C. The strain was maintained in a GPYE medium with glucose (0.5%), peptone (0.5%) and yeast extract (0.3%) with a pH of 5.5. The inoculum is prepared in the same must that is used for the final fermentation. Incubation was performed for 21 days at 12 °C and another 8 days at 28 °C. The final alcohol concentration was 5.6%, which is a sign of good fermentative activity. The final result showed that the wine has radical scavenging activity of 74.53% and assailable iron of 40%, likewise, was free of fuselols like amyl alcohol, which prevents aging in the jar.

Palm Wine It is an alcoholic beverage created from the sap of various palm species such as date, oil and coconut. It is a handmade product that is obtained through the fermentation of the sap that is extracted from the crown of the palm. The sap in the date palm is a white liquid that tends to be very sweet and alcohol-free (Fig. 1.10e), with which a sweet beverage known as neera is prepared in India (Pareek 2015). Due to the natural yeasts in the air, the sap of the palm begins to ferment quickly after its collection, generating in two hours an aromatic wine with up to 5% alcohol (Barreveld 1993). Palm wine can be distilled to create a stronger beverage, known by different names depending on the region that produces it. Excessive extraction of sap in the date palm significantly reduces its fruit yield and can cause death in the short term.

Date Liquor Liquors are alcoholic beverages that are obtained through distillation processes. These are usually brightly colored and flavored, with a sweet and strong flavor, which usually hides their high alcohol content, which can vary between 27 and 55%. Since date fruit is mostly produced in Muslim countries, where alcohol intake is prohibited, the possibility of commercial generation of a date liquor is very small (Aleid 2014). However, in Western countries it is possible to find handmade liquor produced. Being a homemade product, its preparation and additions vary greatly according to its manufacturer. Commonly, this process involves mixing dates with distilled water and yeast, allowing it to ferment for several days, filtering its solids, to finally distill the liquid. In northwest Mexico, homemade liquor is produced, promoting its consumption as a digestive agent (Fig. 1.9a, b).

Date Beer There is a record of the use of dates for the preparation of at least 17 types of beer in ancient Egypt, where the date palm fruit was used as a beer sweetener (Shawky and El-Sharabasy 2015). In beer, commonly the percentage of alcohol is 14–18%. Currently, there is little or no published documentation on the production of date beer. However, in several countries, this beverage is produced in homemade and industrial way. For example, in Spain a date beer is produced as a special edition of winter beer, consisting of dates, juniper berries, five types of malt and sugars, among others, giving it the slight sweetness of dates. In northwestern Mexico, a group of micro-entrepreneurs make homemade beer based on dates, corn and citrus in dark and light versions. In Arizona, USA, a date beer is made, with a bitter touch, caramel flavor, figs and spicy raisins, where only until the end of its intake, does the palate recognize its date flavor.

Date Alcohol The fermentation of fruit sugars is a biochemical process where sugars are converted into ethanol and carbon dioxide by the action of some enzymes. Alcohol can be used in beverages, fuels and medicinal purposes, since it is produced in different purities (Sivakumar

Fig. 1.9 Fermented and non-fermented beverages. **a**, **b** Various presentations of handmade liquor produced in Mexico, **c** coffee-like or infusion of date seed, **d** Medjool date juice, **e** smoothie with dates and banana, **f** date champurrado (traditional Mexican hot beverage) (Figures **a**, **b**, **c**, photographs by Ricardo Salomón-Torres; **d**, **e**, **f**, www.facebook.com)

2002). The alcohol produced during the elaboration of date wine can become an objective product, when separated from the fermented liquor (Barreveld 1993). The production of 95% (v/v) of date alcohol involves, after fermentation, a distillation and rectification process (Sivakumar 2002). Date-distilled alcohol has been used in beverages such as arrak and mahia, resulting in beverages with delicate anise flavor and the balance that gives the date liquor. Likewise, proposals for the development of brandy and vodka have been developed, where the date flavor will be part of the aroma of the final product (Barreveld 1993).

1.4.2 Non-fermented

Date Vinegar Vinegar is a product of the activity of bacteria that generate the chemical reaction of the oxidation of ethyl alcohol (ethanol) to acetic acid (vinegar):

$C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O$ (Schorr et al. 2018)

Acetic acid is possibly the organic acid that causes one of the oldest known chemical reactions, which is vinegar production (Sivakumar 2002). For this to happen, there must be the appropriate conditions of acidity, concentration of alcohol and nutrients in the wine. Date vinegar

generation involves two major processes: first, the transformation of sugar to alcohol by *Saccharomyces* sp. and the second, the conversion of alcohol to acetic acid by *Acetobacter* sp. Date vinegar is dark and fruity and can be used as a substitute for balsamic vinegar (Siddiq and Greiby 2014).

Date Coffee This is a coffee-like beverage prepared from date seeds. Its preparation consists of washing, drying, roasting and grinding the seeds. This infused material has a pleasant, light-colored taste, is free of caffeine, does not cause acidity and may contribute to the elimination of excess weight in the human body (Shi et al. 2014; Abd Rabou and Radwan 2017). The quality characteristics of this beverage against a traditional Arabian coffee were evaluated by Ghnimi and Almansoori (2015). It was found that it has a smaller amount of total phenolic compounds and with less antioxidant power than Arabic coffee. The phytochemical evaluation revealed that date seed contains steroids, tannins and coumarins, while the presence of caffeine, terpenoids, saponins, alkaloids, anthraquinones and anthocyanins was not detected. The identification of minerals is within the ranges contained for an Arabic coffee. Other study suggests the combination of coffee-like and ginger, as a healthy beverage alternative to traditional, a low-cost coffee, which can be mixed with honey and/or milk to improve its flavor (Abdillah and Andriani 2012). In Mexico, this beverage is marketed as an infusion of date seed (Fig. 1.9c).

Homemade Beverages A popular beverage is date juice, obtained by liquefying 200 g of dates in one liter of water, then filtering solid waste and enjoying it with a touch of lemon and ice to taste (Fig. 1.9d). Date shake, commonly combined with banana, is a beverage made with milk, nuts or almonds and cinnamon powder. Two to three dates, a banana and 150 ml of milk are liquefied to generate this beverage (Fig. 1.9e). Atole and champurrado are two traditional winter beverages in Mexico, and these are commonly prepared with corn dough, milk, chocolate, sugar, cinnamon and other ingredients. Recently in the northwest of Mexico (dates are grown), these beverages are being prepared with the date pulp, to replace sugar, known as *date atole* and *date champurrado* (Fig. 1.9f).

Commercial Options Several commercial beverage products have recently appeared, highlighting the beverages produced based on Barhee and Medjool cultivar dates. Some examples of beverages in aluminum cans: one marketed as a date energy drink and a carbonated beverage labeled as date soda (Fig. 1.10a). These beverages have a broad acceptance among the consumer market and opening possibilities of generating new beverages based on the date fruit. There is also a drink made with powdered date, ready to be dissolved in water to prepare date juice (Fig. 1.10b). A common drink is date juice, which is sold in plastic or glass bottles (Fig. 1.10c, d). From the extraction of the sap in the date palm, a sweet drink is prepared, which is common in countries of the Middle East and North Africa (Fig. 1.10e).

1.5 Conclusions and Prospects

The date palm fruit is consumed mostly fresh or dried, with little or no processing. Each year, its production increases and over time there is more research on this fruit, highlighting its health benefits as a functional food. Its high sugar content makes this fruit an ideal ingredient for inclusion in a wide variety of foods and beverages. Dates are being used in gourmet dish, to give a contrast of sweet taste in food. In the absence of a culture of direct consumption and to make its consumption more attractive, in various Western countries, the fruit has been given an added value, covering it with chocolate and various seeds, filling it with cheese, peanut butter and other ingredients. Likewise, an entire industry is expanding with processing this fruit into paste, jam, jelly, syrup, butter, bars, sugar, pickles, candy, among others, which will generate consumption by accompanying it with other foods and being a substitute for other ingredients,

Fig. 1.10 Various non-fermented date beverages. **a** Energy drink and carbonated beverage developed with Barhee dates, **b** date powder to prepare a date beverage, **c**, **d** date juice produced with Barhee dates, **e** date palm sap drink. *Source* www.facebook.com

mainly in the bakery and confectionery industry. Another way of consuming this food is in the form of a drink, since there is a great diversity of fermented and unfermented date beverages, which are produced according to the culture of each country, to satisfy local tastes.

As the date is a functional food, there must be greater dissemination about the great benefits to human health that its consumption generates. This will allow it not only to be an exotic fruit in Western countries and become an indispensable food in the daily diet.

Finally, the expansion of studies derived from the sequencing of its genome could allow the generation of genetically improved cultivars, which induce the production of more and better fruits, for the elaboration of foods with greater nutritional properties.

Appendix: Nutritious Easy Homemade Recipes of Dates

Ingredients	Method	Images
Date balls (laddoos)		
1. Dates 500 g 2. Pistachio 150 g 3. Cashew nuts 150 g 4. Almonds 200 g 5. Walnuts 150 g 6 Pumpkin seeds 50 g 7. Flax seeds 50 g 8. Butter 20 g	1. Grind all nuts and seeds mildly in a grinder 2. Roast nuts on low heat and keep aside 3. Grind dates 4. Melt butter in a pan on a low heat 5. Mix well all ingredients for 5 min 6. Let them cool for 5 min 7. Form the mixture into small balls	
Date milk shake		
1. Dates 100 g 2. Cold milk 500 ml 3. Cashew 5 g 4. Almonds 5 g 5. Cinnamon powder 5 g	1. Soak dates for 2 h and blend them smoothly for 5 min. Other option is avoid soaking dates and blend for 8–10 min 2. Add 500 ml cold milk, dry fruits and cinnamon powder in a blender smoothly for 5 min 3. Garnish it with chopped dates in small pieces 4. Add 1 or 2 scoops of ice cream (optional)	

Courtesy of Anita Jain and Bella Jain, Mumbai, India

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Date Palm Bioactive Compounds: Nutraceuticals, Functional Nutrients, and Pharmaceuticals

2

Ali H. El-Far, Rokaia F. Ragab, and Shaker A. Mousa

Abstract

Date palm (*Phoenix dactylifera* L.) products have been widely consumed for thousands of years in Islamic countries. It has many varieties in different countries including Ajwa, Bouskri, Bousrdon, Bousthammi, Boufgous, Jihl, and Medjool. Date palm is a useful traditional medicinal plant containing numerous bioactive compounds that have free radical scavenging, antioxidant, antimutagenic, antimicrobial, anti-inflammatory, antihyperlipidemic, gastroprotective, hepatoprotective, nephroprotective, anti-cancer, and immunostimulant activities. These biological activities are due to the nutritive and bioactive compounds that are present in its fruits, seeds, pollen, and other parts. *Phoenix dactylifera* contains fatty acids, amino acids, proteins, and steroidal substances of nutritive value. Also, it contains many valuable phytochemical constituents like phenolics, flavonoids, and carotenoids. More research is needed to investigate the pharmacological potentials of *P. dactylifera* bioactive constituents either in free- or in nano-forms. Therefore, we encourage researchers to investigate the molecular and biochemical effects of *P. dactylifera* supplementation on reproduction, hormonal homeostasis, intestinal nutrient transport proteins, apoptosis, and antiapoptotic molecules. In addition, the effect of *P. dactylifera* supplementation on glucose uptake by peripheral tissues with evaluation of glucokinase and hexokinase genes and protein expression is the main future recommendation of our study, in addition to the investigation of gluconeogenic, glycogenic, and glycogenolytic enzymes gene and protein expression. Finally, we encourage researchers to further determine the mechanisms by which *P. dactylifera* induces its biological activities.

2.1 Introduction

It is patently obvious that nature has many medicinal plants with numerous active antitoxic agents that include phytochemicals with powerful pharmacological activities (Ekor 2014). The World Health Organization estimates that up to 80% of humans still rely on traditional medicines (Yadav et al. 2014).

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J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_2

Phoenix dactylifera L. is a versatile common woody herb in the family Arecaceae. The genus *Phoenix* consists of 14 species, including *P. dactylifera* which has been cultivated for at least 6000 years in the Middle East (Copley et al. 2001; Sirisena et al. 2015). Its phytochemical investigation has revealed that the fruits contain anthocyanins, phenolics, sterols, carotenoids, procyanidins and flavonoids, compounds are known to possess free radical scavenging, antioxidant, antimutagenic, antimicrobial, anti-inflammatory, antihyperlipidemic, gastroprotective, hepatoprotective, nephroprotective, anticancer, and immunostimulant activities (Fig. 2.1) (Baliga et al. 2011; El-Far et al. 2016b). Date palm products such as fruits, seeds, pollen, leaves and sap are beneficial to humans, e.g., pharmacology, and to animals. It is desirable for researchers to review the different pharmacological potentials of *P. dactylifera* products. A previous study showed antioxidant and immunestimulant activities of date palm seeds as a feed additive for broiler chickens (El-Far et al. 2016a).

Date palm products are widely consumed food in Islamic countries due to its history of some 6000 years. Ajwa is a date variety grown in Medina, Saudi Arabia which is mentioned by the Prophet as he said *He who ate seven dates (of the land situated) between these two lava plains in the morning, no poison will harm him until it is evening* (Siddiqui 2009).

Date palm has numerous biological activities in addition to its nutritional value. This chapter discusses the biological potentials in detail and directs researchers toward some future research recommendations concerning this palm.

2.2 Active Ingredients

Date palm fruit can be considered as a complete food in addition to its curative effects against numerous health conditions. It is considered a complete diet because it contains different fatty acids, amino acids, proteins and steroidal substances (Ali et al. 2014; Assirey 2015; Boudries et al. 2007; Boukouada and Yousfi 2009). The fruit consists of 76.3% as an average content of sugar of its dry matter, while average protein and lipid contents are 3.23 and 0.21%, respectively, as represented in Table 2.1 (Assirey 2015).

Dates contain phytochemicals like phenolics, flavonoids and carotenoids, which have anticancer and antioxidant properties (Fig. 2.2) (Al-Farsi et al. 2005; Al-Rimawi and Odeh 2015; Kchaou et al. 2014; Naik and Al-Khayri 2016, 2017, 2018, 2020; Shareef and Al-Khayri 2020). The date fruit is believed to have antioxidant properties not only because of food, vitamins and minerals, but also because of a variety of secondary plant metabolites such as flavonoids and phenolics (Benmeddour et al. 2013; Vayalil

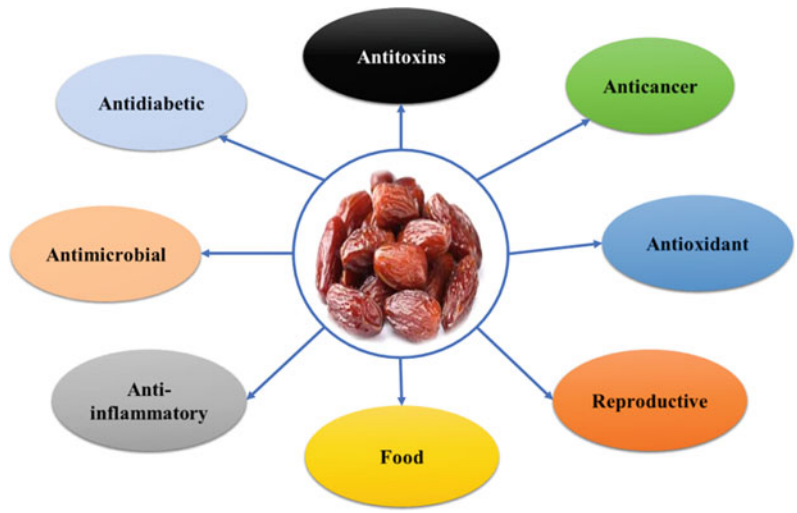

Fig. 2.1 Biological activities of *Phoenix dactylifera* (Figure constructed by Ali H. El-Far)

Table 2.1 Chemical composition (g/100 g dry weight) of flesh dates of 10 varieties

Date variety	Chemical composition			
	Moisture	Protein	Lipid	Ash
Ajwa	22.8 ± 0.1^{ab}	2.91 ± 0.02^{b}	0.47 ± 0.001^{b}	3.43 ± 0.01^{a}
Shalaby	15.2 ± 0.2^{c}	4.73 ± 0.01^{a}	0.33 ± 0.005^{c}	3.39 ± 0.01^{a}
Khodari	19.5 ± 0.1^{b}	3.42 ± 0.03^{a}	0.18 ± 0.004^{d}	3.42 ± 0.04^{a}
Anabarah	29.5 ± 0.2^{a}	3.49 ± 0.01^{a}	0.51 ± 0.004^{a}	2.33 ± 0.01^{b}
Sukkari	21.2 ± 0.1^{b}	2.76 ± 0.01^{b}	0.52 ± 0.001^{a}	2.37 ± 0.05^{b}
Suqaey	14.5 ± 0.1^{c}	2.73 ± 0.04^{b}	0.41 ± 0.005^{a}	2.29 ± 0.03^{b}
Safawy	23.6 ± 0.3^{ab}	2.48 ± 0.02^{b}	0.12 ± 0.003^{d}	1.68 ± 0.01^{d}
Burni	24.4 ± 0.1^{a}	2.50 ± 0.04^{b}	0.67 ± 0.001^{a}	2.02 ± 0.01^{c}
Labanah	10.5 ± 0.1^{d}	3.87 ± 0.05^{a}	0.72 ± 0.002^{a}	3.94 ± 0.02^{a}
Mabroom	21.3 ± 0.1^{b}	1.72 ± 0.05^{c}	0.27 ± 0.001^{c}	2.79 ± 0.05^{a}

Each value represents the mean ± SE
Means with different letters *a*, *b*, *c*, and *d* are significantly different
Source Adopted from Assirey (2015) an open-access article distributed under the Creative Commons Attribution License which permits unrestricted use

2012). Also, the polyphenolic proanthocyanidins may act in combination with other phenolics as free radical scavengers or heavy metal chelators, and, in turn, they can prevent oxidative stress and inflammation (Bladé et al. 2016).

Dates contain considerable amounts of vitamins of water and fat-soluble origin that are very important for vitality. Moreover, they contain vitamins of powerful antioxidant potentials that are capable of chelating different radicals in nonenzymatic reactions such as vitamins A, C, and E (Al-shahib and Marshall 2003; Vayalil 2012), as well as quantities of macro- and micronutrients that have a significant role in many biological functions (Ahmed et al. 1995; Al-Hooti et al. 1997; Ismail et al. 2006). Selenium, copper, zinc, and manganese are of great importance for antioxidant metalloenzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Adelekan and Thurnham 1998).

2.3 Biological Activities

2.3.1 Antioxidant Activity

Studies of the antioxidant effect of dates have shown that oxidative stress induced by reactive oxygen species (ROS) and reactive nitrogen species reduce the body's antioxidant defense system and lead to cellular oxidative damage, as listed in Table 2.2 and shown in Fig. 2.3. *Phoenix dactylifera* has shown antioxidant activities in the Trolox equivalent antioxidant capacity (TEAC) test, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS+) assay, and the ferric reducing/antioxidant power method (FRAP assay) (Biglari et al. 2008). Djaoudene et al. (2019) evaluated antioxidant activity with the xanthine/xanthine oxidase system in a dose-dependent manner. They demonstrated that Tazarzeit and Tazizaout extracts (seed of two date cultivars) were more efficient due to superoxide radical scavenger (IC50 = 9.08 µg/mL) when compared with other types of date seeds because of their abilities to interfere with the formation and propagation of free radicals and protect low-density lipoproteins from oxidation (Habib et al. 2014). El Abed et al. (2018) described the antioxidant effect of the aqueous ethanolic extract of date palm parthenocarpic fruits from 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity with an IC50 value of 0.15 ± 0.011 mg of dry extract/mL and scavenged 94% of superoxide radicals at 0.6 ± 0.03 mg/mL (Naskar et al. 2010). In addition, dates contain a high

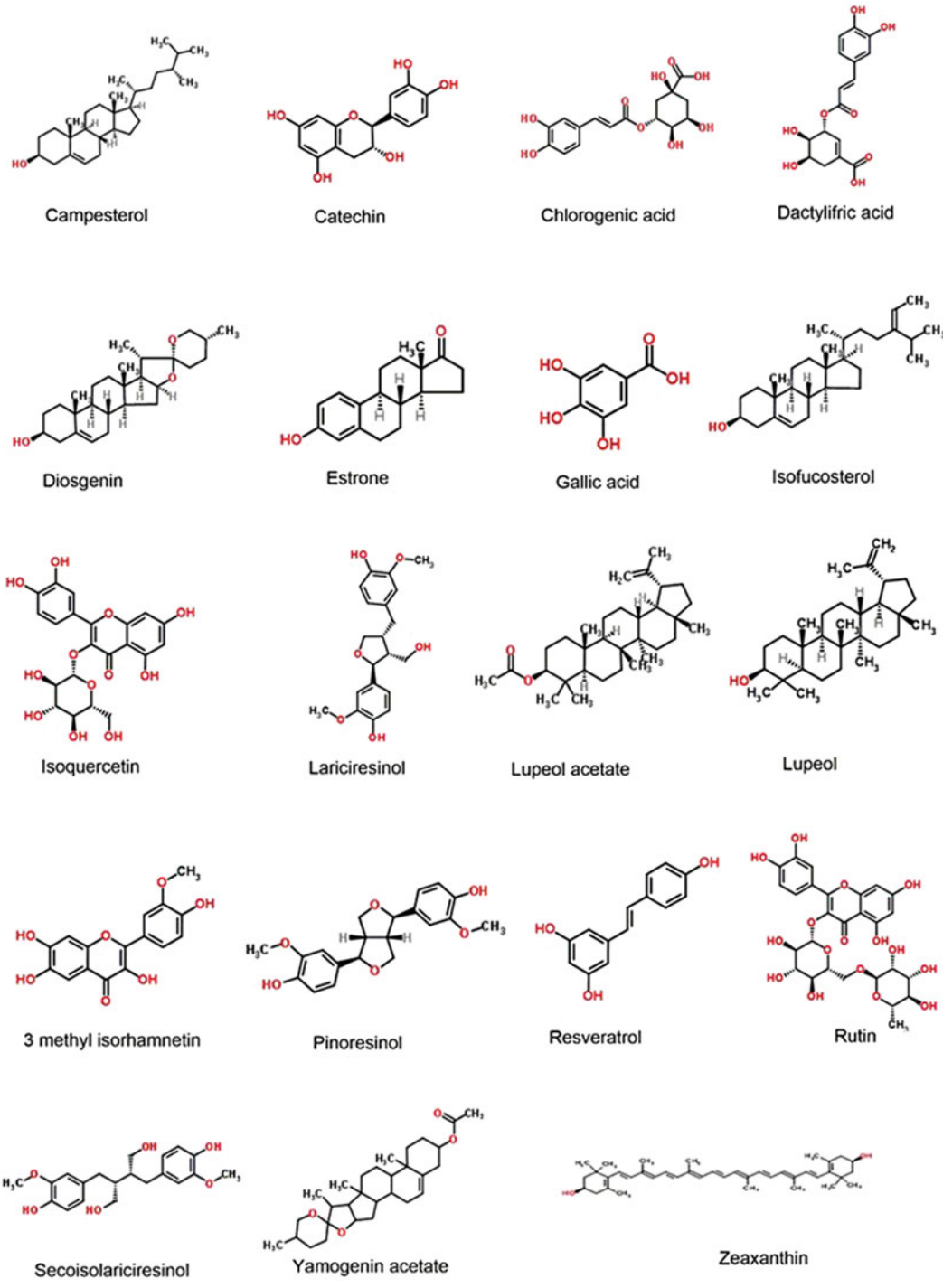

Fig. 2.2 The structures of some secondary metabolites characterized in *P. dactylifera*. *Source* El-Far et al. (2019)

Table 2.2 Antioxidant activity of *Phoenix dactylifera*

Extracts	Study type	Dose/route	Conclusion/mechanism	References
Methanolic and water extracts of *P. dactylifera* pollen	In vivo	500 mg/day/orally	• Normalizing the plasma level of antioxidants	Mohamed and Al-Okbi (2004)
Hydro-alcoholic extract	In vitro		• Trolox assay • $ABTS^{+}$ assay • FRAP assay	Biglari et al. (2008)
Aqueous extract of *P. dactylifera*	In vitro		• FRAP assay • Free radical scavenging activity	Hasan et al. (2010)
Ethanolic extract of *P. dactylifera*	In vivo	Adult male Wistar rats 40 mg extract/kg/orally once daily for 56 consecutive days	• Prevention of oxidative damage to testicular tissues	El-Neweshy et al. (2013)
Aqueous suspensions of raw or roasted *P. dactylifera* seeds	In vivo	Oral dose of 1 g/kg/day	• Ameliorates oxidative damage	Abdelaziz and Ali (2014)
Aqueous extract of *P. dactylifera* fruit	In vivo	Combinations of aqueous date extract (ADE) and trichloroacetic acid (TCA) by several doses/orally	• Potentiates CAT and GPx activities	El Arem et al. (2014)
Aqueous suspension of *P. dactylifera* seeds	In vivo	1 g/kg/day/orally	• Protective effects against early diabetic complications due to the reduction in TBARS and NO levels	Abdelaziz et al. (2015)
Lyophilized extract of *P. dactylifera* seeds	In vivo	250 and 500 mg/kg/orally	• Potentiation of SOD and CAT • Decreasing MDA	Al-Yahya et al. (2016)
Methanolic extract of *P. dactylifera* barks	In vitro		• Antioxidant status improvement	Siahpoosh and Soleimani (2016)
Methanol and acetone *P. dactylifera* extracts	In vitro		• Total anthocyanin content	Samad et al. (2016)
P. dactylifera methanolic extract	In vivo	2.0%, 4.0%, 6.0% of *P. dactylifera* crushed seeds per day orally	• Antioxidant status improvement	El-Far et al. (2016a)
Aqueous *P. dactylifera* extract	In vivo	200 mL/kg orally	• Improves common carp antioxidant defenses, growth-related genes, and immune system	Hoseinifar et al. (2017)

(continued)

Table 2.2 (continued)

Extracts	Study type	Dose/route	Conclusion/mechanism	References
P. dactylifera seeds	In vivo	60, 120, and 180 g/kg per day orally	• Improve antioxidant activity in milk and blood of a dairy goat	Sharifi et al. (2017)
Tazarzeit and Tazizaout extract (date seed)	In vitro		• Inhibition formation of free radicals • Prevent LDL from oxidation • Superoxide radical scavenger	Djaoudene et al. (2019)

ABTS—2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid, FRAP—Ferric reducing/antioxidant power, CAT— Catalase, GPx—Glutathione peroxidase, TBARS— Thiobarbituric acid reactive substances, NO—Nitric oxide, SOD—Superoxide dismutase, MDA—Malondialdehyde, LDL—Low-density lipoproteins

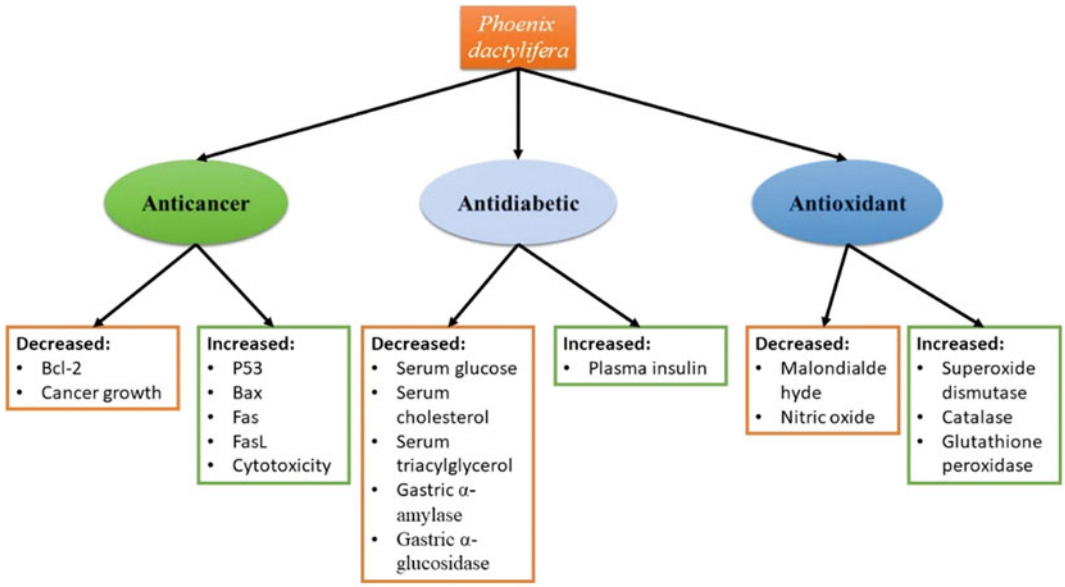

Fig. 2.3 Antioxidant, antidiabetic and anticancer activities of *Phoenix dactylifera* (Figure constructed by Ali H. El-Far)

percentage of vitamins C, A, and E, plus a high total phenolic content (Hasan et al. 2010; Samad et al. 2016). These results of in vitro studies have encouraged researchers to investigate the antioxidant activity of *P. dactylifera* extracts in vivo against different toxicants such as carbon tetrachloride (CCl_4), isoproterenol, cadmium and streptozotocin-induced diabetic rats (Abdelaziz and Ali 2014; Abdelaziz et al. 2015; Al-Yahya et al. 2016; El-Neweshy et al. 2013). The defensive actions of *P. dactylifera* may be correlated with the activation of catalase (CAT), SOD, GPx, glutathione reductase (GR) and glutathione S-transferase (GST), with major reduction in the malondialdehyde (MDA) (Al-Yahya et al. 2016; Baliga et al. 2011; El-Far et al. 2016a; Hoseinifar et al. 2017; Sharifi et al. 2017). In conclusion, dates are a good antioxidant food.

2.3.2 Antidiabetic, Antihyperglycemic, and Antihyperlipidemic Activities

Four studies have dealt with the antidiabetic activity of *Phoenix dactylifera* (Table 2.3 and Fig. 2.3). Some studies showed the antidiabetic effect of dates through elevation of serum insulin in alloxan-induced diabetic rat with normalization of plasma glucose, triacylglycerol, and cholesterol (Mard et al. 2010). Whether the potency of safe β cells or the recovery of alloxan-injured cells improves is attributed to a clear explanation for plasma insulin increases.

Inhibition of either α-glucosidase or α-amylase in vitro can delay carbohydrate digestion and absorption, leading to plasma glucose level normalization and is one of the antihyperglycemic effects of dates. Such an effect was induced by *Phoenix dactylifera* leaf hydro-alcoholic extract (Chakroun et al. 2016) and seed aqueous extract (Khan et al. 2016b). In addition, Hasan and Mohieldein (2016) treated streptozotocin-induced diabetic rats with 10 mL of date seed aqueous extract per day for each rat. The tested extract significantly reverted the elevated serum glucose, cholesterol, and triacylglycerol levels of the diabetic rats to near normal values.

2.3.3 Anticancer Activity

Constituents of *Phoenix dactylifera* have shown antitumor activity, as listed in Table 2.4 and Fig. 2.3. In vitro trials were done to determine the anticancer activity of *P. dactylifera* extracts towards different cancer cell lines such as human epithelial colorectal adenocarcinoma (Caco-2) (Eid et al. 2014) and the human melanoma-derived cell line (IGR-39) (Chakroun et al. 2016). Khan et al. (2016a) demonstrated inhibition of the development of human breast adenocarcinoma by methanol extract from Ajwa variety dates (15 and 20 mg/mL) through upregulation of proapoptotic molecules, p53, Bcl-2-associated X protein (Bax), Fas and Fas ligand (FasL) along with downregulation of B-cell lymphoma 2 (Bcl-2). The antitumor function

Table 2.3 Antidiabetic, antihyperglycemic, and antihyperlipidemic activities of *Phoenix dactylifera*

P. dactylifera/ extracts	Study type	Cell lines/animal	Dose/route	Conclusion/mechanism	References
P. dactylifera leaves extract (PDE) and its fractions	In vivo	Alloxan-induced diabetic rats	200 and 400 mg/kg	• Reduction in elevated serum glucose, cholesterol, and triacylglycerol. • Increase plasma insulin	Mard et al. (2010)
P. dactylifera leaves hydro-alcoholic extract	In vivo	Alloxan-induced diabetic mice	20 mg/kg/28 days Orally	• Antihyperglycemic	Chakroun et al. (2016)
P. dactylifera leaves hydro-alcoholic extract	In vitro	α-glucosidase and α-amylase	Several doses	• Inhibit α-glucosidase and α-amylase	
P. dactylifera seeds aqueous extract	In vivo	Streptozotocin-induced diabetic rats	10 mL/day/rat Stock prepared from 100 g/L	• Normalized serum glucose, cholesterol, and triacylglycerol levels	Hasan and Mohieldein (2016)
P. dactylifera seeds aqueous extract	In vitro	α-glucosidase and α-amylase	5 mg/mL	• Inhibit α-glucosidase and α-amylase	Khan et al. (2016b)

Table 2.4 Anticancer activity of *Phoenix dactylifera*

Extracts	Study type	Cell lines/animal	Dose/route	Conclusion/ Mechanism	References
(1/3)-β-D-glucan from Libyan dates	In vivo	Female CD1 mice	1 mg of (1/3)-β-D-glucan/kg intramuscular injection	• Tumors showed complete regression by day 30	Ishurd and Kennedy (2005)
Freeze-dried form of *P. dactylifera* fruits	In vivo			• Prevention of DMBA-induced mammary cancer	Al-Sayyed et al. (2013)
P. dactylifera pollen	In vivo	Human (head and neck cancers)	2 g pollen daily for 42 days/orally	• Blocking the oxidative free radicals and preventing DNA damage	Elkerm and Tawashi (2014)
Digested date extract Date polyphenol extract	In vitro	Caco-2 cell line	0.2 mg/mL for 48 h exposure	• Caco-2 growth inhibition	Eid et al. (2014, 2015)
Leaves hydro-alcoholic extract	In vitro	IGR-39 cell line	35 and 75 μg/mL	• Cytotoxicity confirmed MTT assay	Chakroun et al. (2016)
Methanolic extract of Ajwa date	In vitro	MCF7 cell line	15 mg/mL and 20 mg/mL	• Upregulation of p53, Bax, Fas, and FasL • Downregulation of Bcl-2	Khan et al. (2016a)
Ethyl acetate extract of date fruit	In vitro	Pancreatic stellate cells		• Antifibrotic agents and active compounds of date fruit could be potent natural anticancer agent	Al Alawi et al. (2020)

DMBA—2,4-Dimethoxybenzaldehyde, Caco-2—Human epithelial colorectal adenocarcinoma cell line, IGR-39—human melanoma-derived cell line, MTT—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, MCF7—human breast adenocarcinoma, Bax—Bcl-2-associated X protein, p53—Tumor protein p53, Bcl-2—B-cell lymphoma 2

of isolated glucans from date products was shown because (1-3)-β-d-glucans exhibit antitumor activity (Ishurd and Kennedy 2005) and were potent against the growth of sarcoma-180 solid tumors implanted in mice (Ishurd et al. 2003). Date fruit extract inhibited the growth of Caco-2 cells. These data suggest that regular consumption of date fruit may reduce colon cancer risk (Eid et al. 2014, 2015). Recently, Al Alawi et al. (2020) demonstrated that an ethyl acetate extract of date fruit reduced the fibrosis of pancreatic stellate cells significantly and acted very well as antifibrotic agents and the active compounds of date fruit could be potent natural anticancer agents.

2.3.4 Anti-inflammatory Activity

The anti-inflammatory effects of *Phoenix dactylifera* were shown as a reduction of paw edema volume (Ali Haimoud et al. 2016), wound healing activities (Abdennabi et al. 2016) and cardioprotection (Al-Yahya et al. 2016). In line with these facts, the anti-inflammatory properties of *P. dactylifera* are summarized in Table 2.5 and illustrated in Fig. 2.4.

Al-Qarawi et al (2005) found that aqueous and ethanolic undialyzed and dialyzed extracts from the fruits of *Phoenix dactylifera* minimized the frequency of gastric ulceration and the rise of histamine and gastrin, and lowered gastric mucin

Table 2.5 Anti-inflammatory activity of *Phoenix dactylifera*

Extracts	Study type	Dose/route	Conclusion/ mechanism	References
Pollen suspension	In vivo	250, 500, and 1000 mg/kg orally	• Anti-inflammatory cytokine expression	Elberry et al. (2011)
Lyophilized pollen extract	In vivo	150, 300, and 600 mg/kg orally		
Khodary date fruit aqueous extract	In vivo	20% of *P. dactylifera* aqueous extract in a dose of 4 mL/kg for 4 successive days	• *P. dactylifera* fruit can protect against coccidiosis-induced inflammation	Metwaly et al. (2012)
Methanolic extract of *P. dactylifera* fruits	In vivo	30, 100, and 300 mg/kg/orally	• Reversing alterations in the biochemical parameters of the brain	Pujari et al. (2013)
Aqueous extract	In vivo	Orally, 300–500 mg of aqueous extract of *P. dactylifera* for 3 weeks.	• Analgesic effect of the extract could be due to the presence of micronutrients in the *P. dactylifera* fruit especially vitamins C and E	Maryam et al. (2015)
Water or methanol extracts	In vitro	Variable inhibition %	• Inhibit production of prostaglandins and thromboxane	Zhang et al. (2017)
Aqueous extract	In vitro cardioprotective activity assay In vivo hemodynamics	250 mg/kg/day or 500 mg/kg/day orally for 21 days	• Anti-inflammatory potential against experimental myocardial damage	Al-Yahya et al. (2016)
Methanolic extracts	In vivo	250/kg orally	• Significant reduction of paw edema volume	Ali Haimoud et al. (2016)
Sap extracted from beser *P. dactylifera*	In vivo	Animals treated topically with a *P. dactylifera* sap twice daily	• Increasing wound healing activity	Abdennabi et al. (2016)
Aqueous ethanolic extract from *P. dactylifera*	In vitro	Dose-dependent (0–200 µg of dry extract/mL)	• Inhibit Phospholipase A2 Activity (anti-inflammatory action)	El Abed et al. (2018)
	In vivo	200 mg/kg		
date seed extracts	In vivo	Dose-dependent	• Protein denaturation inhibition • Lysosomal membranes stabilization • Nitric oxide free radical scavenging ability • C-reactive protein and fibrinogen production inhibition	Bouhlali et al. (2020)

LDH—Lactate dehydrogenase

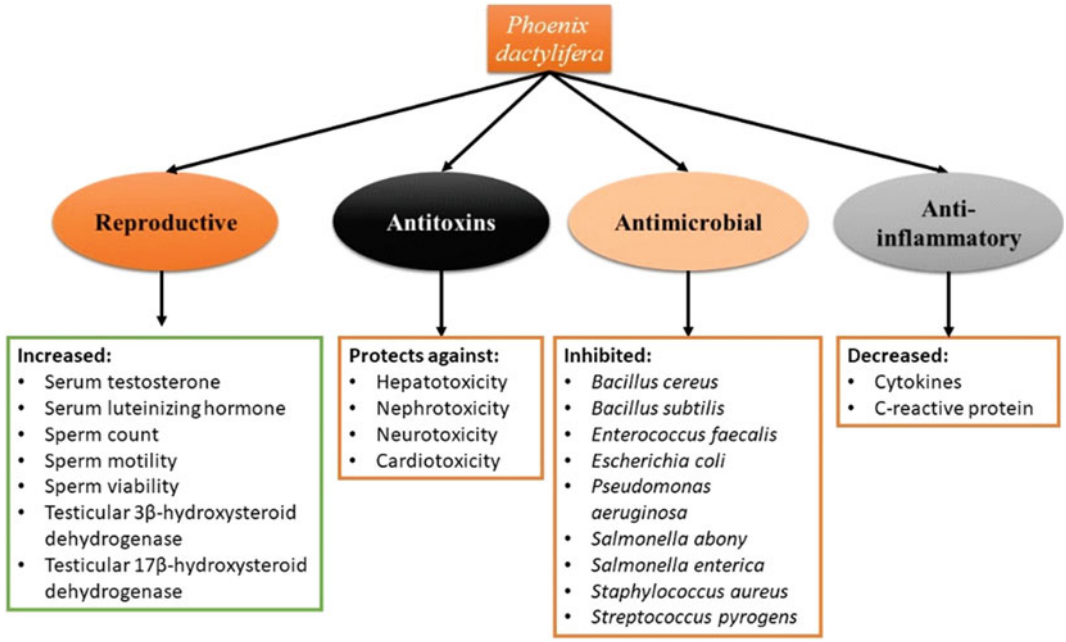

Fig. 2.4 Ant-inflammatory, antimicrobial, and antitoxins activities of *Phoenix dactylifera* (Figure constructed by Ali H. El-Far)

levels in ethanol-induced gastric ulceration. Furthermore, the anti-inflammatory ability of the extract may be due to the antioxidant action of the extract.

The phenolics and flavonoids content could inhibit the formation of prostaglandin endoperoxide, leading to termination of inflammation mediators like prostaglandins and thromboxane (Zhang et al. 2013). Ali Haimoud et al. (2016) tested the anti-inflammatory function of *Phoenix dactylifera* fruit methanol extracts produced in Algeria. The experimental model of carrageen-induced acute paw edema in Swiss albino mice had substantial reductions in the paw volume varying from 35.64 to 67.56% when supplied with oral doses of 250 mg methanol extract per kg.

In traditional medicine, *Phoenix dactylifera* has been used to treat inflammation-associated diseases (Yasin et al. 2015). Recently, it was demonstrated as a pain reliever along with chemical agents such as ibuprofen and paracetamol (Maryam et al. 2015; Sani et al. 2015). There is no clear mechanism for such effects but it has been reported that *P. dactylifera* has active substances that can interfere with prostaglandin synthesis (Taleb et al. 2016a), inhibit the expression of inflammatory cytokines such as interleukin-6 (IL-6), IL-8 and IL-10, tumor necrosis factor alpha (TNF-α), and insulin-like growth factor-1 (IGF-1) (Al-Yahya et al. 2016), analgesic effects due to the presence of vitamins C and E (Maryam et al. 2015), and increased the expression of transforming growth factor beta (TGF-β) (Elberry et al. 2011). On the other hand, the antioxidant mechanism is the base of anti-inflammatory actions of *P. dactylifera*. Abutaha et al. (2018) showed that date palm leaves have higher antiproliferative activity against the HepG2 cell line (82 μg/mL) than against the MCF7 cell line (126 μg/mL) and inhibited the migration of the cell lines, altered LDH levels,

and reduced IL-6 transcript expression on the MCF7 cell line but not on the HepG2 cell line. Such findings show good antiproliferative and anti-inflammatory activities for *P. dactylifera* extract.

El Abed et al. (2018) reported that the aqueous ethanolic extract from *Phoenix dactylifera* parthenocarpic dates with varying concentrations from 0 to 200 µg of dry extract/mL inhibited phospholipase A2 activity, the substrate for pro-inflammatory mediators. Recently, Bouhlali et al. (2020) investigated, for the first time, the process of anti-inflammatory properties of date seed extracts and indicated that these extracts mediated anti-inflammatory activities by inhibiting protein denaturation, lysosomal membranes stabilization, nitric oxide-free radical scavenging and by inhibiting C-reactive protein and fibrinogen development.

2.3.5 Antimicrobial Activity

Different extracts and oils of *Phoenix dactylifera* show strong antimicrobial activities (Table 2.6). Abuharfeil et al. (1999) studied the effect of *P. dactylifera* against the hemolytic activity of streptolysin O and stated that date fruit extract decreased the growth of *Streptococcus pyrogens* by 88.5%, compared with control. In a disc diffusion method, aqueous and ethanol extracts of *P. dactylifera* fruits were highly antibacterial against *Escherichia coli*, *Salmonella enterica* and *Bacillus subtilis*, while *Staphylococcus aureus* and *Enterococcus faecalis* were moderately inhibited by *P. dactylifera* fruits extracts. This antibacterial activity is caused by the inclusion of *P. dactylifera* extracts of esculetin, tannic acid, gallic acid, itaconic acid and ferulic acid (El Sohaimy et al. 2015).

In another study, the antibacterial activity of methanol and acetone extract of the Saudi Arabian *P. dactylifera* varieties Mabroom, Safawi, and Ajwa, as well as Iranian variety Mariami, were evaluated. Mabroom's methanolic extract was better than the Mariami and Ajwa methanolic extracts against *Staphlococcus aureus* (Samad et al. 2016).

Hussain et al. (2019) evaluated antimicrobial activity of the Emirati date pit extract (Ajwa, Fard, Khalas, Khodari, Abu Maan, Lulu, and Mabroom variety date pits) by means of agar-well diffusion assay on *Staphylococcus aureus* (ATCC 29123) and *Escherichia coli* (ATCC 25922). They showed that the *S. aureus* were inhibited with an inhibition zone diameter of 20

Table 2.6 Antimicrobial activity of *Phoenix dactylifera*

Extracts	Study type	Conclusion/mechanism	References
Extracted *P. dactylifera* fruit flesh	In vitro	• Decreased growth of *Streptococcus pyrogens* by 88.5% compared with control with no date extract • Inhibited hemolytic activity of streptolysin O by greater than 90%	Abuharfeil et al. (1999)
Methanol, chloroform, and aqueous extracts	In vitro	• Methanolic and aqueous extracts were similar and had antimicrobial effects against selected gram-positive and negative bacteria and fungi	Shakiba et al. (2011)
Aqueous, ethanol, and ether extracts of three varieties of *P. dactylifera*	In vitro	• Aqueous, ethanol, and ether extracts showed a strong antibacterial effect against *Listeria monocytogenes* and *Staphylococcus saprophyticus*	Saleh and Otaibi (2013)
Methanol-water extract of *P. dactylifera*	In vitro	• Potent antibacterial activity against health hazard pathogens such as *L. monocytogenes, Staphylococcus aureus*, *S. saprophyticus*,	Al-zoreky and Al–Taher (2015)

(continued)

Table 2.6 (continued)

Extracts	Study type	Conclusion/mechanism	References
		Salmonella enterica ssp. *enterica*, *Escherichia coli* and *Psedomonas aeruginosa*	
Aqueous and ethanol extract	In vitro	• Strong antibacterial activity against *E. coli*, *S. enterica*, and *Bacillis subtilis* and moderate inhibition against *S. aureus* and *Enterococcus faecalis*	El Sohaimy et al. (2015)
Bark and *P. dactylifera* fruit extract	In vitro	• Antibacterial activity against *Lactobacillus brevis*, *Salmonella typhii*, *E. coli* and *Pseudomonas* species observed in bark extract but not in fruit extracts	Zehra et al. (2015)
Aqueous acetone extract	In vitro	• *P. dactylifera* extracts displayed antimicrobial activities, particularly at 10 mg/mL against *S. aureus*, *Bacillus cereus*, *B. subtilis*, *E. faecalis*, *Micrococcus luteus*, *E. coli*, *Klebsiella* and *Salmonella*	Kchaou et al. (2016)
Hydro-alcoholic extract	In vitro	• Some extracts had greater potency of inhibitory activities against *B. subtilis*, *B. cereus*, *S. aureus*, *E. coli*, *P. aeruginosa* and *Salmonella abony*	Bammou et al. (2016)
Methanol and acetone extracts	In vitro	• Antibacterial activity against all four tested bacteria	Samad et al. (2016)
Cold methanol extraction of *P. dactylifera* bark	In vitro	• Bark methanol fraction showed excellent antibacterial activity against *E. coli*, *S. aureus*, *Klebsiella pneumonia*, *Salmonella typhimurium*, *Shigella flexneri*, *Vibrio cholerae*, *Proteus vulgaris*, *Salmonella paratyphi*, *S. paratyphi B*, *S. pyogenes* and *P. aeruginosa*	Ravishanker and Raut (2016)
P. dactylifera syrup	In vitro	• Extracted syrup suppresses growth of *E. coli* and *S. aureus*, and the action could be attributed to generating of hydrogen peroxide that mediates bacterial growth inhibition	Taleb et al. (2016b)
Ethyl acetate of Emirati date pits extract	In vitro	• Ethyl acetate extract of Khalas and Khodari varieties induced inhibition of *S. aureus* • Abu Mann cv. pit extract inhibited *S. aureus* and decreased population of *E. coli* • Ajwa cv. pits extracts had inhibition zone 15–18 mm and MIC 5–7.5 mg/mL	Hussain et al. (2019)

DS— Date syrup, MIC— Minimum inhibitory concentrations

mm and MIC by 10 mg/mL of ethyl acetate extract of cvs. Khalas and Khodari. Ajwa cv. extract induced an inhibition zone of 15, 16, and 18 mm in diameter with MICs of 7.5 and 5 mg/mL.

Finally, antimicrobial activities of hydro-alcoholic extract of six Moroccan date fruit varieties including Bouskri, Bousrdon, Bousthammi, Boufgous, Jihl, and Medjool against gram-positive (*Bacillus subtilis*, *B. cereus*, and *Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginos*a and *Salmonella abony*) were examined using a disc diffusion method. All tested date fruit extracts showed antibacterial activity except Medjool and Bouskri cv. extracts,

but this antibacterial effect was still lower than that of gentamicin. For any tested bacterial type, the Bousrdon, and Jihl variety extracts were more active inhibitors with MICs of 2.5–10 mg/mL (Bammou et al. 2016).

As noted above, *Phoenix dactylifera* can be effective against gram-positive and negative bacteria (Fig. 2.4). However, it has been reported that date fruits are more efficient against gram-positive bacteria than against gram-negative bacteria due to the presence of an outer membrane. The most effective substances in *P. dactylifera* are phenolic compounds that account for its antimicrobial effects by generating hydrogen peroxide that mediates bacterial growth inhibition (Taleb et al. 2016b). It has been postulated that phenolic compounds utilize redox-active metals when interacting with bacteria, in particular, gram-positive (Taleb et al. 2016a).

2.3.6 Antitoxic Activities

Studies on hepatoprotective, nephroprotective and neuroprotective effects of *Phoenix dactylifera* against oxidative stress and xenobiotics-induced toxicity are listed in Table 2.7.

2.3.6.1 Antihepatotoxicity

The hepatoprotective potential of *Phoenix dactylifera* was examined by Al-Qarawi et al. (2004) on the effect of aqueous extracts of the flesh and seeds against CCl_4-induced hepatotoxicity in rats. The *P. dactylifera* seed extract was added to drinking water. β-Sitosterol, one of the bioactive compounds present in the extract may be responsible for the observed protective effect against CCl_4-induced hepatic injury in the rat model. In screening and developing of hepatoprotective drugs for the treatment of hepatocellular injuries, the ability of the constituents of such drugs to inhibit the aromatase activity of cytochrome P-450, thereby favoring liver regeneration, is an important factor to consider (Ahmed et al. 2008). Kowalski et al. (1990) suggested that the flavonoids content of *P. dactylifera* could be a factor contributing to its hepatoprotective ability through the inhibition of cytochrome P-450 aromatase.

Saafi et al. (2011) reported a protective effect of the aqueous fruit flesh extract of *Phoenix dactylifera* against dimethoate-induced oxidative stress in rat liver. Oral dimethoate administration (20 mg/kg) led to a rise of all hepatic biomarker enzymes. Furthermore, when animals challenged with dimethoate are treated with aqueous date fruit flesh extract (4 mL/kg), the extract can mitigate diminished hepatic SOD, GPx, and CAT activities, inhibit hepatic lipid peroxidation, and restore various degrees of alterations in hepatic biomarker enzymes, thereby thwarting hepatocellular damage.

Alqarni et al. (2019) reported that oral administration of 25, 50, and 100 mg Ajwa date polyphenol extract per kg body weight to hypercholestrolemic rats significantly reduced their body and liver weights and total hepatic cholesterol. Also, low-density lipoproteins cholesterol (LDL-C), very-low-density lipoprotein cholesterol (VLDL-C), and triacylglycerol was significantly decreased in plasma and liver. Furthermore, treatment with Ajwa date polyphenol extract improved the high-density lipoprotein cholesterol concentration and liver antioxidant enzyme activity in a concentration-dependent fashion, thereby regulating lipid profiles and enhancing the antioxidant defense system (Alqarni et al. 2019).

2.3.6.2 Antinephrotoxicity

Phoenix dactylifera has also been shown to play a key role in treatment and control of complex xenobiotic nephrotoxicity. A hydroacetone extract prepared from seeds of *P. dactylifera* conferred a noticeable protection on the kidney in a dose-dependent manner because the extract contained a high quantity of proanthocyanidins, which exhibited high antioxidant activity (Ahmed et al. 2015). Prolonged administration of aqueous *P. dactylifera* extracts by a dose of 10 mL/day/rat could restore kidney function (Hasan and Mohieldein 2016). This study reported that diabetic rats treated with aqueous *P. dactylifera* extracts were protected from the

Table 2.7 Antitoxic activities of *Phoenix dactylifera*

Activity	Extracts	Study type	Dose/route	Conclusion/mechanism	References
Antihepatotoxicity	Aqueous extracts of fruit flesh and seeds	In vivo/rats	Dose not indicated/orally	• β-sitosterol may be responsible for observed protective effect against CCl_4-induced hepatic injury in a rat model	Al-Qarawi et al. (2004)
	Aqueous fruit flesh extract	In vivo/rats	4 mL/kg daily/orally	• Mitigate hepatic antioxidant activities	Saafi et al. (2011)
	Ajwa date polyphenol extract	In vivo/rats	Oral administration of 25, 50, and 100 mg/kg body weight	• Reduction of total hepatic cholesterol, LDLC, VLDLC, and triacylglycerol in plasma and liver • Improvement of HLDLC concentration and liver antioxidant enzyme activity	Alqarni et al. (2019)
Antinephrotoxicity	Aqueous *P. dactylifera* fruit extract	In vivo/rats	15 g/15 mL/orally	• Observed nephroprotective effect on aflatoxin-B_1 (AFB_1) induced toxicity may be attributed to antioxidant properties of *P. dactylifera* extract	Al-Ghasham et al. (2008)
	Hydroacetone seed extract	In vivo/rats	50 or 100 mg/kg/rat/orally	• Nephroprotective ability of the extract may be explained by its ability to effectively scavenge free radical generated during CCl_4 metabolism.	Ahmed et al. (2015)
	Aqueous seed extract (100 g/L)	In vivo/rats	10 mL/day/rat/orally	• Prolonged administration of aqueous seed extracts of *P. dactylifera* could ameliorate progressive decline in renal dysfunction among diabetic rats treated with the extract	Hasan and Mohieldein (2016)
	Date fruit extract of *P. dactylifera*	In vivo/rats		• Protects the kidney through increasing level of TAS and decreasing levels of TOS and OSI	Celik and Irak (2018)
Antineurotoxicity	Aqueous *P. dactylifera* fruit extract	In vivo/rats	250 mg/kg	• Enhance antioxidant status	Majid et al. (2008)
	Methanolic fruit extract	In vivo/rats	100 and 300 mg/kg/orally	• Polyphenolic constituents may be responsible for the observed neuroprotective effect of the extract in the ischemia hypoperfusion	Pujari et al. (2014)

oxidative stress alterations associated with diabetes.

Al-Ghasham et al. (2008) reported that aflatoxin B1 (AFB1), consistent with renal failure, causes histopathological renal alterations, as shown by increases in plasma creatinine and urea levels. A 2-week treatment of aqueous *Phoenix dactylifera* extract lowered the plasma creatinine and urea levels dramatically, as well as enhanced kidney architecture. They concluded that the observed effect may be attributed to the antioxidant properties of *P. dactylifera* extract. Al-Qarawi et al. (2008) also investigated the effect of *P. dactylifera* extract on gentamicin nephrotoxicity in rats. Toxicity was induced by administration of gentamicin (80 mg/kg/day) intramuscularly. Either *P. dactylifera* extracts were administered to the animals by mixing the *P. dactylifera* flesh extract with their food (50% w/w) or by mixing the seed extract in the drinking water (2:1 w/v). Gentamicin administration was found to cause a significant elevation in the plasma levels of creatinine and urea and induced marked necrosis of the renal proximal tubules. Interestingly, administration of either *P. dactylifera* flesh extracts with the food (50% w/w) or seeds extract in the drinking water (2:1 w/v) resulted in a significant reversal effect in observed indices of toxicity in the kidney. Melatonin, vitamin E, and ascorbic acid that are abundantly present in the extract and that may synergistically act to counteract the overwhelming effect of the free radicals generated, were suggested to be the basis of the nephroprotection.

In another study of gentamicin nephrotoxicity Celik and Irak (2018) investigated the effect of the date extract on certain biochemical parameters and total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) in nephrotoxicity were induced by gentamicin. They also reported that date extract attenuates nephrotoxicity caused by gentamicin and protects the kidney through increasing the level of TAS and decreasing the level of TOS and OSI in rats that received gentamicin plus date extract.

2.3.6.3 Antineurotoxicity

The impact of free radical generation, mediated by oxidative stress in the pathogenesis of various neurological diseases such as a cognitive decline in aging, Alzheimer's disease, Parkinson's disease, and vascular dementia has been widely documented in the literature (Uttara et al. 2009). Nutraceutical antioxidants are also known as potential remedies against solemn neuronal damage owing to their capacity to inhibit and neutralize free radicals by providing neuroprotection (Pujari et al. 2011). Pujari et al. (2014) investigated the neuroprotective and antioxidant effect of *Phoenix dactylifera* fruits against permanent bilateral common carotid arteries (BCCA) in rats. They reported that chronic occlusion of BCCA resulted in a noticeable increase in lipid peroxidation as evidenced by the elevation in MDA levels. They also observed that there was a general decline in the endogenous antioxidants, notably glutathione, GPx, GR, GST, CAT, and SOD. They concluded that 100 and 300 mg/kg of the extract significantly ameliorated these alterations, confirming the protective role of the extract in ischemia hypoperfusion. The polyphenolic constituents in the extract such as flavonoids and plant sterols as well as its ascorbic acid content may account for the observed neuroprotective effect.

A similar study conducted to investigate the neuroprotective effect of aqueous *Phoenix dactylifera* fruit extract in focal cerebral ischemia in rats reported that a 250 mg/kg dose of the extract significantly inhibited neuronal damage induced by cerebral ischemia. Rats were maintained on varying doses of the extract (125, 250, 500, 1000 mg/kg). The extract was administered once per day for 2 weeks; the largest protective effects of the extract were observed at a dose of 250 mg/ kg. At 500 mg/kg, a lower protective effect was observed, and 1000 mg/kg showed a negative effect, which may be attributed to high concentration of antioxidants that may be harmful. They concluded that the extract at 250 mg/kg could protect neurons against ischemia-reperfusion-induced insults (Majid et al. 2008).

Taken together, in search for a promising antitoxic agent in the treatment and management of hepatotoxicity, nephrotoxicity, and neurotoxicity, *P. dactylifera* is a candidate to explore.

2.3.7 Cardiovascular Protective Activity

Phoenix dactylifera fruit has been used as an antihypertensive food for centuries. Because date palm seeds are a source of edible oil and are also used in the pharmaceutical industry, it has been proposed that the oil may exert beneficial effects in cardiovascular conditions (Al-shahib and Marshall 2003). Braga et al. (2007) showed that this fruit is a potent angiotensin-converting enzyme inhibitor. This is an effective strategy for reducing blood pressure. Also, high sodium and low potassium intake have a major role in raising blood pressure (He and MacGregor 2008). Because date fruit contains a high amount of potassium and a low amount of sodium, it can control blood pressure by maintaining the electrolyte balance. Also, the magnesium and calcium contents of the fruit play key roles in this subject (Tang et al. 2013).

Daoud et al. (2017) examined the putative preventive effect of the ethanolic extract of date palm pollen on insoproterenol-induced myocardial infarction in rats and the results proved that date palm pollen extract has preventive effects on the cardiac remodeling process.

Hypercholesterolemia is an important risk factor because of its major impact on the progression of cardio- and cerebro-vascular disorders. It was reported that date fruit supplementation could modulate cholesterol absorption and metabolism (Alsaif et al. 2007). This effect is related to *Phoenix dactylifera* fiber and phytochemicals through reduction in cholesterol absorption and reabsorption of bile acids and inhibition of hepatic cholesterol biosynthesis after production of short-chain fatty acids due to fruit fiber fermentation (Patel and Thompson 2006).

Ahmed et al. (2016) demonstrated that Aseel, the best variety of Pakistani dates, has the same efficiency as atorvastatin with respect to lowering of some markers such as fasting blood sugar, cholesterol, triacylglycerol, LDL-C, and VLDL-C. Generally, this experiment showed that Aseel lowered the blood lipid levels. Some constituents in the fruit such as phytochemicals, β-sitosterol, proanthocyanidin, catechin, quercetin, anthocyanins and selenium may have heart protective and antihyperlipidemic effects (Auger et al. 2005).

2.3.8 Reproductive and Labor Induction Activities

Phoenix dactylifera fruit contains different vitamins, a high percentage of sugar and carbohydrates, proteins, fatty acids, salt and minerals such as potassium and magnesium (Al-Farsi and Lee 2008; Al-Mssallem et al. 2019; Al-shahib and Marshall 2003). The mineral contents of *P. dactylifera* are listed in Table 2.8.

Table 2.8 Mineral composition of date flesh in four cultivars (mg/100 g dry weight). Adopted from Assirey (2015) an open-access article distributed under the Creative Commons Attribution License which permits unrestricted use

Mineral (mg/100 g)	Date variety			
	Ajwa	Kodari	Safawy	Burni
Calcium	187 ± 0.5^{a}	133 ± 0.3^{c}	123 ± 0.4^{c}	168 ± 0.2^{b}
Phosphorus	27 ± 0.01^{a}	16 ± 0.01^{b}	12 ± 0.1^{c}	18 ± 0.01^{b}
Potassium	476.3 ± 0.4^{a}	289.6 ± 0.8^{c}	512 ± 0.6^{a}	422.5 ± 0.5^{b}
Sodium	7.5 ± 0.01^{a}	4.9 ± 0.01^{b}	8.6 ± 0.1^{a}	8.9 ± 0.02^{a}
Magnesium	150 ± 0.7^{a}	60 ± 0.2^{c}	56 ± 0.03^{c}	100 ± 0.6^{b}

Each value represents the mean ± SE
Means with different letters *a*, *b* and *c* are significantly different

Date fruit consumption may be beneficial for women in childbirth because it has a strong supply of energy and has ample calories that can also help to avoid physical fatigue (Alaei and Pakdaman 2009). In addition to generating energy, *Phoenix dactylifera* fruit often contains fatty acids that can generate prostaglandins, which are important for cervix ripening, faster delivery, increased uterine contractions, and induction of labor (Baliga et al. 2011; Odent 1994).

Nasiri et al. (2019) showed that date consumption appeared to reduce gestation and increased cervical dilations in labor. Also, *Phoenix dactylifera* fruit increased the frequency of vaginal delivery and reduced the incidence of caesarean section and its complications (Bagherzadeh Karimi et al. 2020). It has a pivotal role in the induction of labor and enhancement of male fertility (Table 2.9) that may be related to its antioxidant activity (Fallahi et al. 2015; Tahvilzadeh et al. 2016).

An in vivo trial was done to evaluate the effect of the normal and acid-treated powdered date seeds on male rat fertility (Ali et al. 1999). Rats fed with seeds at concentrations of 7 and 14% for 28 days saw a significant increase in the plasma testosterone, while the acid-treated seeds (14%) significantly increased the plasma level of luteinizing hormone (LH). In another study, Ben Abdallah et al. (2009) injected 0.3 mL of diluted seed oil (15 and 20% of oil in saline 0.9%) and reported significant increases in the sperm count, motility and viability in male mice.

Another study (Mehraban et al. 2014) reported the reproductive potential of date pollen through treatment of rats with 1 mL of aqueous extract of pollen using concentrations of 120, 240, and 360 mg/kg body weight administered by gavage. The doses of 120 and 240 mg/kg significantly raised the ratio of testis or epididymis to body weight, sperm count, sperm motility, and estradiol level compared with the control group, with noticeable increases in serum LH and testosterone levels. These findings were confirmed by the study of El-Kashlan, et al. (2015) in which they administered 150 mg ethanolic extract of date pollen per kg in male rats and recorded a marked increase in sperm count and motility, serum levels of LH, testosterone, and estradiol hormones. The authors also recognized potentiation in the activities of testicular 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) that contribute to the testosterone biosynthesis.

2.4 Conclusions and Prospects

Phoenix dactylifera has essential nutritive and curative values because it contains numerous food ingredients besides biologically active compounds giving it diverse pharmacological potentials. For these reasons, we recommend dates as a daily food for human consumption and encourage researchers to investigate the phytochemical constituents of *P. dactylifera* in countries of the Middle East and North Africa and compare results to the phytoconstituents of dates from Aliya and Medina in Saudi Arabia.

Also, we encourage investigations into the effect of *Phoenix dactylifera* supplementation on plasma lipid profiles, nonesterified fatty acids (NEFA), amino acid pool, growth hormone, and insulin-like growth hormone levels. Furthermore, the effect of *P. dactylifera* supplementation should be observed on intestinal amino acid transporters such as oligopeptide transporter (PepT1), excitatory amino acid transporter 3 (EAAT3), Na^+-independent branched-chain, and aromatic amino acid transporter (LAT1), Na^+-independent cationic amino acid transporter (CAT1) and Na^+-independent cationic amino acid transporter (CAT2). In addition to investigation of the effect of *P. dactylifera* on the intestinal monosaccharide transporters such as Na^+-dependent glucose and galactose transporter (SGLT1), glucose transporter (SGLT5), Na^+-independent glucose, galactose and fructose transporter (GLUT2), and Na^+-independent fructose transporter (GLUT5). The future recommendations also could extend to investigate the effect of *P. dactylifera* carbohydrate metabolism through determination of glucose uptake by peripheral tissues with evaluation of

Table 2.9 Reproductive and labor induction activities of *Phoenix dactylifera*

Extracts	Study type	Cell lines/animal	Dose/route	Conclusion/mechanism	References
P. dactylifera seeds	In vivo	Male rat	Normal and acid-treated powdered *P. dactylifera* seeds (7 and 14%)	• Significant increase in plasma testosterone levels • Acid-treated seeds' powder (14%) significantly increased plasma LH	Ali et al. (1999)
P. dactylifera seeds oil	In vivo	Male mice	0.3 mL of diluted DSO/IP 15 and 20% of DSO in saline 0.9%	• Significantly increased sperm count, motility, and viability	Ben Abdallah et al. (2009)
P. dactylifera fruits	In vivo	Women	Six *P. dactylifera* fruits per day for 4 weeks prior to their estimated date of delivery	• Spontaneous labor occurred in 96% of women who consumed dates, compared with 79% of women who did not consume dates • Prostin/oxytocin was significantly lower in women who consumed dates (28%), compared with women who did not consume dates (47%).	Al-Kuran et al. (2011)
Diosmetin 7-O-β-L-arabinofuranosyl (1 → 2) β-D-apiofuranoside (1) and diosmetin 7-O-β-D-apiofuranoside (2) from acetone extract.	In vivo	Alloxan-induced diabetic rats	1.5 mL of (1) and (2) suspensions/100 g body weight	• Highly significant increase in serum testosterone levels • Significantly decreased elevated prostate acid phosphatase in alloxan-induced diabetic rats	Michael et al. (2013)
P. dactylifera pollen	In vivo	Rat	120 and 240 mg/kg doses	• Significantly raised the ratio of testis or epididymis to body weight, sperm count, sperm motility, and estradiol level compared with control group • Increased serum LH and testosterone levels	Mehraban et al. (2014)

(continued)

Table 2.9 (continued)

Extracts	Study type	Cell lines/animal	Dose/route	Conclusion/mechanism	References
P. dactylifera pollen ethanolic extract	In vivo	Rat	150 mg extract/kg orally	• Augmented sperm count and motility, serum levels of LH, testosterone and estradiol • Increased activities of testicular 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD)	El-Kashlan et al. (2015)
P. dactylifera pollen	In vitro	Neonate mouse spermatogonia stem cells (SSCs) and Sertoli cells	0.62 mg/mL concentrations of pollen aqueous extract for 2 weeks	• Increased SSCs numbers	Mahaldashtian et al. (2016)

LH—Luteinizing hormone, DSO—Date seed oil

glucokinase and hexokinase genes and protein expression. Also, gluconeogenic, glycogenic, and glycogenolytic enzymes gene and protein expression should be considered.

We recommend to investigate the effect of *P. dactylifera* supplementation on proapoptotic proteins such as caspase-3, caspase-8, caspase-9, phosphatase and tensin homolog (PTEN), p53 upregulated modulator of apoptosis (PUMA), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), integrins, and E-cadherin. Also, to determine the effect of *P. dactylifera* on the antiapoptotic molecules like mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), actin, cyclin-dependent kinases (CDKs), matrix metallopeptidases (MMPs), mammalian target of rapamycin (mTOR), signal transducer and activator of transcription 3 (STAT3) and receptor for advanced glycation end-products (RAGE) signaling pathways. In the same context, we recommend study of the anticancer effect of *P. dactylifera* in combination with the already established anticancer drugs of either natural or synthetic nature.

The relationship between *P. dactylifera* supplementations and reproduction is an important research point especially in labor induction concerning oxytocin, gonadotropin-releasing hormone (GnRH), and prostaglandin $F_2\alpha$ ($PGF_2\alpha$). Also, investigating the effect of *P. dactylifera* on the molecular evaluation of the gene expression of the enzymes in the testosterone and estradiol biosynthesis and the enzymes included in the female reproduction as LH, follicle-stimulating hormone (FSH), estrogen, and progesterone.

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Green Synthesis of Nanoparticles from Date Palm (*Phoenix dactylifera* L.)

3

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Abstract

Nanoscience is a nascent emerging field in several sectors such as medicine, agriculture, food and textile in recent decades. Nanoparticles (NPs) are considered the basic elements of nanotechnology since they are used for the synthesis of several nanostructured devices or materials. In sustainable agriculture and crops improvement, these NPs are being used mainly as nanopesticides, nanofertilizers and nanosensors. They can be synthesized using different physical and chemical methods such as microwave, sol-gel, co-precipitation and flame spray. These syntheses require the use of reducing and stabilizing agents that can be of great risk to the environment. Thus, biosynthesis of NPs is emerging as another green, safe and cheap method for the synthesis of metal/metal-oxide NPs. Green synthesis could be of bacterial or fungal origin via extracellular reduction or from plant extracts which is widely used. Interestingly, different stable metal NPs were synthesized by various genera of microorganisms especially, *Lactobacillus, Klebsiella, Escherichia, Aeromonas, Bacillus, Corynebacterium, Pseudomonas, Enterobacter, Streptomyces, Rhodobacter and Rhodococcus*. Despite the prevalence of the synthesis of metal NPs by bacteria, their synthesis by fungi is more advantageous since their mycelia offer a large surface area for interaction and secrete fairly large amounts of protein than bacteria. In addition, the successful biosynthesis of NPs from different parts of plants including their seeds, leaves, gums, roots and fruits was widely reported. Hence, the synthesis of NPs from plant aqueous extracts is becoming more appealing and more used since it allows for better control of the shape, size and dispersity of the NPs and does not use toxic organic solvents, compared to the physical or chemical methods. This chapter deals with the green synthesis of NPs (e.g., Ag, Cu, ZnO, CeO_2, Au) from the different parts of the date palm, *Phoenix dactylifera* L., their potential toxic effects and their beneficial applications on date palms.

3.1 Introduction

In nanoscience, a particle is considered as a small object that represents an entire unit regarding its transport and properties (Capaldi Arruda et al.

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J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_3

2015). Nanoparticles (NPs) are considered very special, and they differ from their bulk counterparts because they have the ability to change their chemical, physical and biological properties due to their surface-to-volume ratio (Singh and Kaur 2019). These properties are their small size, surface structure, chemical composition, stability, shape and agglomeration of the NPs (Wang et al. 2016). NPs can be natural or manmade, and they can be either carbon-based or metal-based, with a size smaller than 100 nm in at least one dimension (Siddiqui et al. 2015). Recently, phyto-nanotechnology is increasingly used in agriculture to develop the so-called *smart* crops. For instance, they can provide a time-controlled, target-specific and programmed delivery of certain agrochemicals such as fertilizers, pesticides and herbicides in an *on-demand* manner either to fight against pests and pathogens or for nutritional purposes. This results in less application of agrochemicals which reduces the negative effects on plants and the environment (Wang et al. 2016). Furthermore, the use of nanotechnology appears very promising in the field of medicine where NPs are used as a delivery vehicle to carry diagnostic and therapeutic agents directly to the target cell. They are also used to potentiate the effect and deliver the phytochemical anticancer drugs directly to the cancer cells for effective destruction (Banu et al. 2018).

Nanoparticles are being extensively synthesized through physical or chemical methods that are considered hazardous to the environment. They can be synthesized physically through heat evaporation (Bae et al. 2002) or microwave irradiation (Chikan and McLaurin 2016). This requires high temperature, pressure and expensive materials (Mallikarjuna and Varma 2007). In chemical reactions, the selection of the solvent, the reducing agent and the stabilizing or capping agent are mandatory. The reducing agent is the compound that reduces the metal ions into metallic form. Its active site acts as an electron donor for the production of NPs. The stabilizing agent is defined as the compound that can be used to inhibit NPs agglomeration. The choice of the capping agent also has a great impact on the size range of NPs, morphologies and targeted applications (Annu Ali and Ahmed 2018). When compared to the physical and the chemical methods for the synthesis of NP, the green biological method (a procedure that avoids using harmful organic chemical substances) uses much less energy, is safer to apply, cost-effective, does not produce hazardous products and is environmental friendly (Tri Handok et al. 2019). However, after their synthesis and before their application, NPs must have all the appropriate parameters like size, shape, distribution, surface area, solubility, aggregation, toxicity and biocompatibility (Singh and Kaur 2019). Lately, more focus and interest are directed toward the *green chemistry* in which NPs are developed using a bio template as both the reducing agent and stabilizing agent, instead of inorganic precursors. Green synthesis of nanoparticles can be achieved using five methods as follows: (1) The polysaccharide method using water and polysaccharides that act as a reducing agent, a stabilizing agent, or both reducing and stabilizing agents; an example is the fabrication of silver NPs using starch as a protective agent and β-d-glucose as a reductant in a mild-heating system (Keat et al. 2015). (2) The Tollens method which is carried out in a one-step process and the production of nanoparticles, for example, Ag NPs, is carried out in the presence of ammonia producing silver NPs with different shapes and sizes and here the concentration of ammonia and nature of the reducing agents play a principal role in formulation of the Ag NP size. (3) The irradiation method in which the synthesis of metal nanoparticles can be prepared using various irradiation methods at room temperature without the use of reducing agent, and the size can be controlled depending on the dose and the rate of irradiation. (4) The polyoxometalates method using the polyoxometalates which are a vast family of molecular metal-oxide clusters with a great extent of structures and their reduced forms have a great ability to transfer electrons and protons and/or storage abilities. Hence, they can be employed as efficient donors or acceptors of several electrons without structural change. Thus, they can be used to synthesize noble NPs through stepwise, multi-electron redox reactions inertly.

Finally (5) the biological methods using biological agents as a template that can be used as either a reducing or protective agent for the fabrication of metal nanoparticles such as bacteria (Sunkar and Nachiyar 2012), fungi (El-Aziz et al. 2013; Fatima et al. 2015), algae (Kathiraven et al. 2015) and plant extracts (Mallikarjuna et al. 2011). An example is the synthesis of the unicellular green algae *Chlorella vulgaris* extract which was utilized to synthesize single-crystalline silver nanoplates at room temperature (Annamalai and Nallamuthu 2016). An example of a plant extract is that derived from date palm (Fig. 3.1).

Date palm is a tropical and subtropical tree that belongs to the family Arecaceae. It is widely found in the Middle East region (Saudi Arabia, Iran, Iraq, Egypt, UAE, Tunisia, Algeria, Sudan, Oman), and it is named as the *tree of life* since it provides food, fuel, medicine and construction materials. *Phoenix dactylifera* is a widely distributed tree and has a great economic, nutritional and medicinal importance; its different parts are used to synthesize different kinds of eco-friendly NPs. It is very rich in phytochemicals such as carbohydrates, phenolics, sterols, carotenoids, anthocyanins and flavonoids (Ansari and Alzohairy 2018; Banu et al. 2018; Zafar and Zafar 2019). The procedure of synthesis of NPs is simple and sometimes only requires the purification of NPs through centrifugation methods. Generally, the procedure of bioreduction of metal nanoparticles in plants and plant extracts requires three main phases (Makarov et al. 2014). First, an activation phase where there is a reduction of metal ions and nucleation of the reduced metal atoms occur. Second, a growth phase, where the adjacent nanoparticles of small sizes coalesce spontaneously to form particles of larger sizes accompanied by an increase in the thermodynamic stability. Finally, the termination phase where a final shape of the NPs is obtained (Keat et al. 2015). Through this biobased protocol of nanoparticles synthesis, the process can be highly reproducible and stable. Therefore, this process could be used for large-scale production with more efficiency in cost investment, is eco-friendly and safe for human therapeutic use. For example, the biosynthesis of silver nanoparticles (Ag NPs) reduced the cost of breast cancer treating drug synthesis by up to 60% (Zafar and Zafar 2019).

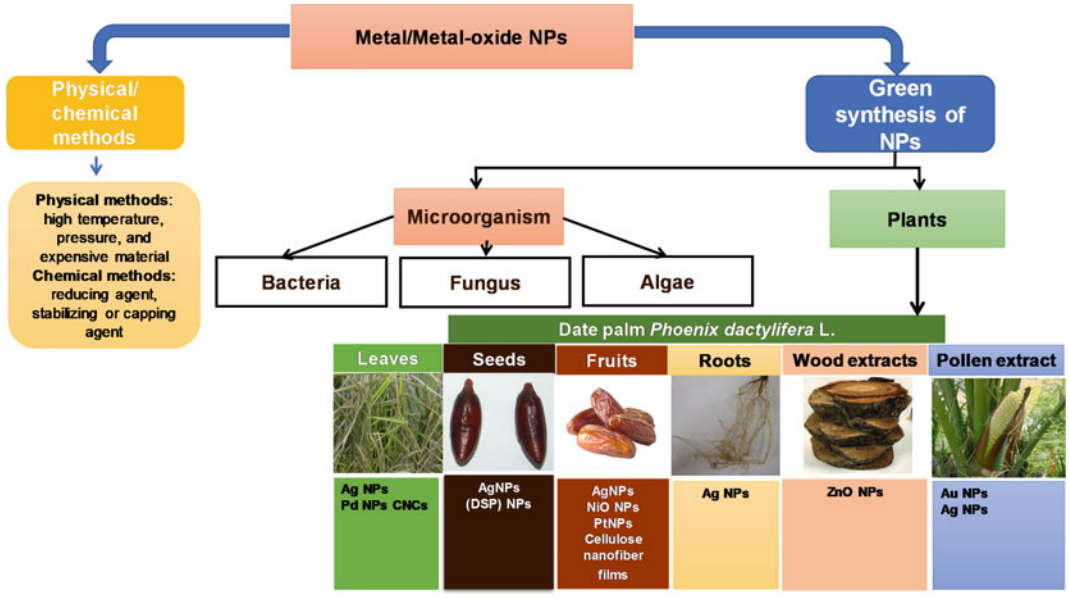

Fig. 3.1 Methods of synthesis of NPs using physical/chemical methods or the green methods like that from the date palm tree (Figure constructed by Besma Sghaier-Hammami)

This chapter deals with the green synthesis of several nanoparticles from the different parts of the date palm, the effect of the application of NPs on that tree and their potential toxic effects.

3.2 Green Synthesis of Nanoparticles

Recently, researchers have started to use different plants to synthesize different kinds of nanoparticles. In nanotechnology, the green synthesis of NPs is classified into three categories: physical, chemical or biological. For silver nanoparticles (Ag NPs), the physical and chemical syntheses tend to be more labor-intensive and hazardous, compared to the biological method which has several attractive advantages such as high yield, solubility and stability (Lee and Jun 2019). *Eucalyptus camaldulensis* Dehnh. (red gum); *Ziziphus spina-christi* (L.) Desf. (Christ's thorn jujube); *Calligonum comosum* L'Hér. (fire bush); *Tagetes erecta* L. (marigold flower); *Ziziphora tenuior* L. (kakuti, in Iran); *Azadirachta indica* A. Juss. (neem); (Ahmed et al. 2016; Bindhu and Umadevi 2015; Mohammed 2015, 2016; Mohammed and Al-Brahim 2014; Mohammed et al. 2018; Padalia et al. 2015; Sadeghi and Gholamhoseinpoor 2015) and *Erythrina indica* Lam. (coral tree) (Rathi Sre et al. 2015) have been used as a mediator for the synthesis of Ag NPs. Since date palms are inherently rich in phytochemicals, they can be used as a novel reducing agent for the synthesis of NPs in large-scale production. Aqueous extracts derived from the date palms are widely reported (see Table 3.1). They are simple, require a single-step method and are being used as a substitute for chemical and physical procedures. A summary of the different NPs synthesis from the different parts of date palm is presented in Table 3.1.

3.2.1 Synthesis of NPs from Fruit

Farhadi et al. (2017) synthesized silver nanoparticles (Ag NPs) by the reduction of Ag^+ solution using date fruit extract (Fig. 3.2). They showed that the green synthesized silver NPs had a considerably high catalytic activity for the reduction of nitroarenes with sodium borohydride (Na BH_4) as the hydrogen donor and a strong antibacterial activity at a concentration of 30 μg/ml. These NPs were spherical in shape and had a size range of 25–60 nm (Farhadi et al. 2017). In another study, the synthesis of silver NPs from date fruit extract showed a potent antimicrobial activity against human microbial strains. As well as an induction of cytotoxicity via necrosis, apoptosis and mitodepressive mechanisms such as the increase in mitotic abnormalities which disturb metaphase and anaphase during cell division and the impairment of mitotic spindle and the orientation of chromosomes that can disturb the cellular components at various stages of the cell cycle. Hence, they were proposed as an adjunct for the treatment of breast cancer. These NPs were spherical in shape and had a diameter range of 20–100 nm (Zafar and Zafar 2019). Date palm fruit extracts were also used to generate silver NPs which were shown to mediate the inhibition of bacterial and fungal growth (Zaheer 2018). Awad et al. (2017) showed that the biosynthesis of Ag NPs from date fruit extract had strong antibacterial and anticancer effects against breast cancer cells (MCF7). They even proposed these NPs as an alternative to anticancer drugs in current chemotherapeutics(Al-Awady et al. 2019). Date palm aqueous and ethanolic extract were also used to synthesize Ag NPs. These silver NPs showed a strong antibacterial effect at a size range of 10–32 nm and anticancer ability at a concentration range of 35.15–56.73 ug/ml against LoVo colon cancer cells, which encouraged the authors to propose them as potential antibiotic and anticancer drugs (Mohammed et al. 2018). Hence, the photosynthesized NPs can kill cancer cells by inducing their cytotoxicity and disrupting their division at various stages of the cell cycle.

Recently, nickel oxide nanoparticles (NiO NPs) have been synthesized using date palm fruit extract as a reducing or stabilizing agent deposited on the surface of graphene oxide sheets. NiO NPs were synthesized with a solution of pH 10

Table 3.1 Summary of the major NPs synthesized from the different parts of the date palm tree (*Phoenix dactylifera*) and their properties

Date palm tissue	Size and properties of NPs	Nanoparticles	References
Date palm fruit	– Spherical, 25–60 nm in size, crystal in nature – Absorption spectrum at ~400–420 nm	Ag NPs	Farhadi et al. (2017)
	– Spherical morphology. – Diameter ranges of 20–100 nm – Surface plasmon resonance (SPR) is at 425 nm	Ag NPs	Zafar and Zafar (2019)
	– Spherical in shape – Size ranges of 3–30 nm	Ag NPs	Zaheer (2018)
	– Average particle diameter is 90 nm – Zeta potential is 25 mV – Maximum wavelength is 400 nm	Ag NPs	Al-Awady et al. (2019)
	– Average size ranges from 67.8 ± 0.3 to 155.7 ± 1.5 nm – Mean zeta potential of −5.4 and −14 mV for aqueous and ethanolic extract, respectively	Ag NPs	Mohammed et al. (2018)
	– Average particles size of 20–30 nm	NiO NPs	Alshatwi et al. (2017)
	– Shape is spherical and homogenous – Size was small and with a range 1.3–2.6 nm – SPR is 321 nm in cv. Ajwa and 329 nm in cv. Barni dates	Platinum nanoparticles	Al-Radadi (2019)
Date palm leaves	– Crystals are of spherical structure – Crystallite size is of 26.3 nm – Zeta potential range of −25.4 to −18.5 nm	Ag NPs	Rashid et al. (2016)
	– Average size of palm tree bark particle is of 32.2 nm – Average size of palm leaves is 119.1 nm – Average size of palm leaves bark extract is 32.2 nm – Plasmon resonance band observed at 430–470 nm for all AgNPs	Ag NPs	Chand (2019)
	– Mostly spherical shaped in the range of 20–60 nm – Average size estimated is 40.13 nm – gNPs have a sharp absorbance peak at 420 nm – Maximum yield is showed at pH = 9	Ag NPs	Aitenneite et al. (2016)
	– Spherical in shape – Particle size ranges of 32–45 nm	Gold (Au) colloids	Zayed and Eisa (2014)
	– Spherical in shape, small-sized and uniformly distributed – Average particle sizes of 13, 5 and 21 nm	Palladium nanoparticles	Tahir et al. (2016)
	– Particles size is in the range of 10–15 nm and 150–200 nm	Cellulose nanocrystals (CNCs)	Jose et al. (2017)
Date palm roots	– Spherical shape – Average particles size of 15–40 nm – SPR curve is between 415 and 425 nm with peak centered at 420 nm	Ag NPs	Oves et al. (2018)

(continued)

Table 3.1 (continued)

Date palm tissue	Size and properties of NPs	Nanoparticles	References
Date Palm pollen	– Almost spherical – Average size of gold NPs is about 95, and AgNPs are 27 nm in diameter – Surface plasmon absorption peaks were strong and broad	Ag NPs and AuNPs	Banu et al. (2018)
	– Average particle size is less than 20 nm	ZnO NPs	Azizi et al. (2015)
Date palm wood extract	– AgNPs dimensions are of 20–60 nm with an average size of 39.57 nm	Ag NPs	Ibtissam et al. (2018)
Date fruit stalks residues	– Size ranges from 81 ± 25 to 60 ± 20 nm	Cellulose nanofibers	Hassan et al. (2014)
Date palm rachis	– Average length and diameter of the particles are around 260 and 6.1 nm	Microfibrillated cellulose	Bendahou et al. (2010)
Date palm seeds	– Shape is spherical – Mean size is of 163 nm in alcoholic dispersion and 92 nm in water dispersion	Nanoencapsulation of date palm pit extract	Bagheri et al. (2013)
	– Size range of 117–347 nm	Date seed powder NPs	Salama et al. (2015)
	– Particles' size vary from about 1–220 nm	Date palm seed nanoparticles	Awad et al. (2017)
	– The shape is spherical – Size is in the range between 1–40 nm with an average size of 27 nm – SPR is of 428 nm	Ag NPs	Khatami and Pourseyedi (2015)
	– Spherical in shape – Size range is of 14–30 nm – SPR peak was observed at 429 nm	Ag NPs	Ansari and Alzohairy (2018)

Fig. 3.2 Photo of date palm fruits (Barhi cultivar) (Photo by Besma Sghaier-Hammami)

so that Ni $(OH)_2$ can be deposited onto the surface of graphene. The biologically synthesized NiO NPs were spherical in shape and had an average size of 20–30 nm and uniformly anchored to the graphene surface. These parameters of size and shape can be controlled by changing the pH of the medium, the zeta potential and the magnitude. Indeed, in silver NPs, by increasing the pH of NPs, the zeta potential also increases resulting in the formation of highly dispersed NPs with small size and spherical shape; however, with an acidic pH, the value of zeta potential decreases and larger-sized ellipsoidal NPs tend to form. Thus, the pH is

responsible for the formation of NPs of various shapes and sizes (Baazaoui et al. 2020; Siddiqui et al. 2015). NiO nanoparticle-graphene nanohybrid exhibit biocompatibility in human mesenchymal stem cells (hMSCs) up to 100 μg/mL and does not cause any significant changes in cellular and nuclear morphologies. Thus, this synthesized nanohybrid represents a promising material for biomedical applications (Alshatwi et al. 2017; Annu Ali and Ahmed 2018).

Platinum is widely applied in medicines, as a catalysis, in pharmaceuticals and in fuel cells (Annu Ali and Ahmed 2018). Platinum nanoparticles (Pt NPs) were synthesized from the extract solution of dates which is a biodegradable surfactant. Polyphenols in the dates of cvs. Ajwa and Barni worked as capping and reducing agents. Under the preparation conditions, Pt NPs had a small size of 1.3–2.6 nm and a homogenous spherical shape. They showed a strong antibacterial activity against the gram-negative bacteria like *Escherichia coli* (RCMB 010052) and gram-positive bacteria like *Bacillis subtilis* (RCMB 010067) and showed a promising result against the hepatocellular carcinoma cells (HepG-2), colon cancer (HCT) and breast cancer cells (MCF-7). Thus, Pt NPs are now easier and safer to use compared to platinum-based commercial chemotherapeutic drugs like carboplatin, cisplatin and oxaliplatin. These drugs are increasingly used to treat cancer but they have too many dose-dependent side effects that could be overcome by the use of the greenly synthesized Pt NPs (Al-Radadi 2019).

Microfbrillated cellulose films were synthesized from date fruit stalk residues, and their size ranged from 81 ± 25 to 60 ± 20 nm. Cellulose nanofiber films made from xylanase pretreated fibers had higher density and tensile strength and lower absorption and air permeability, compared to those made from microfibrillated cellulose films isolated from untreated fibers (Hassan et al. 2014). The different NPs generated from date palm fruits, their size and their successful uses are summarized on Table 3.1.

3.2.2 Synthesis of NPs from Leaves

Phoenix dactylifera produces annually ~20 kg (dry weight) of leaves (Fig. 3.3) which are considered solid waste with no economic value but that are very rich in proteins, carbohydrates, common secondary metabolites, phenolics, flavonoids, sucrose and phospholipids.

These bioconstituents could act as a reducing agent and as a capping agent for Ag NPs without any involvement of additional chemicals (Barreveld 1993; Khiari et al. 2010; Trabzuni et al. 2014). Silver NPs synthesized from the aqueous extract of date palm leaves showed a very strong antibacterial activity. Various concentrations of Ag NPs (0, 8, 16, 32, 64 μg mL^{-1}) were used to study the growth kinetics for *Escherichia coli* and *Klebsiella pneumoniae*. The incubation of silver NPs for 3 h with *E. coli* and *K. pneumoniae* showed a 100% potency of antibacterial activity. It was achieved at a minimum inhibitory concentration (MIC) of 3.6 × 107 cfu mL^{-1} against *E. coli* and of 3.2 × 107 cfu mL^{-1} against *K. pneumoniae* at 8 and 16 μg mL^{-1}, respectively. The shape of Ag NPs was round and spherical with a size of 30–85 nm and the zeta potential increased with the increase of leaf extract concentration. The zeta potential values ranged from −25.4 to −18.5 mV with the highest values achieved when the lowest concentration of leaf extract was used (Rashid et al. 2016).

The synthesis of silver NPs from date palm leaves and tree *bark* was reported by (Chand 2019). NPs synthesized from palm tree bark extract had the size of 32.2 nm, and those from palm leaves extract were of 119.1 nm. Aitenneite et al. (2016) reported that the Ag NPs synthesized from the date palm leaf extract had mostly a spherical shape of 20–60 nm and an average size estimated at 40.13 nm. They had an efficient catalytic activity for sodium borohydride reduction of 4-Nitrophenol to 4-Aminophenol at room temperature. The maximum yield of silver NPs was achieved at pH 9 and a sharp peak of wavelength of about 420 nm (Aitenneite et al. 2016).

Fig. 3.3 Photo of date palm leaves (Photo by Besma Sghaier-Hammami)

Microfibrillated cellulose synthesized from the rachis of date palm increased the stiffness of the natural rubber above its glass–rubber transition temperature upon NPs addition. It further showed a higher adhesion level with the polymeric matrix. NPs had an average length and diameter of around 260 and 6.1 nm, respectively, and an aspect ratio of around 43 (Bendahou et al. 2010). The reductive activity and protective ability of plant biomolecules were also used for the reduction of gold ions (Au) to gold nanoparticles (Au NPs) (Annu Ali and Ahmed 2018). Gold NPs synthesized from aqueous extract of leaves exhibited good catalytic activity for the degradation of 4-nitrophenol. The particle size range is 32–45 nm, and they are spherical in shape (Zayed and Eisa 2014).

Palladium is a silvery white metal that has a high density and potential application in biosensor, pharmaceuticals, catalyst and medical diagnostic (Siddiqi and Husen 2016). Palladium nanoparticles (Pd NPs) were synthesized from the aqueous extract of *Phoenix dactylifera* leaves. The average particle sizes were of 13, 5 and 21 nm and the shape small, spherical and uniform. The Pd NPs showed a strong catalytic activity with a complete reduction of 4-nitrophenol to 4-aminophenol in only 2 min. They were very efficient in scavenging 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals, and they exhibited strong antibacterial activity against *Pseudomonas aeruginosa* (Tahir et al. 2016).

Cellulose nanocrystals (CNCs) were synthesized from leaf aqueous extract. Their size was in the range of 10–15 nm and 150–200 nm, and they exhibited a highly negative zeta potential. It was found that CNC decorated surfaces significantly enhance the coagulation times of blood plasma and whole blood. Cellulose nanocrystals can bear a high amount of negatively charged sulfate groups which mimics the natural anticoagulant heparin. This anticoagulant effect was studied through clinical tests (Jose et al. 2017).

3.2.3 Synthesis of NPs from Roots

The idea behind the use of the date palm roots (Fig. 3.4a, b) to biosynthesize NPs comes from

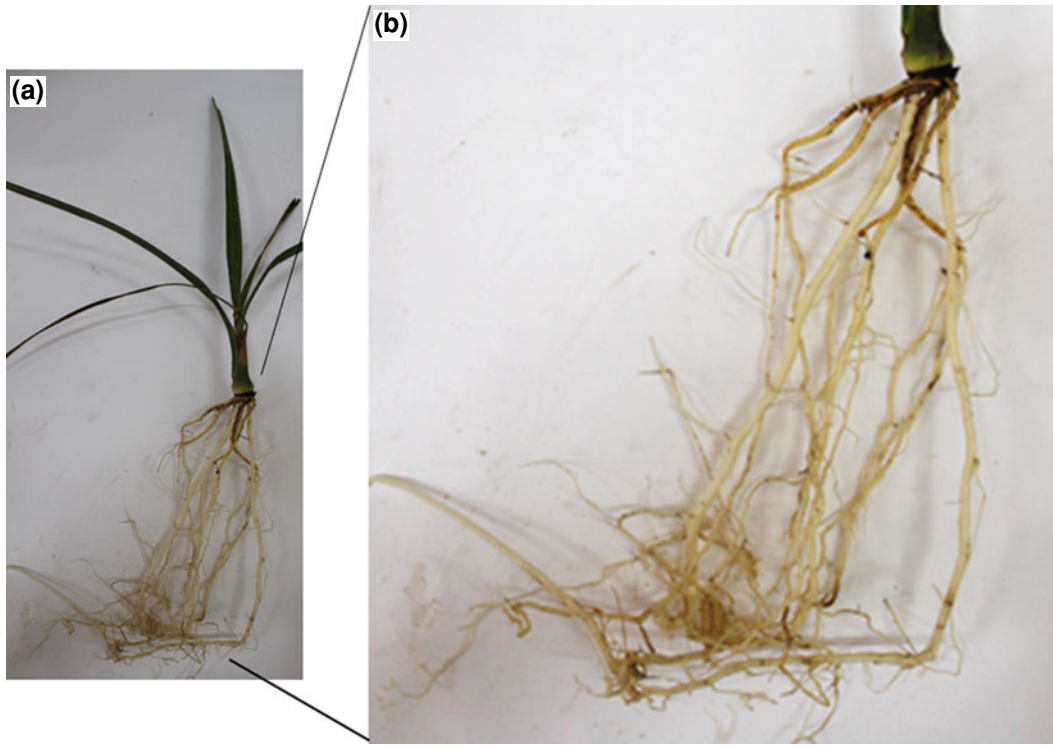

Fig. 3.4 **(a)** whole date palm plant, **(b)** the root system

the fact that they are considered as a solid waste, a cheap material and also because their collection from the adventitious roots aboveground does not damage the date palm.

Ag NPs synthesized from date palm roots were predicted to have a crystal size 15–40 nm and a spherical shape. They showed 100% potency against *Escherichia coli* and *Candida albicans* after 4 h and 48 h incubation, respectively, at 40 µg/ml Ag NPs concentration. A strong effect against the proliferation of MCF7 cell lines in vitro was also evident with IC50 values of 29.6 mg/l. They further showed an ability to control cell viability of human breast cancer cells (Oves et al. 2018).

Fig. 3.5 Date palm seeds (Photo by Besma Sghaier-Hammami)

3.2.4 Synthesis of NPs from Seeds

Date palm seeds (Fig. 3.5) are generally used as animal feed but also in pharmaceuticals and cosmetics because they are a potential source of edible oil. They are also used as alternative medicines to manage diabetes, cancer, liver diseases, gastrointestinal and cardiovascular disorders and to improve the functional integrity of the immune system (Ansari and Alzohairy 2018).

Ag NPs were synthesized from an aqueous extract from date palm pits. It was shown that the Ag NPs had a potent effect on inhibiting the mycelium growth in a dose-dependent manner and a strong bactericide effect at very low concentrations. The shape of the NPs is spherical with a size range between 1 and 40 nm, an average size of 27 nm and an absorption wavelength range of 350–620 nm (Khatami and Pourseyedi 2015).

Seed extracts of *Phoenix dactylifera* are used to mediate the synthesis of silver NPs. These particles have a spherical shape with a size range of 14–30 nm. The surface plasmon resonance (SPR) peak of Ag NPs was of 429 nm. Ag NPs were able to inhibit the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) in a dose-dependent manner, and they had a strong antibacterial activity at very low concentration against methicillin-resistant *S. aureus* strains. This suggests that these NPs could be used as alternatives to treat infectious disease resistant to drugs (Ansari and Alzohairy 2018). Date palm seeds were also used to synthesize date seed powder (DSP) NPs. Salama et al. (2015) synthesized two groups of NPs with a size range of 117–347 and a zeta potential of −4.96 and −5.23. These two groups of NPs named group 1 and group 2 showed strong anti-inflammatory and antihyperlipidemic activities in rats fed a high fat diet. They significantly reduced total cholesterol and inflammatory markers levels (Salama et al. 2015).

Further, it was reported that date palm seed extracts can be used effectively to synthesize Ag NPs. Indeed, they exhibit a unique small-size morphology that is very effective against pathogenic bacteria especially against *Staphylococcus aureus*, *S. epidermidis* and *Escherichia coli* (Salmen 2020).

Date palm seed NPs were synthesized from date palm seeds. They had a size range of 1–220 nm, and they had the ability to destroy or inhibit bacterial growth when applied to a site of bacterial activity (Award et al. 2016).

3.2.5 NPs from Other Parts of the Date Palm

Other parts of the date palm are used for the NPs synthesis like wood extract. This latter was used to synthesize zinc oxide nanoparticles (ZnO NPs) which are the most beneficial metal NPs since they are rapidly formed and nontoxic. Furthermore, they are significantly biocompatible and have a potent antimicrobial and photocatalytic activities. They are the most important NPs because of their increased surface area-to-volume ratio. They are widely used in various industries including medical devices, textile industry and detergent (Ahmed et al. 2017). Their size range is 1–220 nm. These NPs showed a strong antibacterial activity against pathogenic bacteria and a good antifungal activity against *Candida* fungus (Ibtissam et al. 2018). Azizi et al. (2015), also synthesized ZnO NPs from palm pollen grains using a green method that made them very suitable for biomedical use (Azizi et al. 2015).The ZnO NPs were dispersed, and they had an average particle size less than 20 nm. The wavelength of these NPs was more than 360 nm which was attributed to the band gap of the Zn ONPs.

Gold and silver nanoparticles were synthesized from date palm pollen extract. The biogenic NPs showed a dose-dependent cytotoxicity on MCF-7 cells and signs of apoptotic cell death. They further upregulated the proapoptotic protein p53 and downregulated the antiapoptotic protein Bcl-2 (Banu et al. 2018). The gold and silver NPs biosynthesized resulted in the formation of stable and poly-dispersed silver NPs. They showed a broad and a strong surface plasmon absorption peaks with peak characteristic at 659 nm. The shape of the NPs was spherical, and the diameter was 95 nm for gold NPs and 27 nm for silver NPs (Banu et al. 2018).

3.3 Date Palm Response to NPs

The economic importance of *Phoenix dactylifera*, especially in the Arab countries, has led to nanotechnology being applied to improve its productivity and its resistance against pathogens. The response of date palms to NPS treatments is summarized on Table 3.2 and Fig. 3.6.

Marzouk (2017) investigated the impact of the addition of silver (Ag), zinc (Zn) nanoparticles

Table 3.2 Summary of the major NPs synthesized from the date palm tree (*Phoenix dactylifera*) and their positive effects

Nanoparticles	Positive effects	References
Ag NPs	– Strong antibacterial activity at a concentration of 30 µg/ml – High catalytic activity	Farhadi et al. (2017)
	– Significant antimicrobial activity toward human microbial strains – Induced cytotoxicity via necrosis, apoptosis and mitodepressive mechanisms that can disturb the cellular components at various stages of cell cycle – Cost-effective, rapid, nontoxic and sustainable – Can be effectively used as an adjunct for the treatment of breast carcinoma	Zafar and Zafar (2019)
	– Mediation of the inhibition of bacterial and fungus growth	Zaheer (2018)
	– Efficient antibacterial activity against pathogenic methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria – Enhanced anticancer activity against breast cancer cells (MCF7) with high apoptotic effect	Al-Awady et al. (2019)
	– Significant antibacterial ability (10–32 nm diameter) and anticancer ability against a LoVo cell with IC50 ranged between 35.15×10^{3}–$^{5}6.73$ x 10^{3} g/L	Mohammed et al. (2018)
	– 100% potency at 40 µg/ml of Ag NPs concentration was observed against *Escherichia coli* and *Candida albicans* after 4 h and 48 h incubation, respectively – Decrease the cell viability of MCF7 cell lines in vitro with IC50 values of 29.6 mg/l and could act as a control link agent of human breast cancer	Oves et al. (2018)
	– Serve as a fast-acting potent antibacterial agent	Rashid et al. (2016)
	– Method of synthesis is safe without the use of hazardous chemicals	Chand (2019)
	– Ag NPs were used as an efficient catalyst for sodium hydride reduction of 4-nitrophenol to 4-aminophenol at room temperature	Aitenneite et al. (2016)
	– Inhibition of mycelium growth is concentration dependent and greatly increases with the increase in the concentration of Ag NPs in the medium – Synthesized Ag NPs showed a significant bactericide effect on tested bacteria and prevention of the growth of bacteria at very low concentrations	Khatami and Pourseyedi (2015)
	– Inhibition of the growth of methicillin-resistant *S. aureus* strains (MRSA) in a dose-dependent manner – Strong antibacterial activity of Ag NPs at very low concentration against MRSA suggests that these NPs could be used as an alternative approach for the treatment of medical devices associated infections caused by drug-resistant strains	Ansari and Alzohairy (2018)
	– Green synthesized NPs exhibited potent antibacterial activity against the pathogenic bacteria, as evidenced by their zones of inhibition and a good antifungal activity against the *Candida* fungus	Ibtissam et al. (2018)
Au NPs and Ag NPs	– Biogenic NPs showed a dose-dependent cytotoxicity on MCF-7 cells and signs of apoptotic cell death – There is an upregulation of the proapoptotic protein p53 and downregulation of the antiapoptotic protein Bcl-2 after NPs treatment	Banu et al. 2018
Au colloids	– Au colloids showed good catalytic activity for the degradation of 4-nitrophenol	Zayed and Eisa (2014)

(continued)

Table 3.2 (continued)

Nanoparticles	Positive effects	References
NiO NPs	– Graphene-NiO hybrids exhibit biocompatibility in human mesenchymal stem cells (hMSCs) up to 100 μg/mL – Nanohybrids do not cause any significant changes in cellular and nuclear morphologies in hMSCs	Alshatwi et al. (2017)
Pd NPs	– Pd NPs synthesized under optimized conditions exhibited strong catalytic activity with a complete reduction of 4-nitrophenol to 4-aminophenol in only 2 min – These nanoparticles were highly active in scavenging DPPH free radicals – They also exhibited strong antibacterial activity against *Pseudomonas aeruginosa* at a size of 26 ± 0.8 nm	Tahir et al. 2016
Pt NPs	– Hydrophilic antioxidants especially flavanols (polyphenols) in the dates of cvs. Ajwa and Barni worked as capping and a reducing agent – Pt NPs obtained inhibited the growth of gram-negative bacteria *Escherichia coli* RCMB 010052 and *Bacillis subtilis* RCMB – Promising results against hepatocellular carcinoma cells (HepG-2), colon cancer HCT, and breast cancer cells (MCF7) were achieved with cv. Ajwa extract – The dates of cv. Barni were effective against the hepatocellular carcinoma cells (HepG-2), Barni also inhibited the cells of colon cancer (HCT) and breast cancer cells (MCF-7) to a significant extent	Al-Radadi (2019)
ZnO NPs	– Palm pollen was a suitable reaction media and stabilizer in the green technique of synthesis. – This method is suitable and facile method to prepare ZnO NPs for biomedical productions	Azizi et al. (2015)
Cellulose nanofibers	– MFC films made from xylanase pretreated fibers showed higher density and tensile strength properties, lower water absorption and air permeability than those made from MFC isolated from untreated fibers	Hassan et al. (2014)
Cellulose nanocrystals (CNCs)	– CNC decorated surfaces significantly enhanced the coagulation times of blood plasma and whole blood as studied from quartz crystal microbalance with dissipation studies (QCM-D) and simple clotting tests – CNC bear a high amount of negatively charged sulfate groups which resemble the charge density of natural anticoagulant heparin anticoagulant effect of functionalized CNC was demonstrated by clinical test	Jose et al. (2017)
Microfibrillated Cellulose	– Stiffness of the natural rubber was significantly increased above its glass–rubber transition temperature upon NPs addition – Higher adhesion level with the polymeric matrix	Bendahou et al. (2010)
Date seed powder NPs	– Date seed powder preparations had antihyperlipidemic and anti-inflammatory activity in rats fed with high fat diet	Salama et al. (2015)
Date palm seed NPs	– Administration of an effective amount of the date palm seeds NPs synthesized to the site of bacterial activity could be efficient in destroying bacteria and/or inhibiting bacterial growth	Awad et al. (2017)

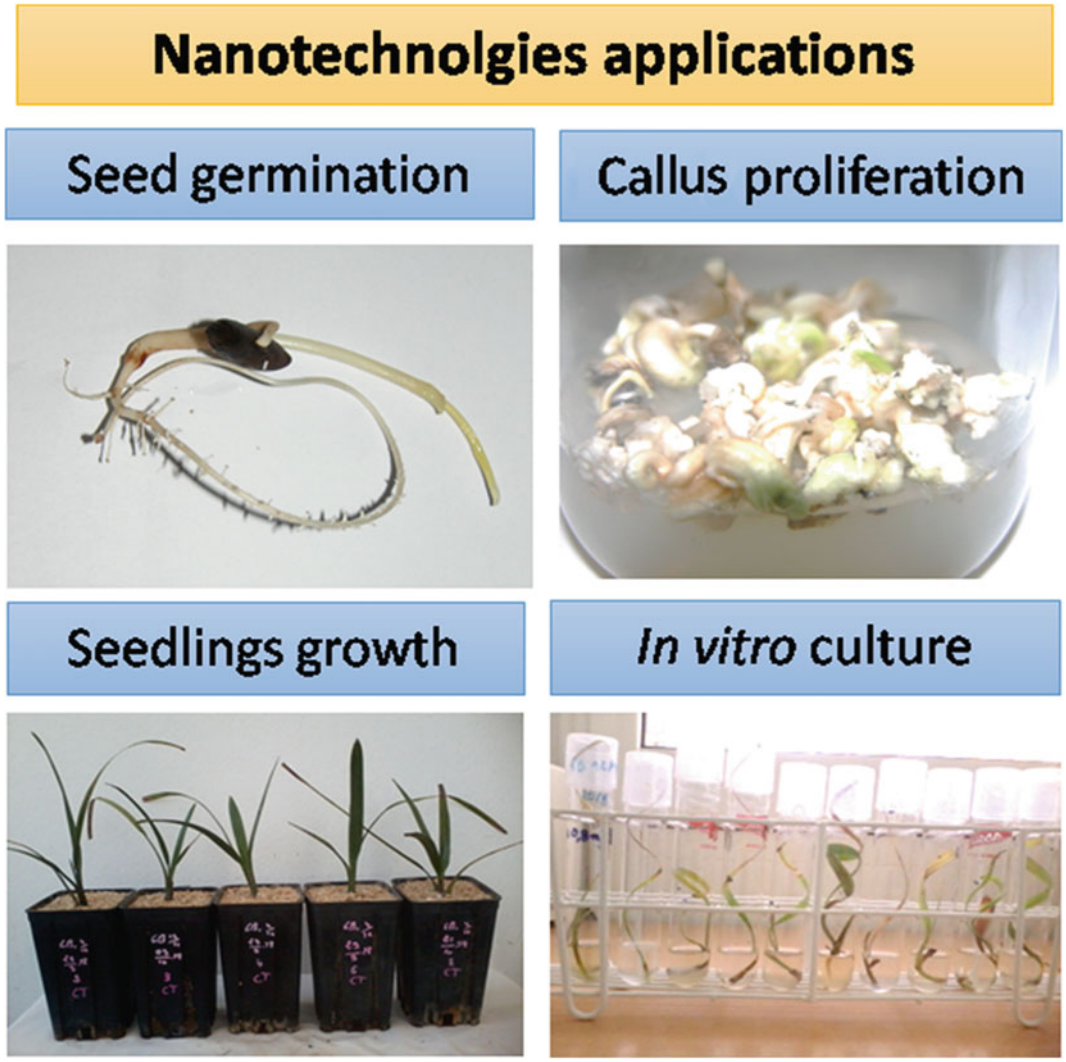

Fig. 3.6 Application of different kinds of NPs to improve date palm seed germination, seedling growth and in vitro culture responses (Photos by Besma Sghaier-Hammami)

and biochar on improving seed germination and early seedling growth rate of cv. Zaghloul. The results showed an improvement of seed germination after using all treatments. Indeed, ZnO NPs improved the seedling early growth after its application. Thus, the use of Ag NPs and biochar at low concentrations combined with nanofertilizers of Zn NPs was recommended to improve cv. Zaghloul seed germination and seedling growth rate. A concentration of 20 mg Ag NPs gave the highest seed germination rate and a concentration of 2 g/petri dish rate of biochar showed promising results to improve seed germination (Marzouk 2017). Further, the application of Zn ONPs on callus production under in vitro conditions showed that they are more effective compared to their bulk counterparts ($ZnSO_4$). Indeed, the results showed that the best callus induction of fresh and dry weight was achieved at a concentration of 2.43 mg/l ZnO

NPs in MS (Murashige and Skoog) medium (Chowdhuri et al. 2004). The use of nanofertilizers (IQ Combi) at three different concentrations (0, 0.5, 1 g/l) has been reported previously (Altemimy et al. 2019). The concentration of 1 g/l was effective in increasing fruit set (77.46%), fresh weight, length, significantly decreased fruit fall (28.09%) and increased the fruit bunch weight (11.329 kg) for both cvs. Khastawi and Zahdi (Altemimy et al. 2019).

Silver NPs were also used to improve the survival of *Phoenix dactylifera* explants and reduce their mortality rate. The results showed that maximum survival of explants 88.89% and 0% mortality were achieved at a concentration of 5 mg/l silver NPs used alone (El-Sharabasy et al. 2017). The in vitro effect of carbon nanotubes (CNTs) on callus, embryogenesis, embryo germination, elongation and rooting stage of *P. dactylifera* was also investigated. The CNTs concentrations used were 0.0, 0.05 and 0.1 mg/l, and the results showed that CNTs affect all stages of micropropagation of date palm. Optimum value of callus fresh weight was reached with the concentration of 0.05 mg/l of CNTs. The number of embryos decreased; while the number of germinated embryos and root number increased during embryogenic stage. An enhancement of shoot length and leaf number was recorded after the application of CNTs in the elongation stage. Further, it increased the root number, root length, plantlet length and hairy roots (Taha et al. 2016). Furthermore, in tobacco it has also been shown that that multi-walled carbon nanotubes were able to induce the growth of tobacco cell culture (with a 55–64% increase over control) using different concentrations (Khodakovskaya et al. 2012). CNTS also showed a significant positive effect on germination and growth vigor of Sewy embryos (Taha et al. 2016), in tomato plants (Khodakovskaya et al. 2009) and in rice (Lin et al. 2009). Likewise, the use of CNTs induced in vitro seed germination for several crops like barley, soybean and maize, and this may be due to the capacity of CNTs to penetrate the seed coats (Lahiani et al. 2013).

3.4 Toxicity of NPs

Several studies showed that the toxicity of NPs is related to size-dependent features. Indeed, the uptake and phytotoxicity of NPs depend on particle size. Particles of small size have a high tendency to accumulate and become toxic compared to their bulk counterparts (Wang et al. 2016). Depending on the concentration used, NPs could exert toxic effects upon application on plants (Goswami et al. 2019). Rico et al. (2011) reported that there is a concentration limit for the application of NPs that beyond it they become toxic to plants (Rico et al. 2011). NPs phytotoxic mechanisms could be through the interruption of electron transport system cycle of chloroplast and mitochondria inside the cell. It could be also through triggering oxidative burst because of the increase in the production of reactive oxygen species concentration (Yan and Chen 2018). Phytotoxicity in plants can be detected through the decrease in germination percentage, root elongation, biomass and leaf number. NPs could cause all the previously mentioned toxic effects even plant death (Lee et al. 2010). However, the toxic behavior of NPs and their phytotoxicity mechanisms are very complicated phenomenon that depends largely on their type, concentration, plant species, tissue exposed and the experimental conditions. Thus, contradictory results were reported in the literature during the last decade (Yan and Chen 2018).

Ag NPs application on radish sprouts crops affected their growth, nutrient content and macromolecules morphology with potential risk to human health. Silver NPs did not reduce seed germination but decreased the total water content, root and shoot length, dry weight, mineral contents and structural proteins (Zuverza-Mena et al. 2016). In onion, Ag NPs caused a decrease in the mitotic index, chromosomal breaks and cell disintegration (Kumari et al. 2009). Silver NPs also negatively affected the growth of mung bean and sorghum cultivated in soil or agar-based medium (Lee et al. 2012). Treatment of tomato plants with silver NPs reduced biomass

and root length (Song et al. 2013). Thus, based on these studies, we can conclude that to exhibit a toxic effect, silver NPs have to penetrate the plant tissue and hinder different metabolic activities.

Further, CuO NPs were found to suppress root elongation in zucchini with no effect on germination (Stampoulis et al. 2009). CuO NPs application was also capable of inhibiting the growth and induced structural changes to roots in wheat plants grown on a sand matrix (Dimkpa et al. 2012). *Arabidopsis* seedlings were also affected by the application of CuO NPs by reducing fresh weight and root length. In the same study, the germination rate and biomass of rice seeds were also affected (Shaw and Hossain 2013). Furthermore, CuO NPs were found to negatively affect root morphology with an almost complete inhibition when they were applied above the concentration limit (Adams et al. 2017; Shaw et al. 2014). Phytohormone and photosynthetic activity were adversely affected following the application CuO NPs (Nair and Chung 2014; Perreault et al. 2014).

TiO_2 NPs were found to negatively affect photosynthesis and the level of antioxidant enzymes like catalase and ascorbate peroxidase (Servin et al. 2013). TiO_2 NPs phytotoxicity was found to be similar to that of Ag NPs or CuO NPs by decreasing plant growth, mitotic index and increase of reactive oxygen species production, antioxidant activity and genotoxicity.

Cerium oxide nanoparticles (CeO_2 NPs) were also reported to have negative effects on treated rice by decreasing mineral contents, several proteins and weakened the antioxidant properties (Rico et al. 2013). In another study, CeO_2 NPs change the content of several plants components such as fatty acids and amino acids (Rico et al. 2014). The application of CeO_2 NPs on cotton raised on hydroponic solution adversely affected the vascular bundles and decreased the level of abscisic acid indole-3-acetic acid (Nhan et al. 2015).

NiO NPs were reported to induce oxidative stress as indicated by an increase in the level of catalase, glutathione (GSH) and superoxide dismutase (SOD), and a decrease in photo pigment levels in tomato seedlings. Thus, it was suggested that the dissolution of Ni ions from NiO NPs triggered cell death through mitochondria-dependent mechanisms (Faisal et al. 2013).

ZnO NPs were found to induce reactive oxygen species production and decrease photosynthetic and antioxidant activities (Tripathi et al. 2017). In another study, fragmentation of DNA after application of ZnO NPs was reported in onion (Ghosh et al. 2016).

Microorganism in the ecosystem can be also affected from the use of NPs because of the generation of oxidative stress (Hong et al. 2014). ZnO NPs were found to be toxic on soil microbes and affect their functions. The application of ZnO NPs on litter-amended soil affected the growth of soil bacteria and fungi compared to only litter-amended soil. They further decreased CO_2 emission and the mineralization of nitrogen and carbon (Rashid et al. 2017).

3.5 Conclusions and Prospects

Recently, more attention is being paid to the green synthesis of NPs since the chemical and physical methods are becoming more toxic to the environment. Because date palm is widely found in several areas and their products are cheap and widely available, they represent a valuable and cheap source of synthesis of NPs. In this chapter, we have tried to summarize the different date palm organs used for the NPs synthesis and their beneficial effects. We reported also the studies concerning the application of the synthesized NPs on the productivity of date palms. Finally, we cited their potential toxicological effects. Future studies should focus more on the synthesis of NPs from the different date palm parts and other plants such as the olive tree. These NPs could be very useful in the amelioration of different plants and cereals to biotic and abiotic stresses with the changing environment in the context of global warming. Indeed, we recently found that NPs were able to increase the tolerance of date palm vitro plants to water and drought stress (unpublished data). The correct use of NPs while respecting several parameters

such as the concentration, size, shape, pH, zeta potential and sonication could help to generate better crops with higher productivity without being toxic to the plants and the environment. Hence, more biosynthesized NPs should be applied instead of fertilizers and pesticides on date palm culture and more investigation should be done to avoid any toxic effects of NPs on crop cultivation and the environment.

Acknowledgements The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through the Small Research group project under grant number (R.G. P.1/195/41) and the Tunisian Ministry of Higher Education, Scientific Research and Technology (LR19 CBBC 02). Besma Sghaier-Hammami was supported by a project titled Programme d'Encouragement des Jeunes Chercheurs (19PEJC07-17).

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Part II
Omics Technologies

Omics Resources and Applications in Date Palm

4

Joel A. Malek and Karsten Suhre

Abstract

Since the release of the first draft of the date palm genome over a decade ago, numerous omics-based resources have been produced for research on this important fruit tree. Large-scale datasets are now available for date palm genome variation, transcriptomics, metabolomics, proteomics and other phenotypes such as imaging of fruit. These have assisted our understanding of date palm biology in the areas of development, population structure, sex determination and fruit qualities such as color and sugar content. Most date palm omics datasets have been applied using a single omics technology. With the increased number of resources across some of the same date palm cultivars, the challenge now is to integrate these resources and apply the outcomes to date palm agriculture in a meaningful way. Here, we explore the development of date palm omics as a field and where it is being and will be applied. We propose a general model for how omics data could be collected and integrated for increased understanding of date palm biology to the benefit of date palm cultivation. We suggest that large-scale omics resources should be collected for the most common cultivars and standard naming and phenotyping processes utilized to ensure that the power of integrated date palm omics research can finally be realized to benefit the field.

4.1 Introduction

The era of omic technologies has brought large-scale research projects to nearly all organisms of scientific and biotechnological interest. The date palm is no exception. This era began with the human genome project when the combination of engineering and robotics, imaging, chemistry and computational improvements allowed experiments to be conducted at an ever-increasing throughput. It then became clear that technologies such as genome sequencing could be used far beyond their original intention of simply decoding a novel sequence. The fast and robust process of deep next-generation sequencing could be utilized for understanding gene expression, epigenetic modifications and transcription factor binding where sequence was used simply to assign reads to a gene and read counts were more important than the underlying sequence per se. Paired-end sequencing allowed for the detection of structural changes. Beyond

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J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_4

this, proteomics and metabolomics increased throughput using automation and parallel computing. The result of these approaches is data intensive and the future benefit will rely heavily on integrating the data to extract the most relevant functional information. Key steps are necessary to ensure that any multiple omics based projects move from descriptive science to application by the research community going forward.

In this chapter, we look at the developments in omics resources and their use in date palm biology. We give a brief state-of-the-field overview and the history of omics use in date palm research. We show how various omics technologies have been used in understanding date palm biology so far. The challenge is for the date palm research community to move beyond single or dual omic-based resources to a well-integrated database that results from multiple omics technologies that can be combined to allow the most insightful research. We therefore propose an approach for future date palm omic research that will allow for integrated omic analysis and identify the challenges, the needed structures and benefits of this type of research.

4.2 Genomics and Phenotypes: Proteomic, Transcriptomic, Metabolomic and Imaging

4.2.1 Genome Resources

The date palm research community entered the genomics era in 2009 with the release of a first draft genome sequence for the Khalas cv. that included gene annotation and genome-wide set of cultivar specific single-nucleotide variants (http://qatar-weill.cornell.edu/research/research-highlights/date-palm-research-program/date-palm-draft-sequence). Importantly, the data was released freely online to the community for others to utilize before its publication (Al-Dous et al. 2011). Prior to the draft genome, only approximately 100 kb of DNA sequence was available at NCBI in the form of phylogenetic and SSR based markers. Since then, multiple date palm genome assemblies and genotype resources have been released. Including the first organellar genomes (Fang et al. 2012; Yang et al. 2010), an improved Khalas reference spanning 560 Mb (Al-Mssallem et al. 2013) including a predicted 90% of genes, a draft genome of the Khanizi cultivar (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA396270), and more recently with the advent of single-molecule-based sequencing a first male genome reference (Hazzouri et al. 2019) and improved Khalas reference sequence (Younuskunju et al. in prep) (Fig. 4.1a, b) spanning 820 Mb of the predicted 900 Mb.

While earlier genome references were ordered using only paired-end reads, the release of the first date palm genetic map (Mathew et al. 2014) has allowed sequences to be assigned linkage groups and dramatically extends the organization of the current set of reference sequences. Indeed, the quality of the date palm genomes available to the community rivals those with larger research communities.

Despite the presence in Genbank of at least four date palm genome references it is clear that the diversity of these references should be increased in the future. Currently, two of the references are from the Khalas cultivar (Al-Dous et al. 2011; Al-Mssallem et al. 2013), one from the Khanizi cv. in Oman and one from a male descendant of four backcrosses with the cv. Barhee female (Hazzouri et al. 2019). With current knowledge of date palm population structure, the fact that the three cultivars with references are from the Eastern subpopulation means that we are still missing a significant view of the date palm genome until a representative from the Western subpopulation is assembled. We believe that significant differences between the two subpopulations exist that are not captured by short-read alignment as is typically done for annotating differences. Indeed, entire genes may be present or absent between the populations that would not be revealed without a Western specific reference assembly.

The date palm genome reference has dramatically improved over the last decade. While the length of the typical contig has likely reached a near maximum given the balance between the benefits of long-read technology and challenges of assembling heterozygous genomes, the level

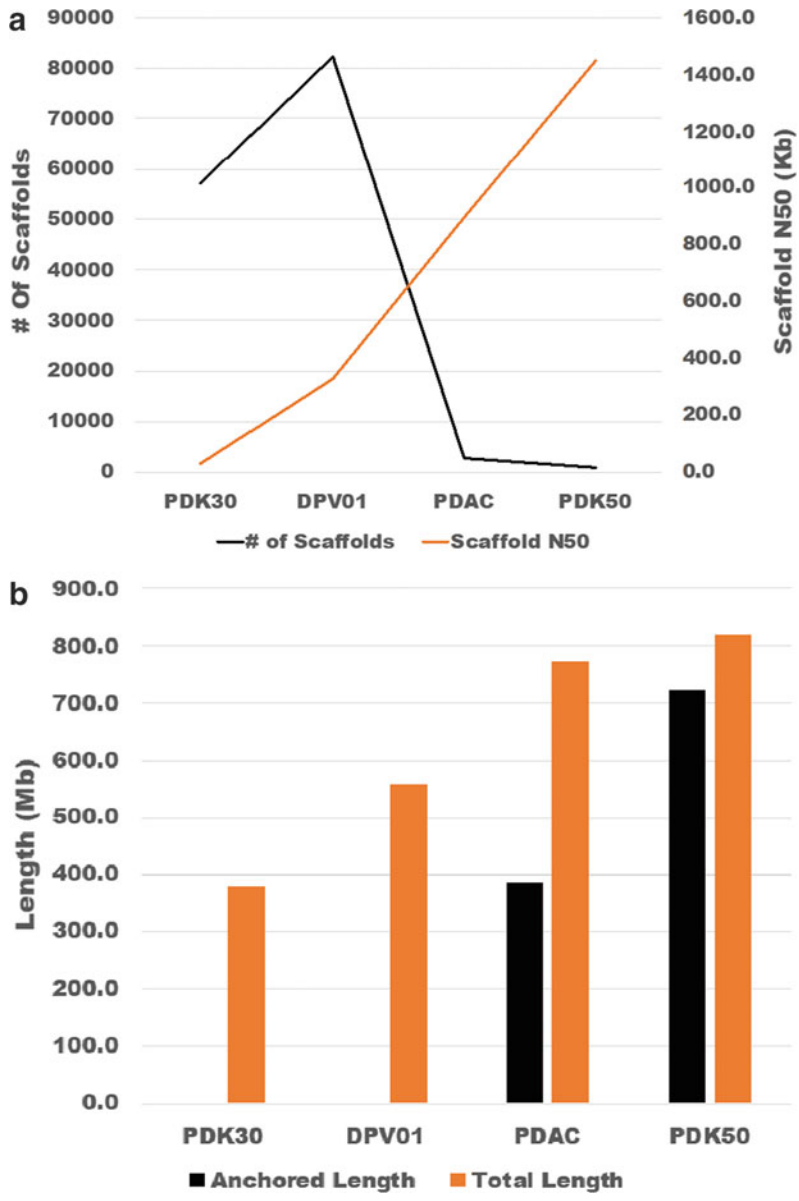

Fig. 4.1 The improvement in the date palm reference genome. **a** Scaffold N50 has increased dramatically while the total number of scaffolds has been reduced significantly, **b** the total length assembled has significantly improved and now seems to be leveling off at the predicted 900 Mb size of the genome. Furthermore, the latest versions of the genome are linked to the genetic map providing added structural organization for the sequence (Figure constructed by Joel A. Malek)

of ordering of the genome still needs further improvement and cross-checking beyond the single genetic map that is currently available. This will likely come from an assembly that integrates chromatin contact (Hi-C) and optical mapping (BioNano) information and would create a more accurately ordered reference that would be considered effectively final. Until whole-genome de novo assembly becomes the norm for every individual, future date palm genome reference improvement will tend more to increasing representation from various subpopulations and further phasing or splitting of haplotypes within the references.

4.2.2 Genotype Resources

While the date palm genome reference sequence offered a glimpse of the gene content and

structure, large-scale genotyping efforts have further increased our understanding of genetic diversity among the date palm population.

The first set of genome-wide single-nucleotide polymorphisms (SNPs) was released with the draft genome of the Khalas cultivar in 2009 and included the results from resequencing seven individuals including cvs. Deglet Noor and Medjool from the Western subpopulation (Al-Dous et al. 2011). This first genome-wide set has since been expanded dramatically to included 60 cultivars from across the date palm growing region (Hazzouri et al. 2015) and related *Phoenix* species (Torres et al. 2018) that together aim to provide a better understanding of the natural and cultivation induced variation in date palms.

Large-scale genotyping projects in date palm revealed that it is highly heterozygous with linkage disequilibrium decaying rapidly (Hazzouri et al. 2019). The implications are that at minimum genotyping ~50,000 SNPs per individual date palm would be required to tag each haplotype block of ~20 kb. To this end, it will be important for groups to continue developing genotyping methods that are consistent and allow labs across the world to coordinate genotyping results for smaller scale projects. Elmeer et al. (2011) predicted a set of 1000 SSR markers and validated a portion of them to allow testing of hundreds of variable loci in the genome. Our group spearheaded the use of genotyping-by-sequencing (GBS) in date palm for projects such as genetic mapping (Mathew et al. 2014) and population structure analysis (Mathew et al. 2015). While GBS offers a relatively cheap way of genotyping across the genome, the genotyping of specific SNPs across different populations requires extremely consistent library construction and is likely not practical when conducted outside one group. For groups that seek to genotype 100–1000 s of variable loci in date palms across a population, we expect that targeted GBS or even genotyping arrays will be developed in the future. While not as comprehensive as genome sequencing these methods could offer more consistent genotyping of the required 50,000 SNPs in laboratories that do not contain the most expensive next-generation sequencing equipment.

4.2.3 Phenotype Resources

Typically omics projects have attempted to link genotype to phenotype. In many cases, the phenotype could be simply a transcriptomic (Al-Mssallem et al. 2013; Bourgis et al. 2011; Xin et al. 2015), proteomic (Marondedze et al. 2014) or metabolomic profile (Diboun et al. 2015; Farag et al. 2014; Stephan et al. 2018) that is related to some physical characteristic in the date palm. For this reason, large-scale datasets of phenotype information as diverse as metabolomics, proteomics and image analysis will be important for future attempts to link genotype and phenotype. There are now multiple resources available with various omic technologies, but their disperse nature, usually on a small subset of cultivars, makes it important that a future direction would be to collect and normalize these datasets in a way that might make them useful beyond the descriptive nature that they currently provide. We refer the reader to other chapters in this book that will deal in more depth with the specific resources for each phenotype/technology, but suffice it to say that for future studies to utilize these data, a linking to other information such as genotype will be of high importance to allow other groups to utilize the data. The growth of disperse omics datasets is encouraging, but challenges in their full use remain. For example, the lack of clarity on naming (transliteration from Arabic) and selection of cultivars discussed below. For omics datasets to be utilized in the future, a consistent naming system will be important.

4.3 The Use of Omics Technology

Once large-scale resources of genotype and/or phenotype have been assembled, the next step is to utilize these in understanding date palm biology and its implication for cultivation. We see a few major areas that date palm genotype information has been utilized and there are early signs of the integration of genotype and other omics technology. These resources have been used in studies on the history of date palm cultivation,

QTL analyses, nutrition and food security, breeding and selection.

Here we present a model for the use of these omics data that suggests a biobank approach (Fig. 4.2). That is, samples of dates or trees are collected and multiple various omic studies conducted on those samples. The combined information is then used to understand date palm biology. Finally, the better understanding of date palm biology is utilized in applications such as cultivation or fruit packaging or other challenges that would benefit from an integrated omics based approach (Table 4.1). The use of the biobank approach ensures that information from multiple studies on the biobank can be cross referenced adding to the potential of information that can be extracted.

As a side note here, the community would benefit significantly from a unified naming system for the cultivars once they are determined to be the same genetically. Different transliterations, both from spoken to written Arabic and from Arabic to English or French have made difficult the comparisons of results across studies. Indeed, the issue was raised and published over 100 years ago as researchers began to study date palm cultivars from multiple regions using non-arabic alphabets (Popenoe 1913). To date, we are not aware of a unified naming system to span transliterations from North Africa to the Arabian Gulf and this will continue to make data integration challenging. We propose that a study be conducted and agreed on by groups for those that have genotypes established to have transliterated names established too. The success of

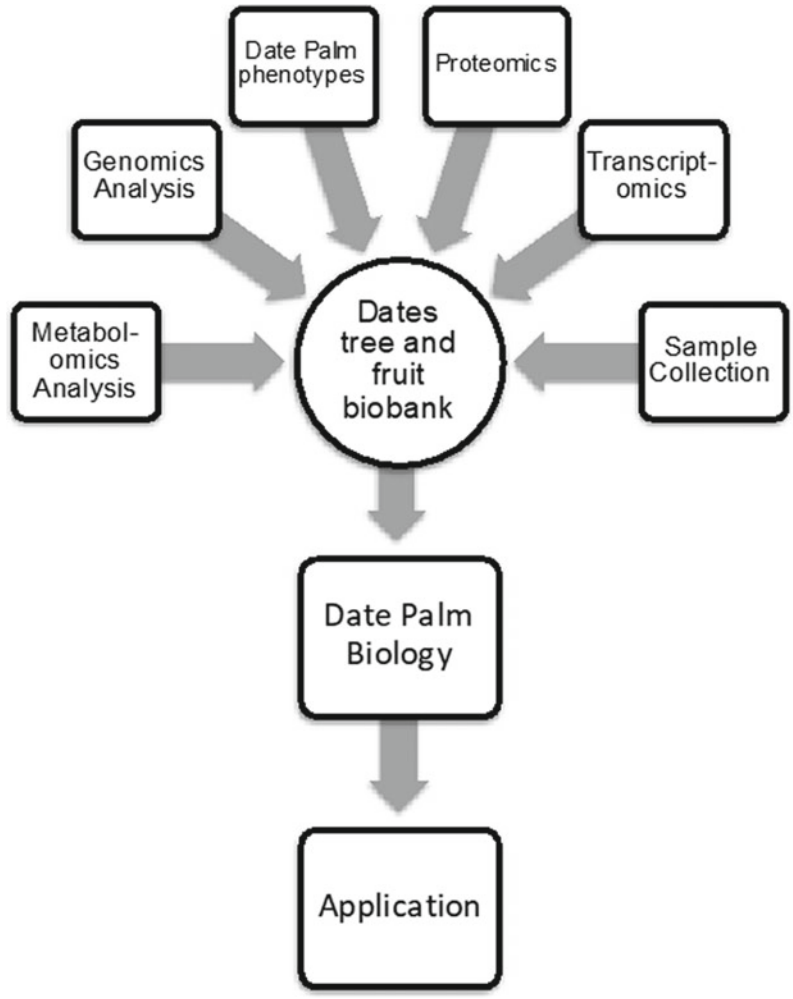

Fig. 4.2 A view of how to utilize omics data in date palm research. To integrate diverse omics data, a biobank should be established with samples that have been deeply phenotyped and analyzed using various technologies. A universal identification/naming system will be critical. This information should then be integrated to better understand date palm biology with the ultimate aim being application at the level of biotechnology (Figure constructed by Joel A. Malek and Karsten Suhre)

Table 4.1 Challenges and opportunities in date palm biotechnology that could benefit from integrated omics research

Challenge	Opportunity
Abiotic stresses	Resistance to salinity and changing environment
Biotic stresses	Bayoud (*Fusarium*) and red palm weevil resistance
Sex determination	Improving genetic diversity
Genetics of flowering/ripening time	Extending date harvest season across the year
Genetic diversity	Food security
Postharvest quality/packaging	Improving commercial success of various cultivars
Metabolite and nutritional analysis	Use of specific dates as functional foods

future cross biobank studies rests on a consistent identification system.

4.3.1 Domestication History

Probably the largest sets of studies that utilize date palm genotype information are those looking at the history of date palm domestication and cultivation. Early, lower throughput studies attempted to simply document the genetic diversity in a country and later these were then extended to better understand genetic diversity across countries and regions (Elshibli and Korpelainen 2009; Haider and Nabulsi 2012; Khierallah et al. 2011; Muhammed et al. 2015; Soumaya et al. 2014). As the number and geographical diversity of the date palms under study increased, it soon became apparent that there were two major subpopulations in date palm with significant genetic structure between Western and Eastern cultivars (Chaluvadi et al. 2014; Hazzouri et al. 2015; Mathew et al. 2015; Zehdi-Azouzi et al. 2015) confirming earlier studies that suggested this from chloroplast markers (Pintaud et al. 2010). The two major identified subpopulations may have further subdivisions in them. Indeed, a recent study using population-wide chloroplast and mitochondrial genotype data showed that there were likely three major and fourth minor maternal contributors to the present-day date palm cultivars and these localized to geographical regions (Mohamoud et al. 2019). Further genome-wide genotyping from under-represented regions such as Libya, Sudan and the Sahel will help clarify whether there are further major subpopulations yet undiscovered.

Studies on the history of date palm cultivation took another significant step forward with the identification of both potentially wild date palms in Oman (Gros-Balthazard et al. 2017) and the discovery that the Western subpopulation was hybridized with *Phoenix theophrasti* Greuter (Flowers et al. 2019). These studies help better explain the population structure of date palm and demonstrate the importance of wide sampling, including from related species when introgression is possible.

The importance of the combined studies on date palm cultivation history and population structure is critical as researchers considered more genome-wide association studies that rely heavily on accounting for population stratification. Indeed, prior to these studies, the view that the date palm population was monolithic would hinder the proper collection for any large-scale genotype:phenotype mapping study. Taking the population structure into account will allow more accurate mapping of genes to traits going forward.

4.3.2 Genotype: Phenotype Analysis with GWAS, QTL and Selective Sweeps

Once genotype and phenotype data have been collected they can be integrated to uncover genetic control of important date palm features. Studies of qualitative and quantitative trait loci

(QTL), genome-wide association studies (GWAS) and/or analyses of selective sweeps rely on such datasets and these are beginning to yield results in the date palm. Early studies showed the association of certain markers with date palm gender although the genome context of these was not well understood (Bekheet and Hanafy 2011).

The first qualitative trait to have its genetic locus identified using genome and genome-wide genotype data was the sex determination locus, to our knowledge. Al-Dous et al. (2011) showed association of a set of scaffolds and SNPs to the male phenotype. They showed strong linkage of the region in a set of related crosses at the USDA date palm collection in California. This was followed up with a deeper understanding of the region across the subpopulations and genus (Cherif et al. 2013, 2016) and has further developed with our group mapping the male-specific region to a set of four genes of which three are present in all males and absent in all females of the genus (Torres et al. 2018). This method, utilizing a kmer counting-based strategy demonstrates the possibility of using distant species within the genus to assist in better understanding of the date palm. Furthermore, the use of the African oil palm genome to understand the changes required for the move to dioecy from monoecy set a precedent for future studies of date palm cultivation under various selective pressures.

This use of oil palm findings was demonstrated by Hazzouri et al. (2015) using a candidate gene approach on the sequences and features of 62 date palm cultivars to detect association of a transposable element insertion in the *virescens* gene to fruit color. This gene had previously been shown to be important for fruit color in the oil palm. This approach of candidate gene checking in large genotype/phenotype datasets provides an example for future studies that can utilize large-scale studies in other monocots such as the oil palm and rice to possibly map genes in date palm.

The first set of GWAS results were recently released for the conversion of sucrose to reducing sugars in date palm (Hazzouri et al. 2019; Malek et al. 2020). A deletion of invertase genes was found to be associated with the presence of high levels of sucrose in the date fruit. This approach that integrated a phenotype from metabolomic and targeted sugar content analysis with population level genotypes is a model for future mapping of date palm phenotypes. In addition to sucrose content, Hazzouri et al. (2019) confirmed association of fruit color to the previously identified transposon insertion and the location of the sex determination region to linkage group 12. However, association to multiple other phenotypes such as fruit length did not reveal significant results. This may be due to the population they utilized not containing the variation needed to map some of the phenotypes and should encourage the continued broad sampling of the date palm population.

It is important to mention that another method for detecting selection, especially cultivation-induced selection, is the search for selective sweeps. Hazzouri et al. (2015) identified regions that appear to have gone through selective sweeps. This field of study has been significantly aided in the finding of potentially wild date palms (Gros-Balthazard et al. 2017). Indeed, if more wild date palms can be identified and sequenced the results would greatly assist the ability to detect regions in the cultivated date palm that have lost significant heterogeneity during cultivation and so likely have been subject to human selection. In the meantime, the recent significant improvements in genome contiguity, combined with a resource of genome sequences from the genus *Phoenix* (Torres et al. 2018), should assist in better understanding and utilizing information from selective sweeps in the date palm.

4.3.3 Diversity and Food Security

As the date palm research community better understands the range of diversity in the date palm population, we believe that one important area of application of omics technology will be to the field of food security within countries and regions. Food security can be seen as applying both to the assurance of food supply, but also to

the quality of that food supply and its provision for nutrition, given climate change, loss of cultivable land and a growing population. The recent findings of at least two major subpopulations, along with subdivisions within those populations (Chaluvadi et al. 2014, 2019; Hazzouri et al. 2015; Mathew et al. 2015; Mohamoud et al. 2019; Zehdi-Azouzi et al. 2015), should encourage the utilization of this diversity against potential disease, climate change and for increased nutritional diversity. Indeed, our own study of genetic diversity led us to conduct a clinical-based test for metabolite differences in the blood of patients who consume dates from one of each of the date palm subpopulations; Khalas from the Eastern and Deglet Noor from the Western population (Mathew et al. 2018). We found significant differences in blood metabolite levels when consuming either date type. These findings highlight the need for using the breadth of date palm genotype diversity in future studies of date fruit human consumption, especially as relates to nutrition.

While utilizing simple genetic diversity is a good start for countries looking to provide security against abiotic and biotic stresses, combining omics resources such as genotype and proteomic/metabolomic databases can reveal important characteristics about date palm fruit that should be targeted for expansion in a country's cultivation. For example, the identification of certain desirable chemicals (Khalil et al. 2017; Malek et al. 2020) in date palm that may be of interest to other industries could suggest opportunity for cultivation of specific cultivars beyond their consumption as dried fruit.

In the area of food security related to changing environmental conditions and disease, it is highly recommended that countries and regions utilize the research on genetic diversity of the date palm population to plan future date palm orchard plantings. The warnings from other major crops such as banana are important to heed for date palm. In the case of banana, the cavendish cultivar makes up 99% of exports because it was originally resistant to *Fusarium* wilt disease. However, new strains of the fungus have been identified (TR4) that can attack the cavendish cultivar and the banana industry is in now in a precarious position (Dita et al. 2018). Studies of the frequencies of certain cultivars in a country (Thareja et al. 2018) highlight the fact that countries tend to focus their cultivation on a very select few numbers of genotypes, dramatically increasing the risk that a single disease or climate event could wipe out a significant portion of the country's cultivation. The best example of the potential risk of cultivating only very few cultivars, such as Medjool and Deglet Noor, was demonstrated by the dramatic loss to *Fusarium* disease in the Maghreb of approximately 15 million trees in the past 50 years (Benzohra et al. 2015). While resistant cultivars have been identified, it will take years to further expand these and shift the cultivation and commercial process to include them (Benzohra 2017). Local preferences for certain flavors and appearances may drive the cultivation, authorities responsible for food security should encourage and subsidize the use of cultivars, at least on a small scale, from the range of date palm genotype diversity.

One immediately relevant area would be a focus on flowering time. Researchers have begun to conduct GWAS studies on important phenotypes (Frederique Aberlenc, pers comm) and application of this information could be used to spread the date palm harvest across multiple months allowing fresh dates to be available during a longer period of the year. On a slower scale, but not less important, are the climactic and environmental changes that put new selective pressures on the quality of date palm harvests. Increased genotype diversity will at least offer a range of alleles that may perform better in the new conditions.

With the newfound population level genome-wide genotype information available to the date palm research community, we hope to see analyses that recommend which cultivars from the population could offer the largest range of allele diversity to certain regions while not requiring 1000 s of foreign cultivars to be imported and spread. Such studies will help ensure the future of date palm productivity.

4.3.4 Breeding and Selection

Ultimately there is a hope that omics data will be utilized to improve date palm breeding and selection. For this to become a reality, research projects will need to better address the pressing needs of date palm cultivation. As mentioned earlier, studies have shown genotype:phenotype relationships for date palm gender, fruit color and sucrose content among others and these are the initial markers that could be used in omics application to breeding. Some initial studies on utilizing transcriptomic data to better understand date palm resistance to salinity are available. However, significantly more markers are needed to ensure that breeding such a long generation tree will ultimately benefit the farmer (Table 4.1). The oil palm research community has conducted breeding and selection experiments (see Barcelos et al. 2015 for review) and that experience may set a good precedence for how the date palm research community designs future studies. It is clear that markers for increased date palm yield, lower water requirements, salinity resistance, disease resistance and date fruit skin separation would likely be the most useful to farmers. Research studies to identify markers for these phenotypes and breeding programs to develop cultivars with the desired features should be the next step in the application of omics research in date palm. In the meantime, the continued input of information in date palm biobanks (Fig. 4.2), resulting in better understanding of date palm biology, will provide future projects with the foundation they need to begin applying this knowledge.

4.4 Conclusions and Prospects

The move from single-tube-based experiments to high-throughput research that yields millions of data points and requires heavy computation has, over the last ten years, come to the date palm research community. The use of these technologies has expanded dramatically and now includes resources from genomics to metabolomics, the gene to the metabolite. The technologies have been utilized to understand important areas of date palm biology including population structure, sex determination, sucrose content, fruit color, chemical changes in ripening and other phenotypes. However, the excitement and potential from these omic technologies will be justified in the eyes of date palm cultivation and farming communities if they can be expedited to yield functional information of benefit to the industry. To accomplish this will require the field move beyond either descriptive omic applications or single phenotype analyses. The date palm research community stands at a critical junction. While the basic science results that general omics technologies provide are of clear interest, it is likely that those results could be more easily obtained in a quick-growing model monocot. Most researchers in the date palm growing countries will need to show a strong justification for the financial investment in date palm omic resources going forward. To justify the challenges in applying omics technologies to date palm will require that a clear benefit to the farmer ultimately be shown. The date palm research community should take this as a challenge to develop a good portion of future research around this goal.

Our goal in this chapter has been to provide a history of the rapid developments in the use of omics technology in date palm research, while suggesting a framework for future research that will allow the maximum benefit of data from these technologies to be integrated and applied. We believe the future of omics research in date palm is bright; however, there are challenges to its implementation that we have raised including technical challenges and the justification of expense to the long-term benefit of the farmers. Despite these, we believe that such research has a potential to reveal insight that is specific to date palm biology, that may or may not be immediately applicable, but should be pursued nonetheless. The potential for results from a well-integrated database of a few hundred date palm cultivars is much larger than the sum of the parts. The use of a biobank approach will assist in ensuring that ongoing studies and rapidly improving technologies can most benefit from the foundation that has already been laid.

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5 Proteomic Insights of Date Palm Embryogenesis and Responses to Environmental Stress

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Abstract

Date palm (*Phoenix dactylifera* L.), a dioecious species, represents a major agricultural component of arid and semiarid regions. It plays a fundamental role in the socioeconomic balance. Dates, indeed, represent a major income and food source for local populations in the Middle East and North Africa. A better understanding of the plant's response to biotic and abiotic stress may help in improving date production, especially of elite cultivars. For this reason, molecular tools including genomics, transcriptomics and proteomics were developed to realize this response. Recently, proteomics has become widely used in biological research to explain several unknown biological functions. In the date palm production field, it is being used to elucidate the mechanism of plant resistance to environmental stress. The present chapter describes proteomic approaches used to compare the proteome of zygotic and somatic date palm embryos. Additionally, an explanation is given about the effect of the addition of different supplements to medium culture on the protein content and profile of somatic embryos. Furthermore, the response of date palm to abiotic stress including brittle leaf disease, salinity and drought at proteomic levels is described and discussed.

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5.1 Introduction

Date palm is an important plant that grown in several subtropical and tropical regions of the world, especially the Middle East. It has traditional and socioeconomic importance (El Hadrami and Al-Khayri 2012). Besides being a major income source for the producing country, date consumption is known to be beneficial for medicinal purposes because of its richness in tannins that relieve intestinal disorders. Roots are also used for toothache treatment, and pollen is rich in estrogenic compound such as estrone.

J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_5

Furthermore, leaves and roots are rich in nanocellulose and natural fiber (Alotaibi et al. 2019).

Date palm reproduction can be performed using either one of these three methods: (a) offshoot separation and transplanting, (b) seed propagation and (c) micropropagation. Micropropagation represents the fastest and the most reliable method to generate large numbers of plants (Hadrami et al. 2011), especially through somatic embryogenesis (Fki et al. 2011, 2017).

In order to estimate the quality of the regenerated seedlings from somatic embryogenesis and to evaluate the property of this biological system, powerful tools like genomic, transcriptomic and proteomics need to be used. Recently, protein signatures experimentally determined by proteomics are used to identify the phenotype, characteristics and properties of biological systems and processes. The coupling of gel electrophoresis with mass spectrometry represents a powerful method to study the somatic embryogenesis, seed maturation and germination, and plant response to biotic or abiotic stress (Jorrín-Novo 2020; Rey et al. 2019; Sghaier-Hammami et al. 2020). Advances in proteomics and other omics have and continue to contribute to crop and plant breeding programs through the identification of new gene products linked to desired agronomic traits and productivity (Jorrín-Novo 2020).

The present chapter describes studies of the proteome of zygotic and somatic embryos at different developmental stages. Proteins were extracted using the TCA phenol methods (Maldonado et al. 2008) and then separated by one and two electrophoresis gels. Differential proteins were subjected to matrix-assisted laser *desorption* ionization/time of flight (MALDI-TOF-TOF). The identification of proteins involved in the maturation and germination showed that somatic embryos lack those that are involved in the dormancy process and the storage proteins. The effect of the addition of different supplements (ABA, sucrose, arginine) to medium culture of somatic embryos showed an improvement of the protein content and especially the induction of storage proteins in treated somatic embryos. Proteomic approaches are also being used to study the response of the date palm seedlings to drought and salinity stress.

5.1.1 Proteomics Importance for Biological Research

The term *proteomics* was first given during the 1994 Siena meeting by Marc Wilkins, derived from *PROTein complement of a genOME* (Wilkins et al. 1996). The proteome should be understood as the total set of proteins or gene products present in a biological unit at a specific developmental stage and under determined external biotic and abiotic conditions. Proteomics allow the study of the proteome present in a biological assembly along with its description, quantification, genotype dependent variations, its implication in developmental and environmental related changes, post-translational modifications (PTMs), as well as its interaction with other proteins and molecular assemblies (Jorrín-Novo 2020).

Proteomics represents an important approach and a fundamental discipline in the postgenomic era, and it could answer the questions concerning production and function of proteins, such as regulation, mechanism of action, location and interaction with other proteins or molecules. This is of great relevance, taking into account that proteins are the molecules that exert the most relevant biological functions. Like genomic and transcriptomics approaches, proteomics incorporates highly developed techniques and protocols that made the analysis of a large number of proteins easier and faster (Wolters et al. 2001). The first generation of proteomics research used the two-dimensional (DE) protein separation coupled with mass spectrometry (MS) analysis of spots, and then, the second generation was based on the liquid chromatography (LC)-based shotgun strategies, and finally, the third generation was based on quantitative approaches including label and label-free variants (Jorrín-Novo et al. 2019). Therefore, it is advisable for novice proteomics users to start with the simplest

methodology using one-dimension (1D) gel electrophoresis (shown to be very important in the analysis of simple proteomes) and then move to the most sophisticated methodology such as 2-DE and gel-free. Traditional 2-DE coupled to MS is still the most frequently used platform (Heinemeyer et al. 2009).

All or most of the following steps may be included in a standard proteomic experiment, including the experimental design, sampling, preparation of tissue, cell or organelle, extraction and fractionation/or purification of proteins, labeling or modification, separation, MS analysis, identification of proteins and, finally, statistical analysis of data and validation (Jorrín-Novo 2014). Each biological system (i.e., plant species, organ, tissue, cells) has its characteristics, and each research subject has its objectives. Therefore, the most appropriate protocol should be used accordingly. In fact, a good experimental design is essential for the success of any proteomic experiment. For any differential expression proteomics design, a sufficient number of replicates are a prerequisite. It should be set up according to the dynamic nature of the proteome. Hence, a correct interpretation of the results could be reached and therefore a confident assignment of any protein as variable. This is very useful in case of the identification of the proteins as disease markers or as markers to develop plant breeding programs (Valledor et al. 2014).

5.1.2 Proteomics for Embryogenesis Studies

Somatic embryogenesis represents a successful technique for monocotyledon and dicotyledon multiplication. It is a good alternative for clonal propagation and breeding of elite plants that have limited multiplication using zygotic embryogenesis (Fki et al. 2003, 2017; Othmani et al. 2009). Several reports have described embryogenesis at the molecular level using proteomic and transcriptomic approaches (Aguilar-Hernández and Loyola-Vargas 2018; Gallardo et al. 2007; Imin et al. 2004; Roja Rani et al. 2005; Stasolla et al. 2004). A deeper knowledge of proteins involved in zygotic and somatic embryogenesis could be very useful for the improvement of the latter since the resulted seedlings are less vigorous than those from zygotic embryogenesis (Sghaier et al. 2008). Proteomics studies may also help in improving the quality of somatic embryo derived seedlings and in developing new strategies for plant multiplication using in vitro culture. In conclusion, proteins can be considered as biomarkers for embryo maturation, seed acquisition of desiccation tolerance and seedling vigor.

5.1.3 Proteomics for Plant Stress Research

The concept of *stress* is used by many biologists, but it is very elusive since it can be put in different contexts and in many ways in the scientific literature. It can further be attributed to the stressor (the environmental component) as well as to the stressed (the biological component). Environmental stresses can represent a physical response to a changing environment. The most deleterious environmental stresses, for example, are those that result from naturally occurring or man-made changes in abiotic factors including temperature, other climatic factors and chemical components. Biotic agents, like bacterial, fungal, algal and viral diseases, may also cause biotic stress in plants. Hence, plants have developed several adaptive mechanisms to confront these stresses. It is now becoming widely known that proteins are the main mediators of this resistance by playing a major role in the activation of genes and thus the control of the genome leading to physical features (such as in xerophytes) or by directly defending the plants from the stressors (i.e., antioxidant enzymes and chapronins) or indirectly (like key enzymes in osmolyte synthesis) (Bona et al. 2007; Pandey et al. 2008; Sghaier-Hammami et al. 2013; Xiong et al. 2017).

5.2 Importance of Date Palm and Methods of Multiplication

Date palm (*Phoenix dactylifera* L.), mostly cultivated in both Old World (Middle East and North Africa) and New World (American continent), constitutes the major crop in arid and semiarid areas, mainly in the regions of Southwest Asia and North Africa. It is a very important species that belongs to the palm family (Arecaceae) and has about 200 genera and more than 2500 species (El Hadrami and Al-Khayri 2012).

In addition, to the impractical multiplication of date palm via seed propagation (sexual propagation), offshoot propagation also (asexual or vegetative propagation) produces a limited number of plantlets (20–30 at most) depending on the variety, fertilization treatment, irrigation and earthing-up around the trunks (Zaid and De Wet 2002). Therefore, in vitro culture represents an effective potential alternative for date palm multiplication (Fki et al. 2003, 2017); especially somatic embryogenesis which is well-established in date palm (Al-Khayri 2003). However, somatic embryos seedlings are less vigorous than those raised from natural seed. The poor vigor of somatic embryo (SE) derived seedlings seems to be related to their incomplete maturation process (Roberts et al. 1990). Moreover, date palm SE differs from the zygotic embryos (ZE), as it lacks a seed integument and endosperm (Fig. 5.1), which are critical for seed survival and germination (Brownfield et al. 2007).

A more thorough investigation of the proteins that are involved in zygotic embryogenesis would be very useful for the improvement of micropropagation techniques of elite genotypes that represent a challenge for date palm and other woody plant species (Cairney and Pullman 2007; Chin and Tan 2018).

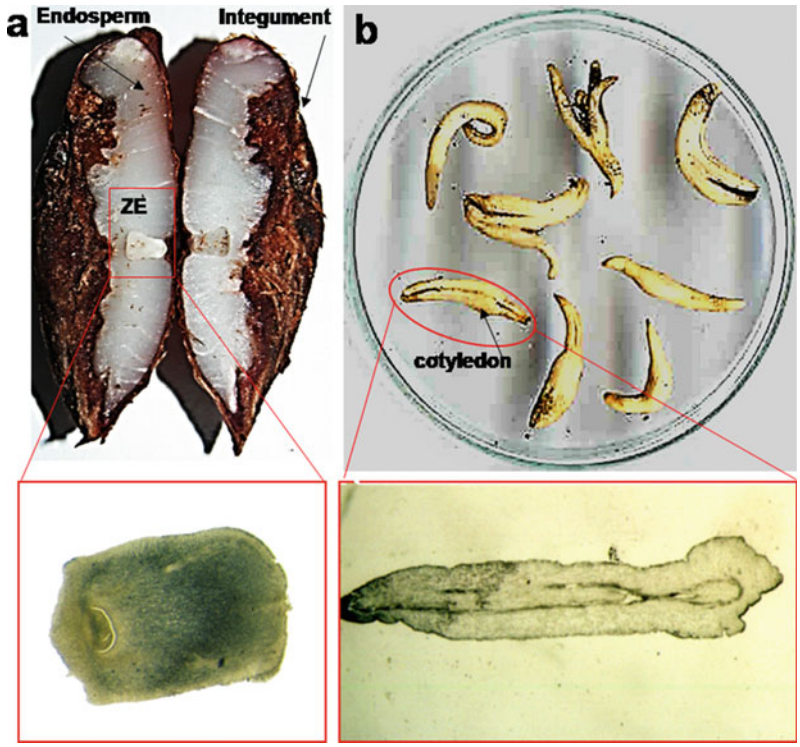

Fig. 5.1 Date palm embryos. **a** Zygotic embryo (ZE) in the middle of the seed and below it histological section, **b** Somatic embryo (SE) resulted from somatic embryogenesis and its histological section (Photos by B. Sghaier-Hammami)

5.3 Proteomics Study of Date Palm Zygotic Embryo During Development, Maturation and Germination

In order to define specific markers that characterize the different phases of zygotic embryogenesis, the accumulation of total proteins during ZE development (12–17) weeks after pollination (WAP) until maturation stage (23 WAP) and during germination (9–15 days of germination (DG) were monitored (Sghaier-Hammami et al. 2009a). Proteins (500 mg) were separated by 2-DE with IEF carried out at the 5–8 pH range (Fig. 5.2).

Following a traditional univariate analysis made with 2-DE data, 194 spots, differentially expressed in the different developmental stages, were determined. They were either qualitative or quantitative. Sixty-five variable spots, including those that showed qualitative and quantitative changes underwent through MALDI-TOF analysis, using the nonredundant National Center for Biotechnology Information (NCBI).

Seed development and germination showed that storage proteins (glutelin, prolamins) were accumulated during embryogenesis and then consumed during germination. Protein content declined during germination, especially from 9 DG–12DG (Fig. 5.2), which corresponds with the emergence of the cotyledonary leaf (Sghaier-Hammami et al. 2009b). These findings agree with those reported by Lai and McKersie (1994) and Sghaier-Hammami (2020), which revealed that starch reserves and storage proteins were quickly hydrolyzed following the germination of embryos.

Starch reserves were accumulated at the early embryogenic stages which indicate an active starch synthesis during embryonic development. After germination, changes in energy metabolism

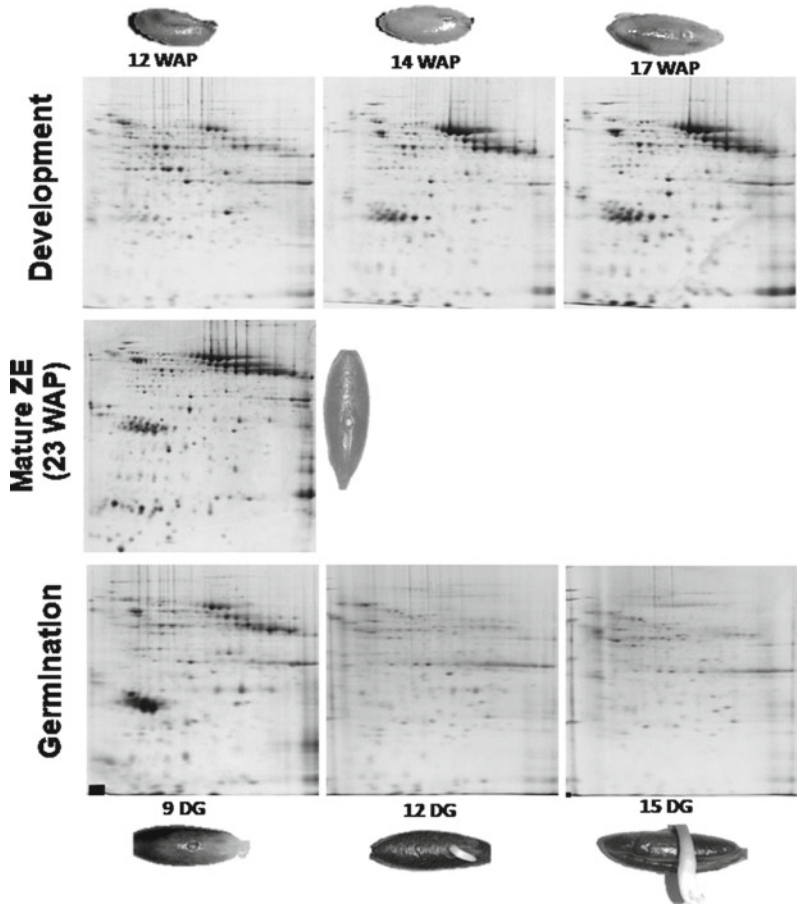

Fig. 5.2 Real gel 2-DE map between zygotic embryo at different stages development, once mature and during germination; 500 μg of protein was separated in the first dimension on an immobilized, linear 5–8 pH, gradient then separated in the second dimension on a 12% acrylamide-SDS gel. Gels were stained with Coomassie (Photos by B. Sghaier-Hammami)

(from fermentation to respiration) were observed during zygotic embryogenesis. The evolution of glycolytic and tricarboxylic acid cycle enzymes, during seed development, indicates active energy metabolism during embryogenesis.

Stress-related proteins (HSP family) are highly expressed during the early developmental stages and then decreased at germination stages. These members of the HSP family are highly expressed during zygotic embryogenesis (DeRocher and Vierling 1994).

To conclude, dramatic changes in the ZE proteome during embryogenesis and germination levels were observed. ZE continuously accumulates proteins from early to late embryogenesis stages, and these values are maximal at mature or close-to-mature embryos. A comparative study between protein content of date palm ZE and SE will be of great interest to explain the weakness of SE seedlings.

5.4 Comparison Between Protein Content of Zygotic and Somatic Mature Embryos

Somatic embryogenesis could be a good alternative for clonal multiplication, crop improvement and breeding of recalcitrant plant species (Merkle et al. 1995). However, SE seedlings are less vigorous than those raised from true seed. The quality of SE seedlings could be improved using proteomics, and a new in vitro culture for plants propagation and manipulation could be developed. A comparative proteomic study between ZE and SE protein contents was carried out.

Using SDS-PAGE and 2-DE (Sghaier et al. 2008, 2009), different proteins from ZE and SE were analyzed and identified in both embryo types (Fig. 5.3).

SDS-PAGE analysis showed a poor protein profile for SE, compared to that of ZE. Data analyzed using 2-DE electrophoresis revealed that ~60% of the spots were differently expressed in SE and ZE (335 spots out of the total 559). The variable spots were either absent in ZE or SE (qualitative variable spots; in number of 263) or differentially accumulated between the two types of embryos (quantitative variable spots; 72 spots). The differential proteins (63 variable spots) were subjected to MALDI-TOF-TOF mass spectrometry analysis combined with the nonredundant NCBI.

Most of the identified somatic embryo specific proteins belong to glycolysis pathways and amino acids metabolism, whereas those of the zygotic embryos belong to a mixture of proteins

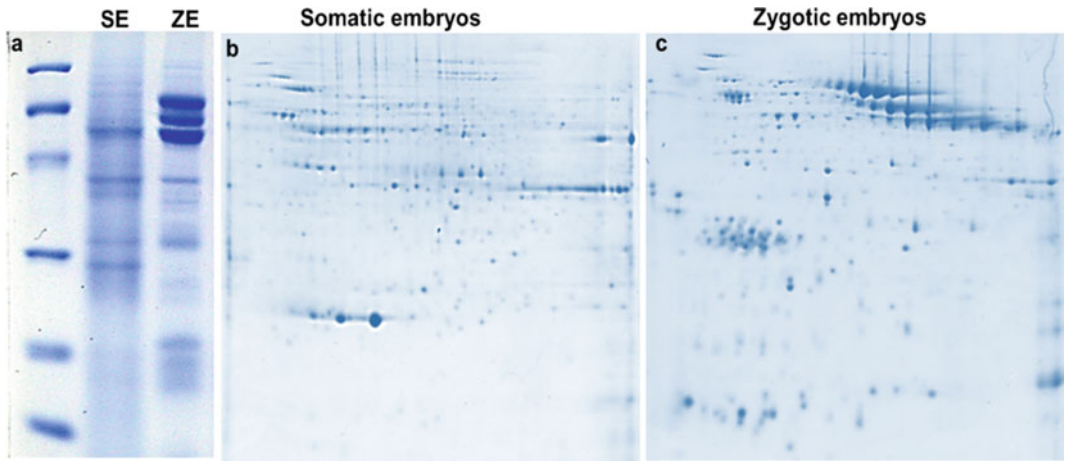

Fig. 5.3 Analysis of total proteins from mature somatic embryos (SE) and zygotic embryos (ZE) using SDS-PAGE (**a**), and 2DE (**b**, **c** for SE and ZE, respectively), 500 μg of protein was loaded on strips (5–8 pH gradient) and then separated on a 12% acrylamide-SDS gel. Gels were Coomassie-stained (Photos by B. Sghaier-Hammami)

families (storage and stress-related proteins, carbohydrate biosynthesis) (Sghaier et al. 2009). Hence, the involvement of more proteins in energy metabolism and ATP demand is being required by the SE compared to the ZE, which may facilitate rapid germination in the former without undergoing the dormancy phase.

Glutelin proteins are abundant in ZE and absent in SE, which indicates that the SE lacks reserve proteins compared to the ZE. Stress-related proteins (HSP family) are more abundant in ZE than in SE. Their accumulation was most likely induced as a consequence of seed dehydration and mediated desiccation tolerance acquisition (Karuna Sree et al. 2000). According to the previously mentioned results, HSP can be used as a maturation marker of ZE. In contrast, in SE, the degree of maturation is different which may cause desiccation sensitivity. This may allow the SE to enter rapidly in germination without undergoing the dormancy phase. For that, plantlets that are derived from SE are less vigorous than those coming from ZE (Roberts et al. 1990). Overall, a low rate of protein accumulation observed in somatic embryos was due to a lack of precursors in the medium (Komamine et al. 1992; Misra et al. 1993). It may also be the result of the absence of inducing signals (e.g., hormones and/or desiccation), which are required to stimulate the synthesis of specific molecules.

5.5 Effect of Abscisic Acid, Sucrose and Arginine on Somatic Embryo Protein Content

Maturation of somatic embryos may be induced by the application of exogenous abscisic acid (ABA), which promotes embryonic maturation and supports the accumulation of storage proteins (Corredoira et al. 2003). In addition, previous studies showed that the addition of a high concentration of sucrose to the culture medium of cucumber (Lou et al. 1996) and melon (Nakagawa et al. 2001) can enhance the induction of somatic embryos. Sghaier et al. (2009) and Sghaier-Hammami et al. (2010) described the effects of additives (ABA, arginine, sucrose) on the induction and accumulation of proteins of date palm somatic embryos reported in the current chapter. Indeed, ABA, arginine and sucrose were used separately as previously described by Morcillo (1998). There was no difference in length and width between all the treated embryos whatever the treatment used except for the 20 µM ABA treatment, which caused a significant width increase by around 1.5 times more than the untreated ones (Fig. 5.4).

Proteomics analysis showed that ABA arginine and sucrose have an important effect on date palm protein content. The amount of total proteins increased significantly after almost all treatments, and especially in those treated with 20 µM of abscisic acid (ABA), where a double amount of proteins was observed. Although, the different treatments significantly enhanced protein synthesis, still the reached levels were much lower than those found in the date palm ZE (120 mg g^{-1} FW) as previously described (Sghaier et al. 2009).

The 1-D and 2-DE protein profiles showed qualitative and quantitative differences between the untreated and treated SE (Fig. 5.5).

All 34 variable spots were subjected to MALDI-TOF-TOF mass spectrometry analysis, including those that showed qualitative and quantitative changes with the highest maximum/minimum value ratios. Identified proteins belong to the following functional categories: energy metabolism, protein translation, folding and degradation, redox maintenance, cytoskeleton and storage proteins. The major proteins implicated in energy metabolism (glycolysis, citrate cycle), protein translation, folding and degradation, redox maintenance and cytoskeleton were downregulated in SE treated by ABA or sucrose. Most proteins that belong to the translation and degradation categories were suppressed under the influence of sucrose. In contrast, storage proteins were more abundant in SE treated by ABA than the untreated ones. Arginine treatment (10 mM) led to the appearance of new proteins homologous to peroxyredoxine and 7S globulin. In fact, addition of nitrogen sources into the culture medium

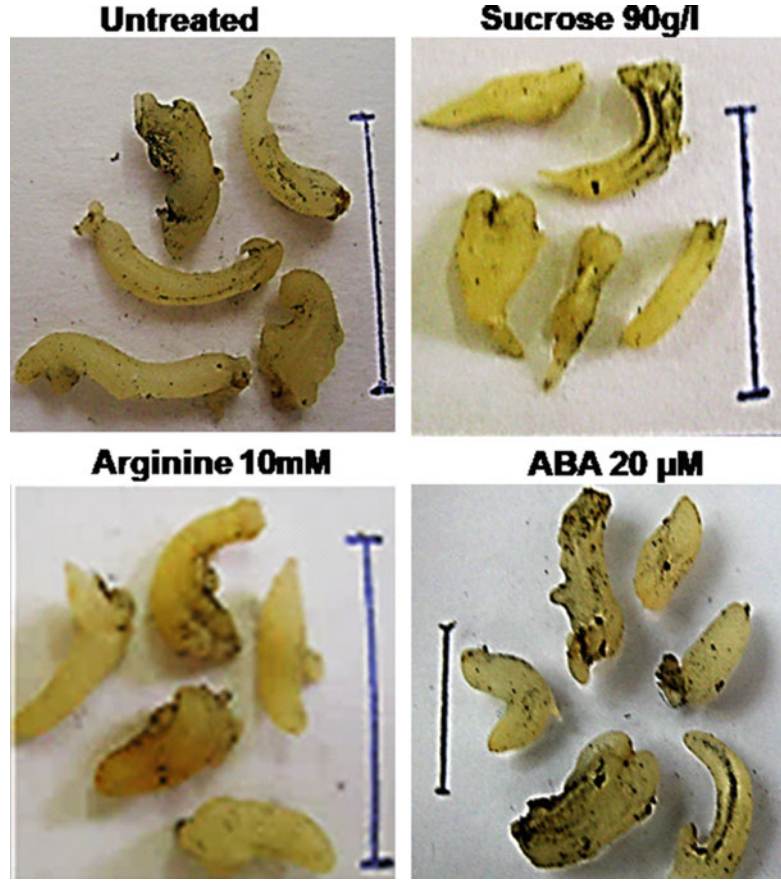

Fig. 5.4 Photos of somatic embryos matured in culture medium supplemented with sucrose, arginine and ABA (bars = 1 cm) (Photos by B. Sghaier-Hammami)

enhances storage protein accumulation and has positive effects on somatic embryogenesis in oil palm and other species (Lai and McKersie 1994; Morcillo et al. 1999).

To conclude, the application of additives contributed to the activation of storage and defense protein synthesis as well as the inhibition of other metabolic pathways that can be involved with the seed. Therefore, this would allow the preservation of the embryo in an anabolic phase. This can be confirmed by the number of down-regulated proteins which were more important than the upregulated ones for all the treated SE. Although additive treatments did not reach maximal efficiency to increase SE quality, a higher germination capacity could be acquired, and better conversion rates into a vigorous plantlet similar to those derived from the ZE was reached.

5.6 Proteomics Studies of Date Palm Response to Brittle Leaf Disease

As do most crops, date palm culture suffers from several destructive diseases. Brittle leaf disease is classified as one of the most serious. Indeed, since its appearance in Tunisia in 1980, it became more serious and epidemic, spreading to nearby regions such as Eastern Algeria (Saadi et al. 2006; Triki et al. 2003). According to previous studies, three stages of brittle leaf disease (BLD) were defined: a) Stage S1: characterized by chlorosis of a few fronds, b) Stage S2: leaflets become brittle, twisted and frizzled and c) Stage S3: the entire plant stops growing and finally dies. The timeline of the development of the disease could last 4–6 years from the appearance

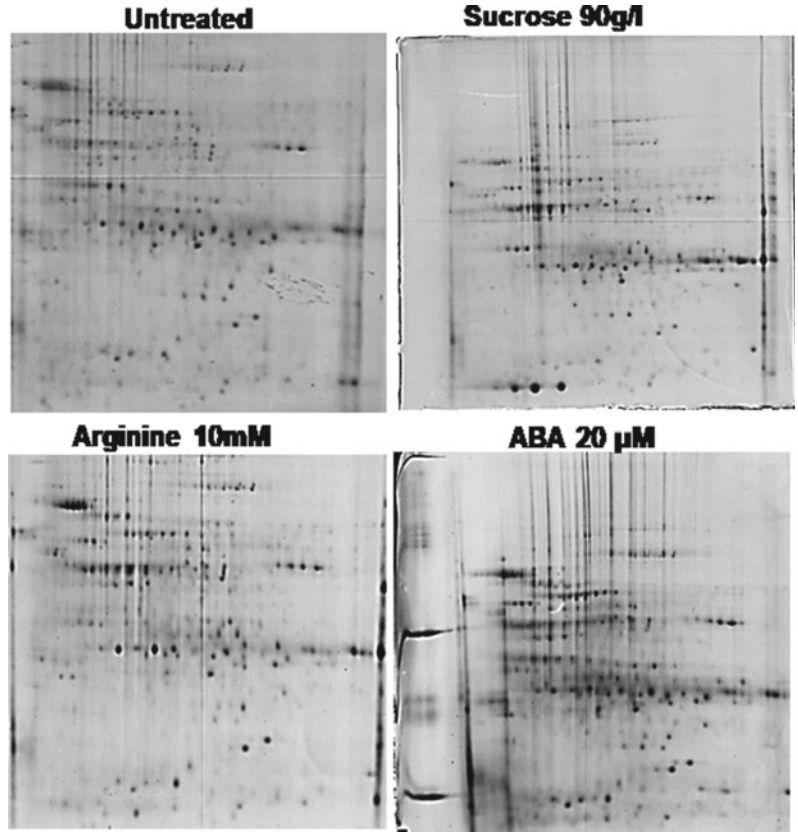

Fig. 5.5 Areal gel 2-DE map of untreated and treated SE by sucrose, arginine and ABA. 400 µg of protein was separated using the first dimension gel on an immobilized, linear, 5–8 pH gradient and in the second dimension on a 12% acrylamide-SDS gel. Gels were Coomassie-stained (Figure constructed by B. Sghaier-Hammami)

of the first symptoms (S1) to the death of the tree (S3).

Although, the BLD epidemiology could be caused by a pathogen, no biotic agent has yet been identified as the main causal agent (Triki et al. 2003). Leaflets of affected palms have been shown to contain significantly lower manganese concentration than those of healthy controls (HC). Furthermore, soils where BLD develops contain about one-half the manganese content compared to normal soils (Namsi et al. 2006).

Proteome of BLM-affected palms leaflets was reported in other studies (Marqués et al. 2011; Sghaier-Hammami et al. 2012). Different proteins were separated by 2-DE at the three stages, with IEF carried out at the 3–10 pH range (Marqués et al. 2011) and at 5–8 pH range (Sghaier-Hammami et al. 2012). The separation of proteins at the 5–8 pH range seems to be more efficient than the 3–10 pH range since the use of this latter resulted in obtaining protein spots that are concentrated in the 5–8 pH regions (Gómez-Vidal et al. 2009).

The 2-DE protein profiles of the HC and BLD-affected palm leaflets showed 364 resolved spots in the master gel (Fig. 5.6), and most of them (297 spots) were variable between samples (Sghaier-Hammami et al. 2012). The 2-DE protein profile of the HC seems to have less protein spots than BLD-affected palm leaflets at S1, S2 and S3 stages, which may be explained by an easier extraction of proteins from the affected leaves by BLD. The number of proteins in BLD-affected palm leaflets at S2 were higher than those affected at S1 and S3 (Fig. 5.6).

The major group of identified proteins corresponded to chloroplastic metabolism (60%). They belong to photosynthesis and the Calvin cycle, carbohydrate metabolism, amino acid biosynthesis, protein fate (synthesis and turnover), stress-related proteins and cytosqueleton. Actually, proteins belonging to photosynthesis

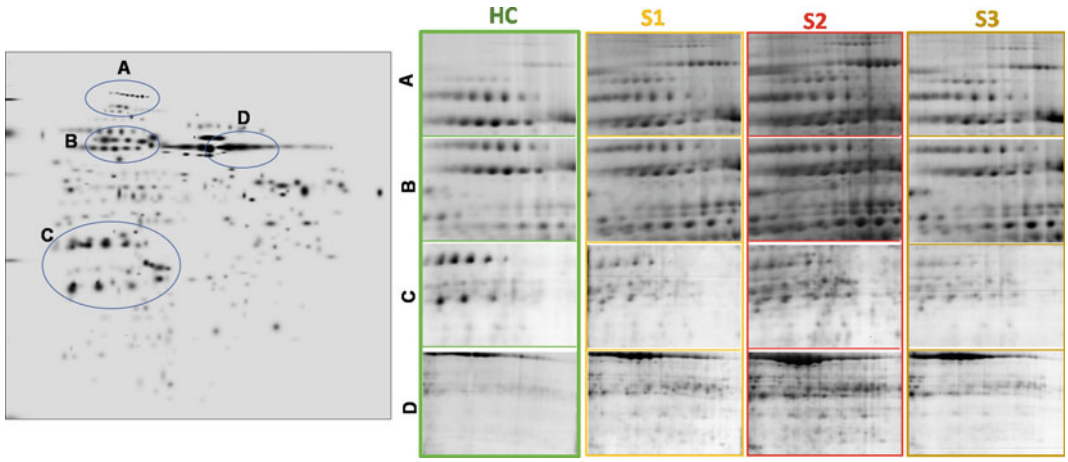

Fig. 5.6 Representative 2-DE master gel of healthy control (HC) and diseased leaves representing different disease stages (S1, S2 and S3). 500 μg of proteins were separated by 2-DE, with IEF carried out in the 5–8 pH range. Gels areas (**A**–**D**) are magnified to visualize different protein spots between samples (Figure constructed by B. Sghaier-Hammami)

electronic chain had a low intensity in the different BLD-affected palm leaflets at the three stages of disease. On the other hand, proteins belonging to proteolysis group and stress-related proteins increased in BLD-affected palm leaflets and especially at stages S2.

Seven RubisCO protein species were identified, where six of them seem to be a product of protein degradation processes (Sghaier-Hammami et al. 2012). The observed leaf chlorosis during the S2 disease stage was most likely related to the RubisCO degradation. Previous studies (Saidi et al. 2012) showed that BLD led to a decrease in the photosynthetic activity and the reduction of chlorophyll content. This may be tightly related to the increase of protease proteins observed in the BLD-affected palm leaflets (Sghaier-Hammami et al. 2012). Similarly, Adam et al. (2006) demonstrated that chloroplast proteases may participate in chloroplast biogenesis during adaptation to environmental condition changes through the degradation of some proteins.

Other proteins (MSP-33 kDa subunit) that belong to photosynthetic chains decreased in BLD-affected palm leaflets. The manganese-stabilizing protein (MSP-33 kDa) subunit has a fundamental role in the maintenance of the integrity and the activity of the manganese cluster (Seidler 1996). Furthermore, as was reported previously the decrease in the MSP-33 kDa protein subunits and the luminal oxygen-evolving system (OEC) proteins (PSBO2, PSB, PSBP, PSBO1) corroborates the hypothesis of the relation between the MFC and manganese deficiency (Marqués et al. 2011). We thus suggest here, that the decrease in MSP-33 kDa protein subunits and the degradation of RubisCO proteins can be used as biomarkers of manganese deficiency BLD-affected palm leaflets.

The induction of stress-related proteins is an immediate response of the plant to stress. The chloroplastic heat shock proteins (Hsp70 kDa), the chaperonin 60 subunit beta1 and the peroxiredoxin proteins were reported to increase in the BLD-affected palm leaflets. Additionally, transcriptomic analysis demonstrated that Hsp70kDa-related cDNA was upregulated in BLD-affected palm leaflets (Saidi et al. 2010).

The chaperonin 60 subunit beta 1 has been reported to be implicated in cell death and systemic acquired resistance and to play a role in the acclimation of photosynthesis to heat stress, possibly by protecting Ribulose-1.5-bisphosphate carboxylase/oxygenase large subunit (RubisCO) activase from thermal

denaturation (Salvucci 2008). In addition, plant 2-Cys peroxiredoxins are targeted to chloroplasts following post-translational modifications in which they have a fundamental role in protecting the photosynthetic membrane against photooxidative damage (Baier and Dietz 1997, 1999).

To summarize, in the present proteomics, the BLD disease work was thoroughly investigated during disease development from the earliest to the latest stages (demise of the date palm tree). Changes in the 2-DE protein profile start at early disease stage (S1), in which a decrease in MSP-33 kDa subunit proteins was observed. At the S2 stage, a degradation of the RubisCO proteins was found when leaflets became chlorotic. This showed that this disease is not only specific to manganese deficiency, but it confirms that BLD is an abiotic stress.

5.7 Proteome Analysis of Response to Salinity and Drought

Salinity and drought represent the two major environmental stresses that adversely affect crop production in the world. Date palm responds differently to both types of stresses. The palm is endowed with great tolerance to extreme drought, and relatively high levels of soil salinity (Yaish and Kumar 2015). Proteome analysis are a convenient tool for testing the response of these plants to abiotic stress (Fercha et al. 2014; Mostek et al. 2015). Indeed, this technique was used previously by other investigators in 18-month-old date palm seedlings under severe salt (48 g/L NaCl) and drought (without irrigation or 82.5 g/L PEG) stresses during 1 month (El Rabey et al. 2016).

Drought stress induces the accumulation of photosynthesis-related proteins (RubisCO) and glycolysis pathways, whereas salt stress (NaCl) induces the accumulation of stress-related proteins (chaperonin proteins) and inhibits eight proteins involved in photosynthesis. Under the above conditions, the levels of ATP synthase CF1 alpha chain were significantly changed. Salt stress and severe drought stress (without irrigation) caused changes in the abundance of RubisCO activase and one of RubisCO's fragments in the same spots. A high concentration of NaCl had an inhibitory effect on the date palm biosynthesis.

In conclusion, following proteomic analysis, drought and salt stress were found to cause differential expression of genes that resulted in high or low protein abundance of the chosen protein spots. In addition, drought stress under lack of irrigation caused inhibition of the expression of all genes controlling drought tolerance.

5.8 Conclusions and Prospects

Proteomics, like other omics technologies, including transcriptomics and metabolomics, is currently playing a very important role in plant research. The combined use and integration of all of them, in the systems biology direction, will allow a deeper understanding of the different biological processes, to identify key genes linked to and relevant in plant productivity, fitness and resilience. In fact, ~80% of the researches in the plant field use this approach frequently and with successful results.

This chapter highlights the importance of the use of the proteomic approach in dissecting the proteome of the date palm somatic or zygotic embryo and the characterization of the proteins involved in stress response. Using proteomic approaches, we have shown the following:

(a) During zygotic embryo development and germination, storage proteins and stress-related proteins were most abundant during the early developmental stages and then their presence decreased during germination.
(b) Somatic embryo contains lower proteins content than zygotic embryo and lacks proteins involved in the maturation process like storage proteins and proteins related to stress tolerance which may be due to the lack of precursors in its medium culture.
(c) The application of exogenous abscisic acid (ABA), sucrose and arginine to medium culture of somatic embryos improves their content in proteins, especially those related to storage and stress.
(d) Upon abiotic stress, date palm leaves and roots showed a reduction in proteins related

to energy metabolic pathways, especially of photosynthesis and the induction of proteins relation to stress and defense.

Future research on date palm somatic embryogenesis should be based on other proteomics platforms, such as shotgun, to increase the proteome coverage as well as its integration with other omics and classic approaches in the system biology direction. The comparison between zygotic and somatic embryos will reveal which genes and gene products determine the difference and could be considered as markers of viability and vigor. In addition, new chemicals should be tested and incorporated into the in vitro culture medium at the different developmental stages, getting the somatic embryo proteome closer to that of zygotic, and ensured the success in the propagation program.

Acknowledgements The authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through the Small Research group project under grant number (R.G. P.1/195/41). This work was supported by the Tunisian Ministry of Higher Education, Scientific Research and Technology (LR19CBBC02). Dr. Besma Sghaier-Hammami was supported by the project Program d'Encouragement des Jeunes Chercheurs (19PEJC07-17).

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Date Palm Metabolomics

6

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Abstract

Metabolomics identify and analyze, in a comprehensive and high throughput manner, all the metabolites of an organism including amino acids, organic acids, sugars, fatty acids, lipids, steroids, small peptides, vitamins and phenolics. By measuring global sets of low molecular weight metabolites, metabolomics provide a *snapshot* readout of metabolic activity status in relation to the genetic makeup of the variety, its natural gene expression or its response to external stimuli encountered where it is grown. When the snapshot readout can be associated with the outcome phenotype (e.g., healthy vs diseased; high vs low fruit sugar content), more high-throughput analytical techniques and tools can be combined with metabolomics to provide a better understanding of the physiological processes occurring in the studied variety. In date palm, the metabolome representing the pool of small molecules chemically distinct through their structure (fingerprint) or biochemically involved in certain pathways (precursors, substrates, products) has traditionally been used to identify biomarkers for diagnosis and prediction. In recent years, metabolomics has become essential not only to the identification of simple biomarkers but as a technology for discovering actives that drive physiological processes, help create new cultivars with desired attributes, or add value to harvested dates.

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6.1 Introduction

Metabolomics is an emerging science that allows for a comprehensive identification and analysis of metabolites produced by an organism. These include amino acids, organic acids, sugars, fatty acids, lipids, steroids, small peptides, vitamins and phenolics. Depending on the nature of the experiment or analysis, the readout can provide either a snapshot of response to a specific growing condition or an over-time variation linked to a change in the environment. Given the wide range of molecules targeted in the analysis

J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_6

markers and identifiers that remain stable regardless of the growing conditions or the environmental stimuli exerted on the variety become identifiers of the variety. However, the fraction of metabolites that changes in response to specific environmental stimuli can be correlated to a specific condition and become a marker of specific physiological condition. For instance, comparing the metabolome of a healthy versus diseased plant from the same variety can help establish a fingerprint that can be used for either diagnosis or prediction. Likewise, the analysis of the metabolome at a critical stage of growth and development such as the transition from vegetative to reproductive can create a signature that can be used to predict flowering and maturity. When comparing various varieties with different fruit attributes one can establish correlations between the phenotype and the metabolic profiles detected.

This chapter provides an update on the progress made studying date palm using metabolomics. It also explores the various ways metabolomics can help to identify biomarkers and discover actives or pathways that could guide the breeding programs in creating cultivars with desired attributes that could improve the value added of dates.

6.2 Metabolomics: Principles and Expected Outcomes

Metabolomics is the science that analyzes the metabolome within the cells and tissues of a living organism (Fig. 6.1). It qualitatively identifies and quantitatively analyzes, in a comprehensive and high throughput manner, all endogenous and exogenous low molecular weight metabolites or molecules of the organism studied. These include amino acids, organic acids, sugars, fatty acids, lipids, steroids, small peptides, vitamins, phenolics and other metabolites (Diboun et al. 2015; Stephan et al. 2018). Metabolomics have several applications, especially in date palm, in terms of metabolic phenotyping of varieties, adding value to the dates, deciphering their compositions and highlighting health benefits and nutritional values, or, alongside other integrative omics, helping improve the

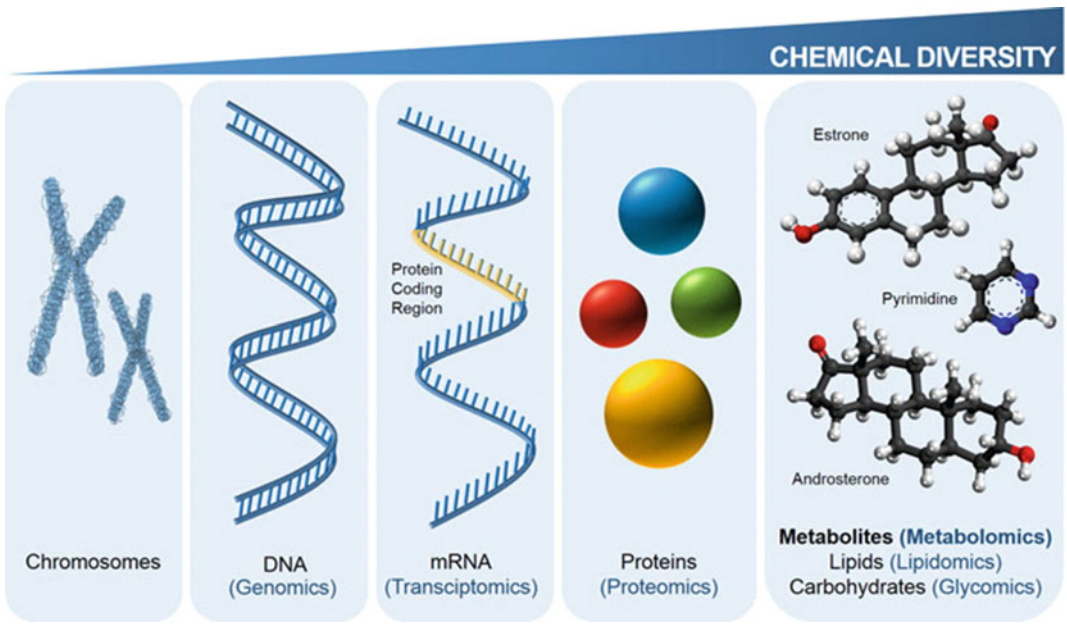


Fig. 6.1 Metabolites as active modulators of genes and proteins activity. *Source* https://www.vanderbilt.edu/cit/introduction-metabolomics-research/

biotechnology and bioengineering of new cultivars or hybrids (Al-Khayri et al. 2017; El Hadrami et al. 2011a; Stephan et al. 2018).

In many plants, including date palm, the metabolome representing the pool of small molecules chemically distinct through their structure (fingerprint) or biochemically involved in certain pathways (precursors, substrates, products) has traditionally been used to identify biomarkers for diagnosis and prediction (Hamed et al. 2017; Riekeberg and Powers 2017). However, in recent years, the value of metabolome analysis (metabolomics) has gone far beyond the identification of simple biomarkers to become a technology for discovering actives that drive physiological processes, help create new cultivars with desired attributes or add value to harvested dates. This stems from the fact that the metabolome affects cellular physiology through the modulation of the genome, proteome and transcriptome.

By analyzing amino acids, organic acids, sugars, fatty acids, lipids, steroids, small peptides, vitamins and other secondary metabolites (El Hadrami et al. 2011b), metabolomics provide a *snapshot* readout of metabolic activity status in relation to the genetic makeup of the variety, its natural gene expression or its response to external stimuli encountered where it is grown. When the snapshot readout can be associated with the outcome phenotype (e.g., healthy vs diseased; high vs low fruit sugar content), more high-throughput analytical techniques and tools can be combined with metabolomics to provide a better understanding of the physiological processes occurring in the studied variety (Fig. 6.2).

6.2.1 Why Apply Metabolomics Approaches to Studying Host *x* Environment Pathways?

Since the measured metabolites can reflect alterations or dysfunctions in the metabolic fluxes of tissues and cells in the plant, metabolomics is an appealing application to evaluate the host x environment interaction and help the breeding process. In case of disease, metabolomics can identify biomarkers of resistance or susceptibility to elucidate the pathways involved. Important limitations do exist, although with regard to the experimental design restrictions and the fact that the metabolic profile of an organism is dynamic. Metabolomics experiments are also restricted by methodological challenges linked to

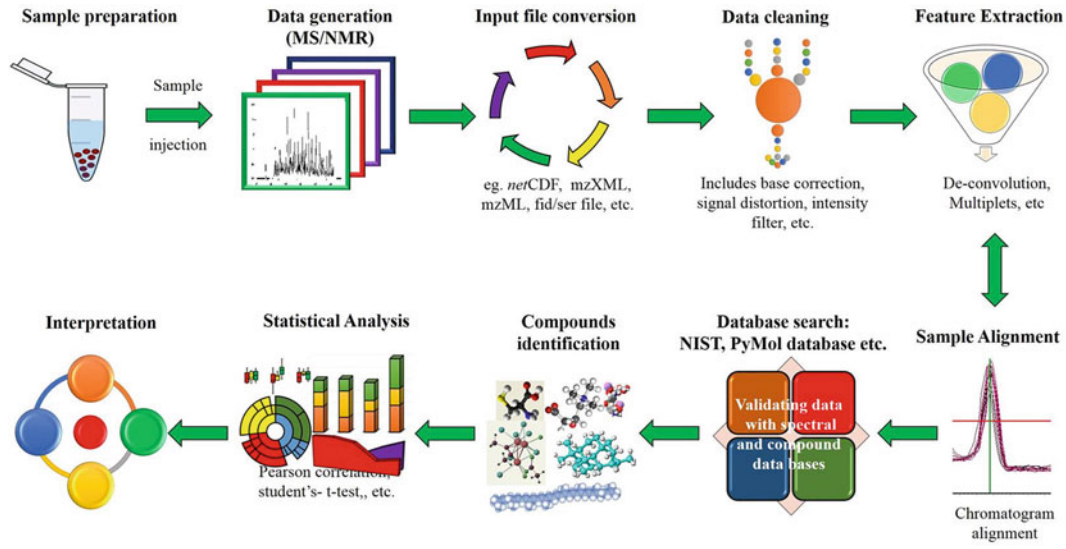


Fig. 6.2 Example of metabolomics platform. *Source* Kumar et al. (2017)

the very low concentrations of the detected molecules, the variability and lack of standardized sampling, as well as the complexity of methods used to process and analyze the data.

6.2.2 Metabolomics Experimental Approaches

Metabolomics approaches can be either targeted to test a given hypothesis or untargeted for an open discovery (Fig. 6.3). The complexity of sample preparation, precision in the experimental design, level of quantitation and number of metabolites detected can be different between the two approaches. Untargeted metabolomics provides impartial quantitative and qualitative analyses of the metabolites of an organism. It faces the challenges of detecting and analyzing unknown metabolites present at low quantities. Targeted approaches, on the other hand, take advantage of focusing on known biochemical pathways, the enzymes involved and end products. For both methods, the development of the triple quadrupole mass spectrometer (TQMS) had made it possible to detect and identify low-abundance molecules and metabolites. In addition, it allows an optimization of the samples so that the dominance of high-abundance metabolites can be reduced.

6.2.3 Metabolomics Analytical Technologies

Metabolomics rely on the use of two main analytical methods: nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). NMR is a spectroscopic technique that measures energy absorption and reemission by an atom's nuclei in response to a variation in the external magnetic field (Farag et al. 2012a; Marshall et al. 2015). It produces spectral data, which allow both the quantification of concentrations and the characterization of chemical structure of metabolites. Mass spectrometry, on the other hand, acquires spectral data in the form of a mass-to-charge ratio (m/z) and a relative intensity of the ionized compound (Draper et al. 2009). MS-based metabolomics is generally combined with a separation step to reduce the complexity of the biological sample and allow the analysis of various sets of molecules at different times (Alonso et al. 2015). The most common separation techniques used are liquid chromatography (LC) and gas chromatography (GC), termed LC-MS and GC-MS techniques (Farag et al. 2012a, b; Theodoridis et al. 2011). The choice of metabolomics techniques is often based on the objective of the study, the type of sample and resource availability (Farag et al. 2014).

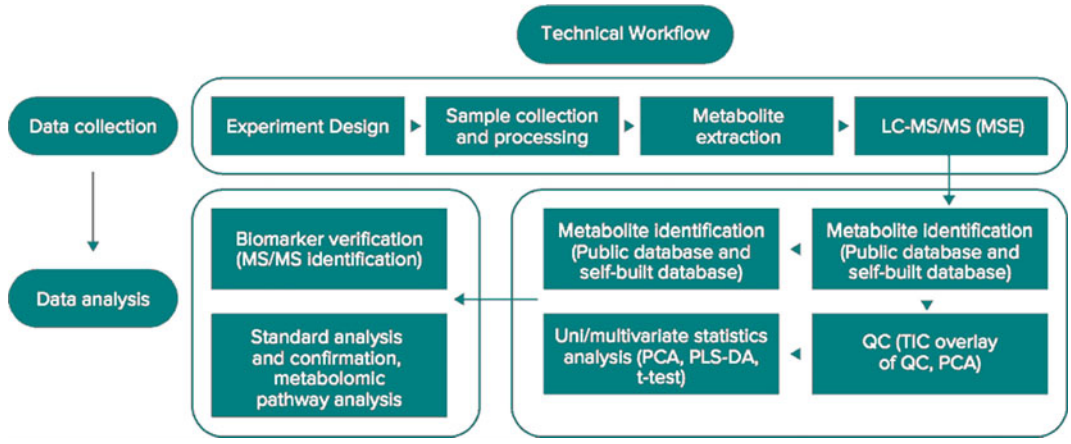


Fig. 6.3 Workflow for metabolomics analysis. *Source* https://www.vanderbilt.edu/cit/introduction-metabolomics-research/

NMR requires limited sample preparation and produces spectra that directly and linearly correlate with metabolite concentration (Beckonert et al. 2007), which makes it highly selective and reproducible. However, NMR sensitivity is relatively low and in only the most abundant species can generally be detected (Beckonert et al. 2007; Farag et al. 2012a, b; Theodoridis et al. 2011). Mass spectrometry, applied to adequately prepared samples that went through separation by chromatography, can also generate a specific and highly sensitive signal. This signal is also dynamic and very useful targeted metabolomics. However, quantification of metabolomics with MS is still one of the weaknesses as it is dependent on the type of sample preparation and the molecular environment, both of which affect signal intensity (Theodoridis et al. 2011).

6.2.4 Metabolomics Data Preprocessing and Analysis

Metabolomics data processing and analysis is still a challenge. Some of these challenges are common to other omics while others are unique to metabolomics. These include the multidimensional nature of the collected data, the use of multiple analytical methods (e.g., NMR, LC-/GC-MS), the high degree of collinearity between features, nonlinearity and non-normality of the data, the treatment and handling of missing data.

High-throughput NMR and MS spectral data need to undergo pre-processing in order to ensure their quality and replicability (Berg et al. 2006). Once the metabolite is detected, quantitative and qualitative data analysis can be performed (Fig. 6.4). Parametric and nonparametric statistical tests can be applied to identify biomarkers associated with the outcome of interest (Bartel et al. 2013). Other multivariate analyses such as principal component analysis (PCA), hierarchical cluster analysis (HCA) and self-organizing map (SOM) are also often used to classify data in clusters and highlight patterns while testing the robustness of the data (Misra and Van der Hooft 2016; Worley and Powers 2013). Supervised methods such as partial least square discriminant analysis (PL-SDA) and orthogonal PLS-DA (Barker and Rayens 2003; Trygg and Wold 2002) can be applied both to concentration tables and spectra for discriminating between disease phenotypes and endotypes, prediction and biomarker identification. In large samples, more sophisticated data mining such as hidden Markov and Bayesian chains, support vector machine (SVM), random forest and neural network are used to identify and predict biomarkers (Bickel et al. 2009; Trygg et al. 2007).

6.2.5 Metabolomics Databases

A series of public and private metabolomics databases has been developed in recent years to serve as a repository for the massive datasets of proteins, peptides, nucleic acids and other biomolecules resulting from metabolomics studies. These databases (e.g., UniProt, EntrezGene, ENZYME, CO, KEGG, LMPD, GMD) help store the MS, LC-/GC-MS and NMR information of metabolites and allows researchers to browse, search, retrieve and annotate new structures and molecules to ultimately permit their identification (Fig. 6.5).

6.3 Metabolomics in Date Palm

6.3.1 Metabolomics Activity Screening for Identifying Metabolites That Modulate Phenotype

Dates have important nutritional and health benefit values (El Hadrami and Al-Khayri 2012; El Hadrami et al. 2011a) that warrant an investigation of their metabolomics profile and the composition of their fruits and ripening processes. Previous attempts at characterizing dates metabolome were constrained by the size of samples analyzed and the limited access to a wide set of varieties representing the major geographical areas where date palm is grown. Diboun et al. (2015) used two large cohorts of mature dates exhibiting substantial diversity in

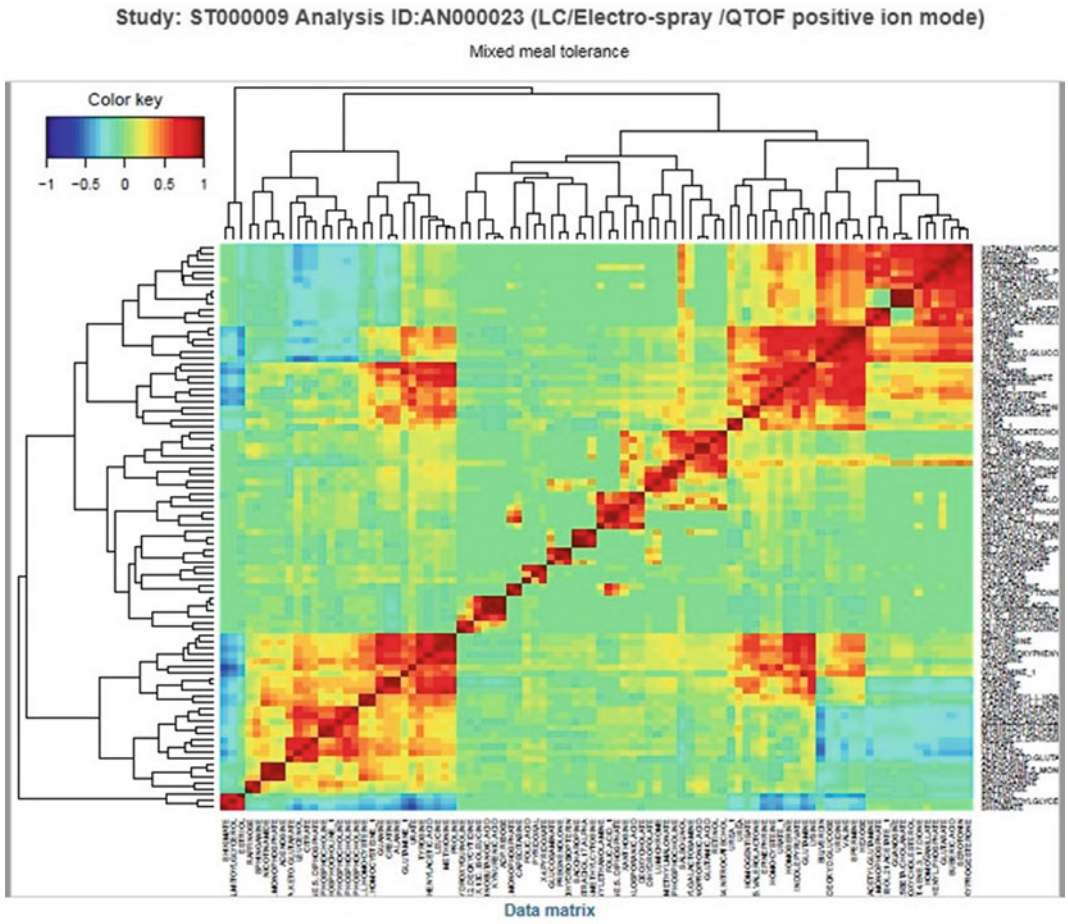


Fig. 6.4 Cluster correlation analysis using metabolomics data generated through LC-ESI-QTOF. *Source* https://www.metabolomicsworkbench.org/

origin, varieties and fruit processing conditions to conduct a metabolomics study and identify major determinants of the fruit metabolome.

Multivariate analysis applied in this study (Diboun et al. 2015) revealed a first principal component (PC1) significantly associated with the region of origin (North African or Gulf region). On top of this distinction, and based on the ripening profile, dry dates (North African) were represented at one end of PC1 while the majority of the analyzed dates, with a soft type and a late ripening profile (Gulf), were represented at the other end of PC1.

Studying a subset of the samples, varying in their ripening process, revealed correlation between the metabolites detected in PC1 and the loading values and enrichment patterns of dates for each stage of ripening. The first phase of ripening is characterized by enrichment in regulatory hormones, amines and polyamines, energy production, tannins, sucrose and antioxidant activity. The second phase is dominated by the activation of the phenylpropanoid pathway and the phospholipid metabolism. The third and final phase is marked by sugar dehydration activity and degradation reactions leading to the increase in volatile synthesis.

Data from this study also revealed that the ripening and maturation process of dates was an important factor in the variation of metabolome, which affects the nutritional and economical values. In addition, the study detected changes in

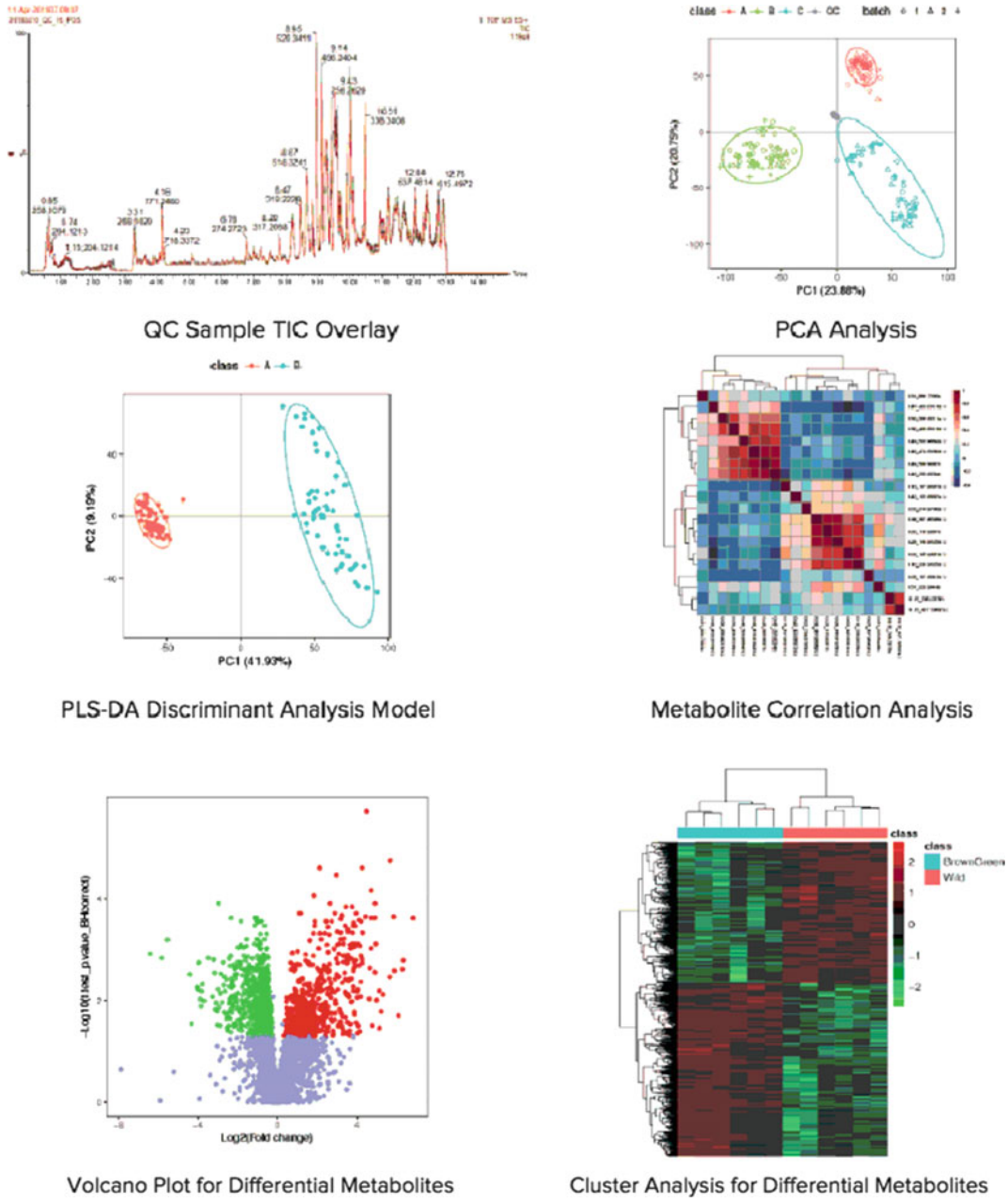
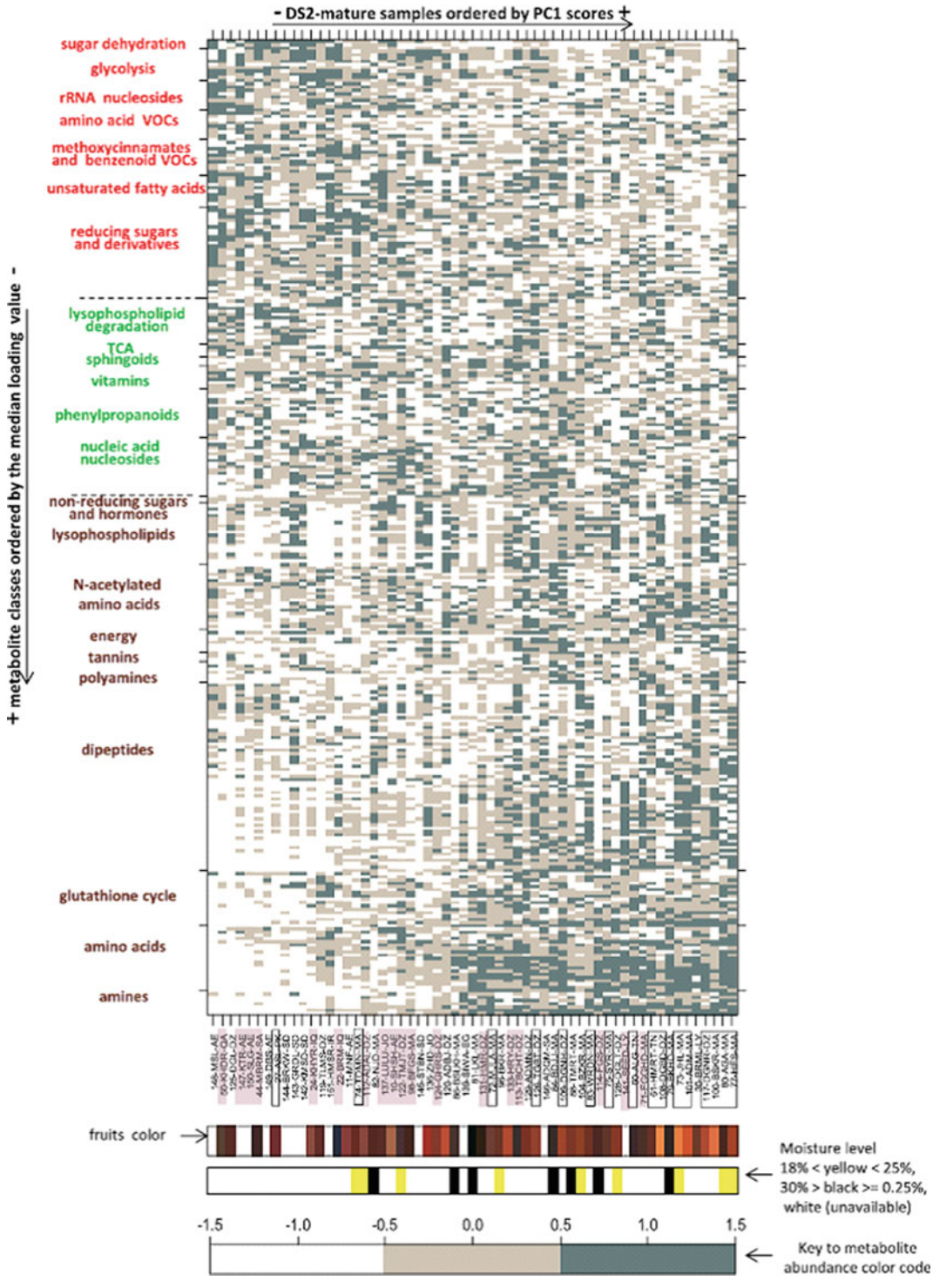


Fig. 6.5 Type of analysis, clustering methods and correlations used for metabolomics. *Source* https://www.metabolomicsworkbench.org/

the metabolomes that are linked to the uniqueness of dates as compared to other fruits. Dates share similar biochemical processes of ripening with other fruits but exhibit an additional process of natural dryness that prevents degenerative senescence following the ripening (Fig. 6.6).

A comprehensive metabolomic dataset of date palm fruit was published by Stephan et al.


- DS2-mature samples ordered by PC1 scores +
+ metabolite classes ordered by the median loading value -
sugar dehydration
glycolysis
rRNA nucleosides
amino acid VOCs
methoxycinnamates and benzenoid VOCs
unsaturated fatty acids
reducing sugars and derivatives
lysophospholipid degradation
TCA
sphingoids
vitamins
phenylpropanoids
nucleic acid nucleosides
non-reducing sugars and hormones
lysophospholipids
N-acetylated amino acids
energy
tannins
polyamines
dipeptides
glutathione cycle
amino acids
amines
fruits color
Moisture level
18% < yellow < 25%,
30% > black >= 0.25%,
white (unavailable)
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
Key to metabolite abundance color code

◀ **Fig. 6.6** Heatmap analysis based on DS2-mature data showing the abundance level of metabolites arranged in biological classes by increasing PC1 loading values (*Y*-axis) along date samples arranged by increasing PC1 scores (*X*-axis). Metabolites classes are shown on the left in different colors to reflect various biochemical phases of the ripening process in dates: (brown) early ripening khalal (green) ripening underway corresponding to rutab and (red) overripening. The positive range of PC1 shows increased discoloration among dates many of which belong to the dry type (black framed rectangles). The soft type (highlighted in purple rectangles) is enriched at the negative range. Information on the dry/soft phenotype is variety specific and was collected from the literature where possible. Low moisture fruits and relatively moist fruits appear randomly scattered along PC1. *Source* Diboun et al. (2015)

(2018). This study provided detailed information on the phenotypes and metabolomics profile of 196 dates from 123 distinct varieties originating from various countries where the date palm is grown. It also highlighted the high level of diversity of the dates per country of origin, variety and post-harvesting conditions. The study used a non-targeted mass spectrometry-based metabolomics approach to characterize 427 metabolites of the fruits from a wide range of pathways. The data have been deposited in the http://www.metabolomicsworkbench.org database.

Based on the metabolites super- and sub-classes dataset of date palm, fruit can be categorized by type and abundance (Figs. 6.7 and 6.8). The pie charts below highlight some of these classes and their importance.

The PCA analysis revealed several grouping based on the country of origin, the variety and type of fruit (Fig. 6.9). Similarities and differences were detected among varieties from the same country as well as among varieties from separate countries. The type of fruit associated with the harvesting and storage conditions was a major contributor in grouping varieties into ripe, unripe fruit and intermediate group.

The amino acid profile of the 247 varieties analyzed was very similar, in the 100–500 m/z range. In the low and high range of m/z, some distinct differences were differential for a few varieties (Fig. 6.10). The same analysis can be conducted for other metabolites such as fatty acids, terpenoids, sugars, sterols and flavonoids (El Hadrami and Al-Khayri 2012; El Hadrami et al. 2011b).

A volcano plot has been generated from the metabolomics data gathered (Fig. 6.11). This scatter-plot is typically used to quickly identify meaningful changes in the large dataset with replicated data points. It plots statistical significance (i.e., p values from an ANOVA) on the Y-axis versus fold change on X-axis (metabolites), highlighting the ones with the large magnitude

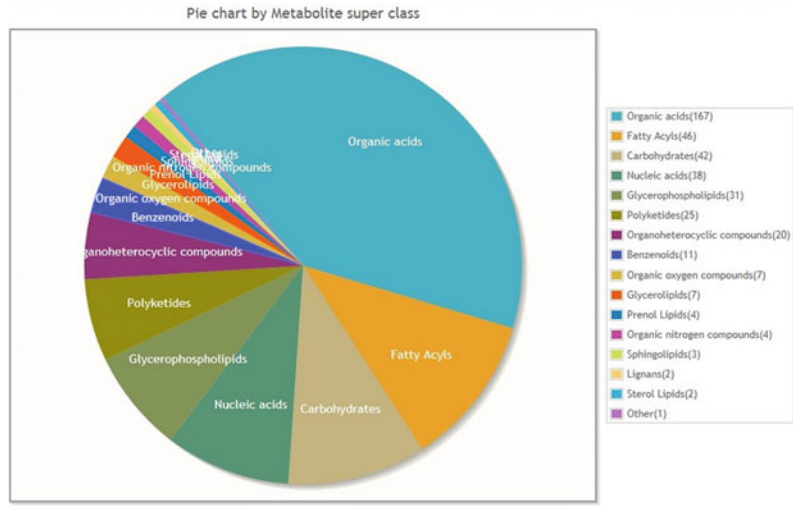


Fig. 6.7 Pie chart by metabolite superclass. *Source* Stephan et al. (2018); http://www.metabolomicsworkbench.org

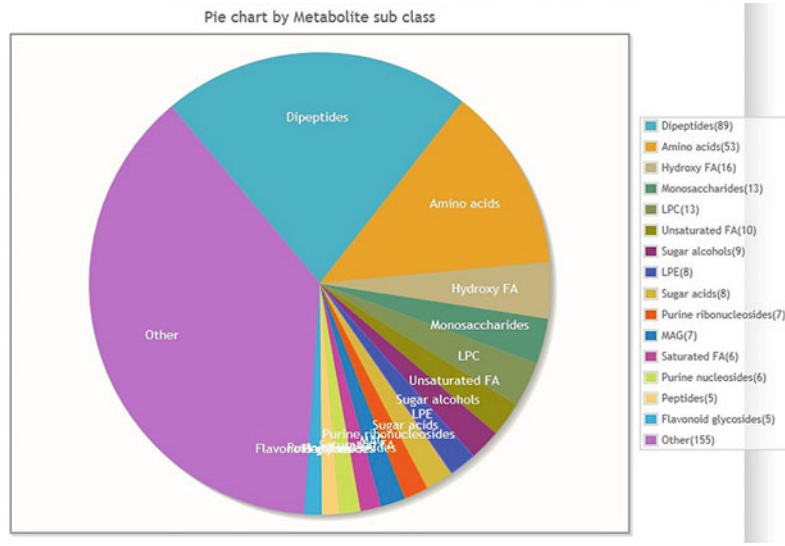


Fig. 6.8 Pie chart by metabolite subclass. *Source* Stephan et al. (2018); http://www.metabolomicsworkbench.org

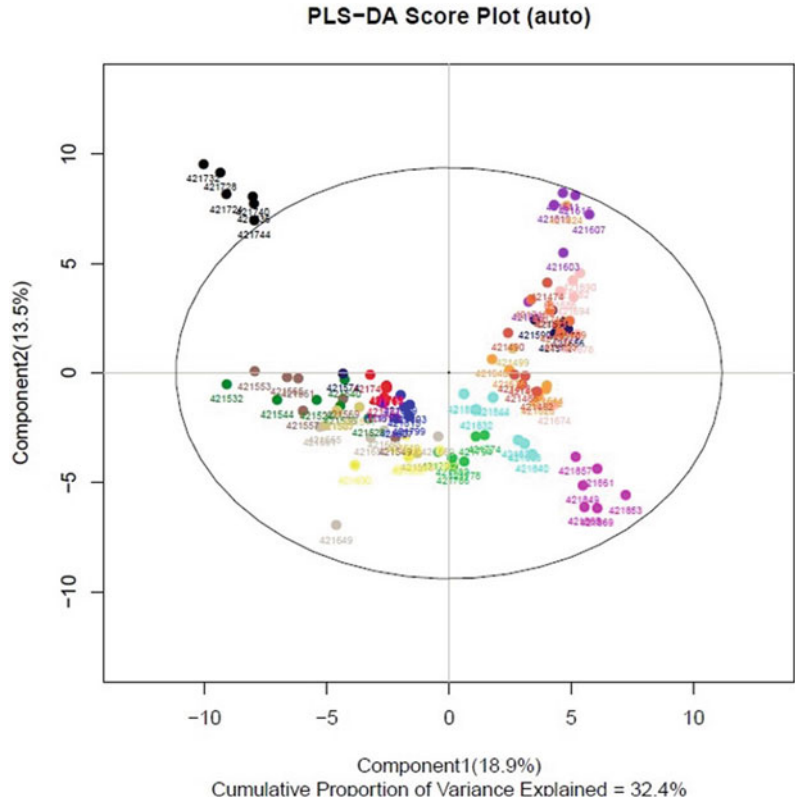


Fig. 6.9 PCA analysis output of a metabolomics study in date palm. *Source* Stephan et al. (2018); http://www.metabolomicsworkbench.org

changes. Plotting the negative log of the p values on the Y-axis on the base of 10 results in data points with low p values (highly significant) appearing toward the top of the plot. The X-axis is the log of the fold change between the two conditions (ripe vs unripe). The log of the fold change is used so that changes in both directions appear equidistant from the center. Plotting

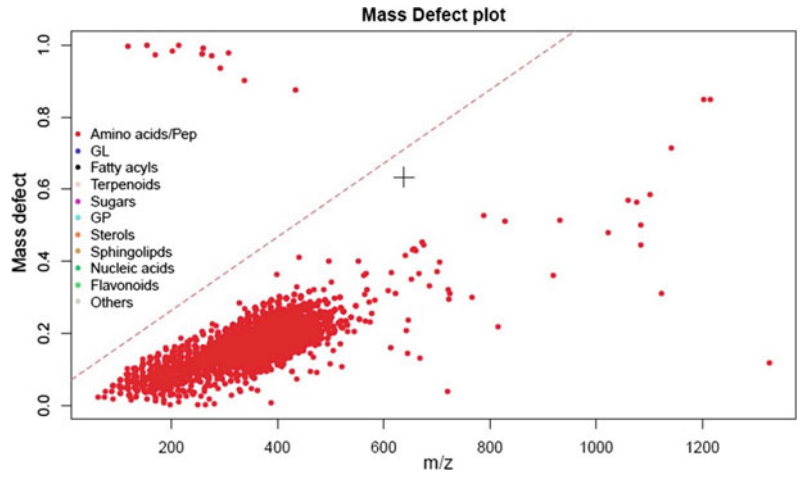


Fig. 6.10 Mass defect plot for a metabolomics study in date palm. *Source* Stephan et al. (2018); http://www.metabolomicsworkbench.org

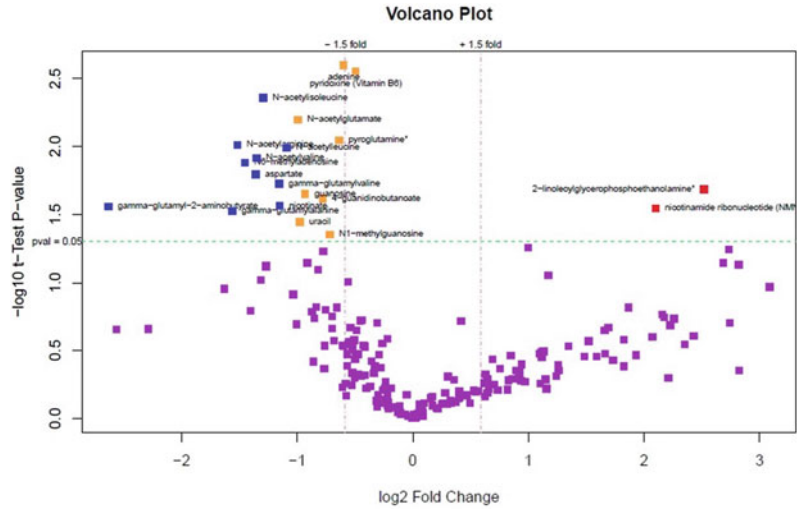


Fig. 6.11 Volcano plot generated using a large sample of date palm varieties. *Source* Stephan et al. (2018); http://www.metabolomicsworkbench.org

points in this way results in two regions of interest in the plot typically found toward the top of the plot either the left- or right-hand sides. These represent values that display large magnitude fold changes, hence being left or right of center, as well as high statistical significance toward the top.

Focusing on the subclasses displaying the large magnitude fold changes and high statistical significance several metabolites of interest can be plotted and classified by importance (Fig. 6.12).

Based on the ripening habit of the fruit, three separate groups are distinguishable (Fig. 6.13). These include ripe and unripe fruits and a third group with a mixed feature where the fruits are mid-ripe.

Analyzing the dataset of metabolites associated with the two distinct features of the fruit (ripe vs unripe) highlighted the importance of at least 20 metabolites (Fig. 6.14). These included amino acids such as histidine, polyamines such as putrescine, secondary metabolites (El Hadrami et al., 2011b) such as apigenin and vitamins such as B6 and B12.

The 427 varieties were separated based on their metabolomics profile into separate groups depending on the country of origin and the type of fruit (Fig. 6.15).

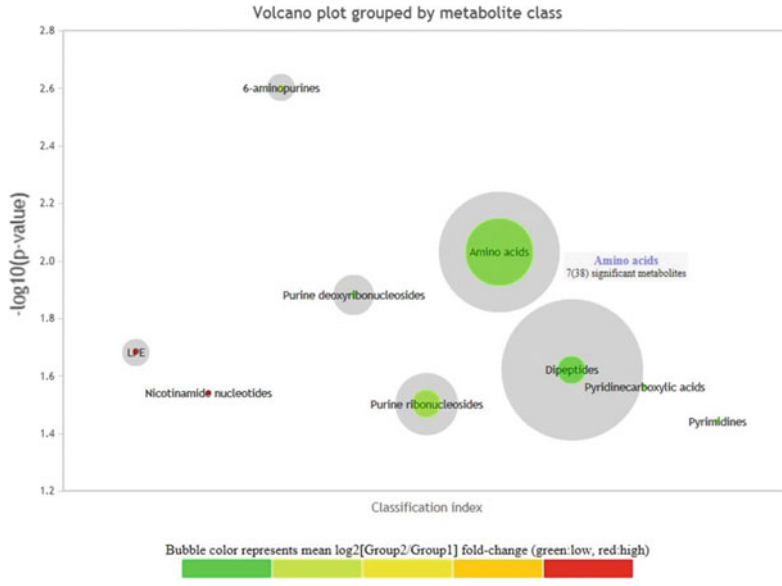


Fig. 6.12 Volcano plot grouped by metabolite class. *Source* Stephan et al. (2018); http://www.metabolomicsworkbench.org

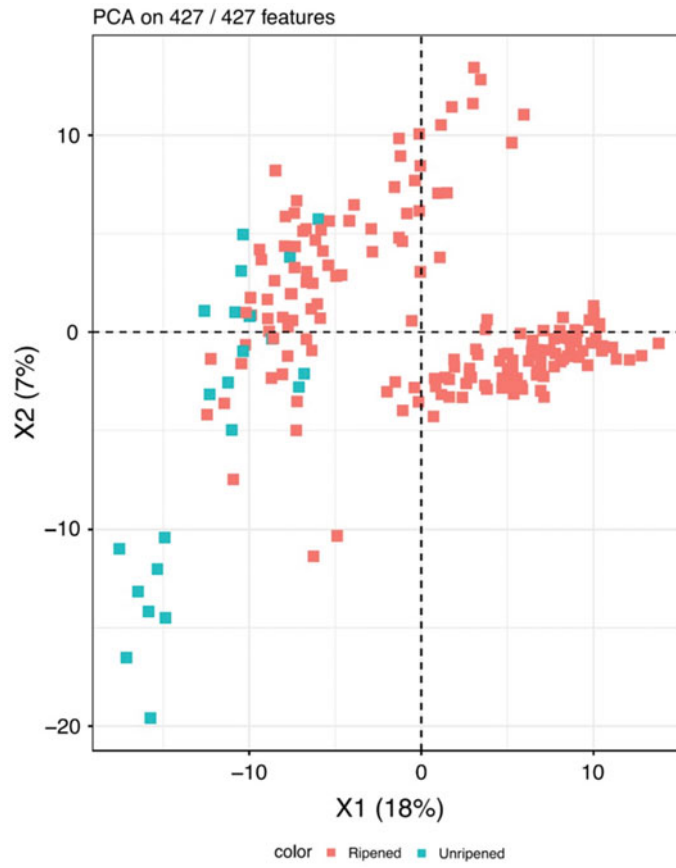


Fig. 6.13 PCA sample plot showing metabolites of ripened and unripened dates. *Source* Stephan et al. (2018); http://www.metabolomicsworkbench.org

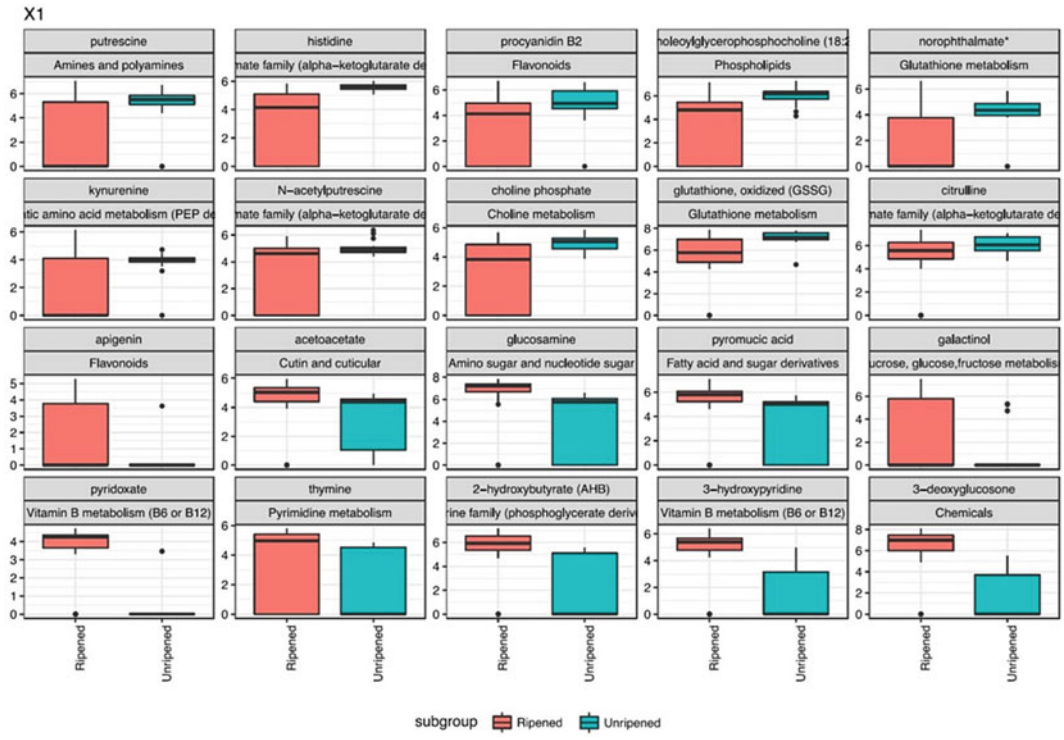


Fig. 6.14 Box plot showing the top 20 PC1 metabolites. *Source* Stephan et al. (2018); http://www.metabolomicsworkbench.org

6.3.2 Metabolomics: Beyond Biomarkers and Toward Functional Food and Nutraceutical

Metabolomics in date palm has made it possible to screen the metabolome and highlight the value added of several metabolites with a potential use as functional foods or nutraceuticals. Hamad et al. (2015) analyzed the metabolic profile of 12 varieties of dates and performed the free radical scavenging and anti-lipid peroxidation activity of their extracts. Significant differences were detected among these cultivars in terms of the chemical composition of phenolics, amino acids and minerals. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) revealed similarities and variations among the tested varieties that can be associated with the effect of the local growth environmental conditions on the metabolism.

The local growth conditions were also associated with the variation in content and profile of flavonoids and phenolic acids in dates (i.e., luteolin, quercetin; apigenin, *p*-coumaric, ferulic, sinapic acids; cinnamic acid derivatives) El Hadrami et al. (2011a, b). The antioxidant activity of date is highly correlated to their contents in flavonoids and phenolics. This helps prevent the accumulation of free radicals, which can damage cells, nucleic acids, membrane lipids and proteins, leading to cancer and aging-related diseases (Bilgari et al. 2009; Shinmoto et al. 1992; Wuytac et al. 2013). For instance, a 54% inhibition of the formation of free radicals was recorded with extracts derived from Deglet Noor variety (Chaira et al. 2009). Table 6.1 summarizes some of the recent studies highlighting the nutraceutical and pharmaceutical effects of dates (Al-Alawi et al. 2017; Otify et al. 2019).

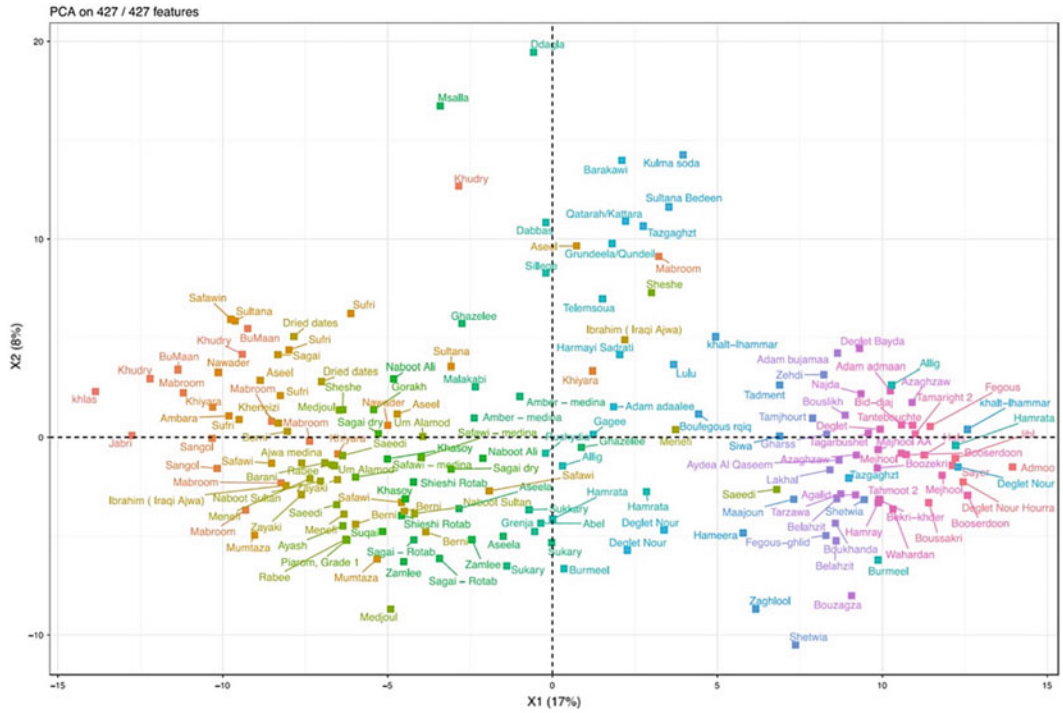


Fig. 6.15 PCA sample plot of dates. PC1 score was mapped to colors so that the dates with similar metabolite profiles have a similar color. *Source* Stephan et al. (2018); http://www.metabolomicsworkbench.org

6.4 Conclusion and Prospects

The recent developments of omics approaches including in metabolomics in date palm have led to the discovery of functional compounds and the identification of metabolic and biosynthesis pathways. This has several impacts in terms of the: (a) improvement of the varieties grown; (b) understanding of regulatory mechanisms involved in the ripening and maturation of dates; and (c) development of new functional foods and nutraceuticals derived from dates.

In terms of improving varieties, now that the full genome of date palm has been sequenced and annotated, an approach using metabolomics would guide the breeding programs at selecting the right parents and progenies with the desired attributes that would guarantee an added value to their fruit. Used alongside classical breeding methods, metabolomics has the ability to provide the quantitative markers that could be turned in quantitative trait loci (QTL) to accelerate the production of varieties in this perennial crop with many challenges in terms of its breeding.

Similarly, deciphering the regulatory mechanisms controlling fruit production and maturation can lead to an enhancement of yield and quality and an improvement if the shelf life of the harvested fruits. This can be even more important for varieties with smaller fruit sets or fruit prone to early decay in storage that were discarded from many breeding programs despite their other attributes and traits.

One of the fields that will be benefiting metabolomics is the ever-expanding market of functional foods and nutraceuticals. Metabolomics will help expand the opportunities offered by this sector to add value to dates. Research is already on its way exploring the various uses and health benefits of the classical and newly discovered metabolites extracted from dates.

Table 6.1 Summary of some of the published pharmacological potentials of date palm

Activity	Extract	Study type	Main conclusions	References
Antioxidant activity	Aqueous extract (fruits)	In vitro	Hydroxyl radical scavenging potential	Vayalil (2002)
			Superoxide scavenging potential	
	Phenolic and flavonoid fractions (fruits)	In vitro	Antioxidant capacity	Borochov-Neori et al. (2015)
			Radical scavenging potential	
	Aqueous-methanol extracts (fruits)	In vitro	Total phenolic contents	Chaira et al. (2009)
			Total flavonoid contents	
	Methanolic extract (fruits)	In vitro	Antioxidant capacity	Mohamed et al. (2014)
			Total phenolic contents	
Anti-cancer activity	Date extract (DDE) and polyphenol-rich extract (DPE)	In vitro	Growth inhibition of Caco-2 cells	Eid et al. (2014)
	Fruits	Mice	β-glucan anti-tumorigenesis	Ishurd and John (2005)
Hepatoprotective activity	Aqueous extract (fruits)	Rabbit	Elevation in serum GSH level	El-Gazzar et al. (2009)
			Serum MDA level, ALT and AST activities restoration	
			Elevation in serum IgM, IgG and IgA levels	
	Aqueous extract (flesh and seeds)	Rat	Reduction in elevated serum activities of ALT, AST and ALP due to CCl_4	Al-Qarawi et al. (2004)
	Aqueous extract (fruits)	Rat	Significant reduction in thioacetamide-induced elevation in plasma bilirubin concentration and enzymes activities of AST, ALT, LDH and γ-GT	Ahmed et al. (2008)
	Aqueous extract (fruits)	Rat	Pretreatment with date palm fruit extract restored the liver damage induced by dimethoate, as revealed by inhibition of hepatic lipid peroxidation, amelioration of SOD, GPx and CAT	Saafi et al. (2011)
	Aqueous extract (fruits)	Rat	The aqueous extract attenuated oxidative stress induced by trichloroacetic acid by decreasing the extent of hepatic TBARS (thiobarbituric acid reactive substances) formation, restoring the activities of SOD, CAT and GPx	
Nephroprotective activity	Proanthocyanidin-rich seed extract	Rat	Significant decrease in MDA in renal tissue	Ahmed et al. (2015)
	Aqueous extract (fruits and seeds)	Rat	Significant decrease in plasma creatinine and urea levels due to gentamicin nephrotoxicity	Al-Qarawi et al. (2008)
	Aqueous extract (fruits)	Rat	Significant reduction in renal MDA due to dimethoate	Saafi-Ben et al. (2012)

(continued)

Table 6.1 (continued)

Activity	Extract	Study type	Main conclusions	References
Neuroprotective activity	Methanolic extract (fruits)	Rat	Attenuation of GSH levels and SOD and CAT activities depletions induced by ischemia	Pujari et al. (2011)
	Aqueous extract (fruits)	Rat	Significant reductions in grooming frequency and sciatic motor nerve conduction velocity	Zangiabadi et al. (2011)
Gastrointestinal protective activity	Various extracts (fruits and seeds)	Mice	Aqueous and ethanolic extracts were emptied from the gastrointestinal tract contents	Al-Qarawi et al. (2003)
	Phoenix dactylifera sap	Rat	Significant increase of GIT transit time	Souli et al. (2014)
	Aqueous extract (pulp)			
	Aqueous and ethanolic non-dialyzed and dialyzed extracts (fruits and seeds)	Rat	Marked amelioration of gastric necrosis, hemorrhage, congestion and edema in stomach histological sections and biochemical levels of plasma gastrin and histamine in gastric mucosa induced by ethanol	Al-Qarawi et al. (2005)
	Ethanolic extract (leaves)	Rat	Oral administration of extract or its fractions in alloxan-induced diabetic rats significantly reduced serum glucose, triacylglycerol and cholesterol	Mard et al. (2010)
	Aqueous, ethanol, methanol and acetone extracts (seeds)	In vitro	All extracts possess significant antidiabetic activity	Khan et al. (2016)
Antihyperlipidemic activity	Methanol–water extract (leaves)	Rat	Significant decrease in serum LDL-C levels	Abuelgassim (2010)
Antimicrobial activity	Aqueous extract (fruits)	In vitro	Significant reduction in *Candida albicans, C. tropicalis and C. kefyr* adhesion to human buccal epithelial cells	Abu-Elteen (2000)
	Aqueous extract (fruits)	In vitro	Weakness and distortion of *C. albicans* cell wall	Shraideh et al. (1998)
	Aqueous extract (fruits)	In vitro	Growth inhibition of *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, and *Pseudomonas aeruginosa*	Sallal and Ashkenani (1989)
Fertility improvement	Ethanolic extract (pollen)	Rat	Restoration to the significant reduction in sperm count and motility and with sperm abnormalities along with increased testicular MDA and decreased GSH levels due to cadmium chloride	El-Neweshy et al. (2013)
	Ethanolic extract (pollen)	Rat	Alleviation of significant lowering in genital sex organs weight, sperm count and motility, serum LH, FSH and testosterone due to induced hyper- and hypothyroidism	Mehraban et al. (2014)
	Fruits	Human	Significant increase in the mean cervical dilatation, proportion of intact membranes and spontaneous labor in comparison to control	Al-Kuran et al. (2011)

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Part III

Molecular Breeding and Genome Modification

Induced Mutagenesis in Date Palm (*Phoenix dactylifera* L.) Breeding

7

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Abstract

Date palm (*Phoenix dactylifera* L.) is an economically important crop in the oases agroecological zones. In vitro mutagenesis has been an effective strategy for genetic improvements in several traits of crop plants. However, studies related to the genetic improvement of this fruit tree are very limited. Several conventional approaches including physical and chemical mutagens, insertional and somaclonal mutations have been practiced creating the desired traits. However, contemporary site-directed mutation approaches, like TALENS, ZNFs and CRISPR-Cas, have not yet been put into practice for the date palm. The prospects and applications of currently accessible mutagenesis methods for date palm genetic improvement are discussed in this chapter. New breeding tools (NBTs) for targeted mutagenesis through CRISPR-Cas-based genome editing (GE) and its base editing (BE) versions can be very effective to engineer date palm genomes. However, with a large and complex genome, heterozygosity and outcrossing, somaclonal variation during in vitro regeneration, the presence of single-nucleotide polymorphism (SNP) and ultimate genetic instability caused by these SNPs pose challenges. Such challenges could be addressed effectively by the execution of site-specific CRISPR-Cas versions, like BEs, coupled with high-throughput screening techniques. Finally, the hierarchy of targeted mutagenesis over random mutagenesis is addressed as a potential approach for futuristic studies of date palm genetic improvement.

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7.1 Introduction

Date palm (*Phoenix dactylifera* L.) is an iconic fruit plant species having one of the oldest domestication histories in the Middle East and North African regions, with a good economic return and a rich source of nutrition, feed and livelihoods. Date palm is a diploid

J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_7

(2n = 2x = 36), dioecious, monocotyledonous and perennial woody fruit crop with remarkable socioeconomic values in the agricultural context. Date palm fruit is a rich source of vitamins, minerals and carbohydrates, besides possessing medicinal importance for cardiovascular conditions (Alhaider et al. 2017). Date palm can withstand extreme drought, heat and soil salinity and thus a good candidate to study stress tolerant genes, underlying mechanisms and interactions with the surrounding ecosystem (Ram et al. 2019). Despite its rich history and economical importance, thus far the potential of date palm molecular genetics is poorly explored. Developments in genetics and genomics of this important fruit crop are very limited. However, during the last decade, date palm genomic data has been the major focus in a number of research studies (Sattar et al. 2017). Subsequently, the date palm whole genome was completely sequenced in 2013 (Al-Mssallem et al. 2013), following the sequencing of chloroplast (Yang et al. 2010) and mitochondrial genomes (Fang et al. 2012). After advancements in second and third-generation sequencing techniques, the whole genome of many date palm cultivars has been re-sequenced (Hazzouri et al. 2015). The average size of a date palm genome is projected at 550–658 Mb (Al-Mssallem et al. 2013; Hazzouri et al. 2015; He et al. 2017). The availability of publicly accessible genomic databases, more advanced bioinformatics approaches, high-throughput sequencing platforms and current insights in novel plant transformation techniques, can be effectively used to explore genetic diversity, construction of advanced genetic linkage maps, development of molecular markers and a build-up of functional genomics research in date palm (Sattar et al. 2017).

7.1.1 Problems Facing Date Palm Genetic Improvement

Complete sequencing of the entire genome is fundamental to an understanding of the molecular basis of complex genetic traits in plants. The first date palm nuclear genome assembly was partially (~60%) assembled using genome-wide high-throughput sequencing (Al-Dous et al. 2011). Later, Al-Mssallem et al. (2013) identified > 3.5 million polymorphic sites in the 605 Mb date palm nuclear genome. The whole genome was further refined with maximum coverage by Hazzouri et al. (2015). In a recent study, He et al. (2017) deciphered the population structure and genetic diversity of 62 date palm cultivars using simple sequence repeat (SSR) and single-nucleotide polymorphism (SNP) markers. These studies provided genome-wide genetic structure, gene duplication history, cultivar genetic diversity and categorization of key functional genes. Concomitantly, many related studies were also carried out in date palm. These included genome-wide association mapping (Hazzouri et al. 2019), miRNA profiling (Xiao et al. 2013; Xin et al. 2015), transcriptome delineation (Yin et al. 2012), comparative genomic synteny with oil palm (Mathew et al. 2014) and genetic diversity modeling using SNP data (Mathew et al. 2015; Sabir et al. 2014). Recently, the available complete whole-genome sequence data of date palm cultivars have been used to develop an online date palm genomic resource database (DRDB) to provide a comprehensive platform to explore genetics, genomics and molecular breeding in date palm (He et al. 2017). This database is based upon SSR and SNP markers and can be freely accessed online (http://drdb.big.ac.cn/home). The Plant Genome and System Biology (PGSB) is another online available database of different plants, but date palm genome database is one of its important integral parts; it is a joint venture of Weill Cornell Medical College, Qatar and the German Research Center for Environmental Health, Germany. It can be accessed at http://pgsb.helmholtz-muenchen.de/plant/pdact/index.jsp or https://qatar-weill.cornell.edu/research/research-highlights/date-palm-research-program. The Date Palm Molecular Markers Database (DPMMD) is a more comprehensive type of database than the two abovementioned databases. It harbors basic and applied research information related to date palm genetics, genomics and molecular breeding. It contains more than 3,611,400 DNA markers including SNPs, SSRs, SSR-SNPs information

and provides genetic linkage maps, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, DNA barcodes and date palm markers related articles indexed in PubMed journals, along with a list of SSR and SNPs primers integrated from previously published data (Al-Dous et al. 2011; Mokhtar et al. 2016). This can be accessed freely at: http://dpmmd.easyomics.org/index.php.

7.1.2 Genetic Diversity and Erosion

Date palm is one of the oldest domesticated fruit crops and has been grown for ~7100 years (Tengberg 2012). Archaeological records show date palm cultivation during the early Bronze Age in the Arabian Peninsula and later from North Africa at the end of the second millennium BCE (Flowers et al. 2019). Date palm has rich genetic diversity due to its long domestication history and, as a result, more than 3000 date palm cultivars have been recognized today. These cultivars have considerable variations in fruit color, size, and moisture and sugar content. It has been suggested that interspecific hybridization between Middle Eastern cultivars and the wild *Phoenix theophrasti* Greuter contributed to the diversification of date palm (Flowers et al. 2019). Date palm has long been an important part of the sustainable economy and food security of the Middle East and North African countries. Due to its socioeconomic importance, there has been increasing interest in the genetic variation of important traits to support future breeding and domestication. Nevertheless, the induction of genetic variations through conventional breeding in date palm is complicated for a number of reasons, such as long development time (~5 years to first flowering), offshoot propagation and at least 10–15 years to achieve maximum yield. Development through molecular markers is also restricted due to the scarcity of mapping populations for quantitative trait loci (QTL) mapping and related breeding efforts for crop improvement. Unlike other fruit crops, only a few date palm populations are suitable to apply QTL mapping and, therefore, breeding efforts are barely managed or completely terminated (Hazzouri et al. 2019). The development of genetic and genomic resources in date palm is thus encouraged because applying traditional plant breeding and genetics approaches are so difficult. Over time, date palm cultivation has shifted from traditional to monocultural cultivation, which has led to the erosion of many potential genetic resources and ultimately impacting the agrobiodiversity in date palm cultivation areas (Jain et al. 2011). In addition, severe environmental disasters and abrupt changes in agroclimatic conditions have further aggravated the date palm biodiversity situation.

7.2 Mutation Breeding

The changing climate, population growth, economic development and increased demand for bioenergy are some of the global causes for food security issues (Ye et al. 2016). In response to global food security, the development of genetic resources necessarily requires identification of inter- and intra-species variations, which is key to successful crop breeding programs. An alternative to limited genetic resources is mutation breeding using a variety of mutagenic resources to generate diversified mutant resources. Mutations are the key force for genetic variations and directly drive the evolution of an organism. In addition, researchers have utilized induced mutations to bring genetic improvements in all three domains of life: animals, microbes and plants (Ram et al. 2019). In agriculture, mutation breeding plays a critical role in the development of new cultivars with better performance in terms of yield, quality and resistance against biotic and abiotic stresses through novel tools for induced and targeted mutagenesis (Fig. 7.1). The role of mutagenesis is expanding in plant biology to study certain gene functions through forward and reverse genetics. Some other functional genomics approaches include loss- or gain-of-function of the targeted genes to investigate gene functions.

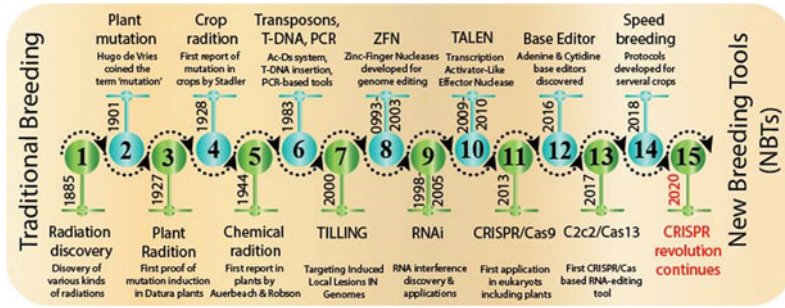

Fig. 7.1 Timeline of historical developments of induced mutagenesis in plants. Induced mutagenesis was employed as a breeding tool after the invention of X-rays during the late nineteenth century or beginning of the twentieth century. The critical inventions/discoveries and their subsequent applications for plant mutation breeding are summarized, respectively. A boom of inventions in mutation breeding occurred during the last three decades after the development of PCR-based molecular tools. The primary stimulus for a paradigm shift from random to targeted mutagenesis is the introduction of site-directed nucleases during the 2000s. Finally, it involves the CRISPR-Cas9-based new breeding tools (NBTs), which have been steadily progressing since their first application in plants in 2013 (Figure constructed by M.N. Sattar in Adobe Illustrator 2019)

7.3 Approaches of Mutation Induction

Several techniques have been empirically applied to induce mutations in crop plants, such as chemical, physical, insertional and targeted mutagenesis. However, these mutagenic techniques are usually costly and require tedious processes to detect successful mutational events in the targeted plants. Meanwhile, progress in next-generation sequencing (NGS) techniques has revolutionized mutagenesis by providing cost-effective screening of millions of mutants in a short time period. Moreover, coupling induced mutations with NGS approaches provide a powerful platform to study forward and reverse genetics for crop improvements. Discrete technological advancements in molecular biology have strengthened the mutation breeding program. For example, the complete genomes of a large number of crops are now available, enabling site-specific mutagenesis using various genome editing (GE) techniques to bring desired mutations at a targeted locus in the genome. Such site-specific mutations have been successfully induced using various GE approaches zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the most recent clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated9 (Cas9) endonucleases.

7.3.1 Chemical Mutagenesis

Chemical mutagenesis is a simple and the most convenient option to induce genetic mutations in a variety of plant species, but it may create serious environmental and health hazards. Chemical mutagens are mostly alkylating agents, which are more gene-specific than physical mutagens. These may include colchicine, diethyl sulfate (DES), ethyl methanesulfonate (EMS), ethylenimine (EI) and sodium azide. Colchicine is the most widely used commercial chemical for the induction of polyploidy in plants. The primary source of colchicine is meadow saffron (*Colchicum autumnale* L.). The application of colchicine causes chromosomal arrest during the segregation of chromosomes in the metaphase stage of meiosis and ultimately results in chromosomal doubling followed by the induction of tetraploids (Fatima et al. 2015; Rana et al. 2020). EMS (an ethylating agent) is mostly effective for the induction of point mutations and deletions in certain chromosomal segments (Kostov et al. 2007). EMS populations are the most

advantageous functional genomics tool to be used in crop breeding as they cause the induction of dominant mutant alleles. EMS alkylation usually mutates the affinity of guanine (G) bases to thymine (T) instead of cytosine (C) by forming O6-ethylguanine, which results in C/G to T/A transitions, respectively. It can also induce the transition of G/C to C/G or G/C to T/A through 7-methylguanine hydrolysis. EMS-based mutagenesis has wide applications that are equally effective for a wide range of crop genomes. For example, it has been shown to induce similar mutations in *Arabidopsis* and maize genomes despite a 20-fold difference in their genomic size (Till et al. 2004); therefore, EMS-based mutagenesis is preferable in plants.

7.3.2 Physical Mutagenesis

The use of radiation to develop elite crop cultivars and to study functional genomics in crop plants has been in use for the last 90 years. Initially, only X-rays and gamma rays were the primary sources of radiation; however, these have been subsequently replaced with fast neutrons, UV and ion beams, respectively (Tanaka et al. 2010). The use of chemical mutagens (such as EMS) produces more frequent mutations, but the mutation induction through radiation results in a higher number of mutations and thus, more loss-of-function mutations occur for a targeted trait (Shirasawa et al. 2016). About one-half of the mutant crop varieties registered in the FAO/IAEA mutant variety database have been generated through gamma irradiation mutagenesis (Beyaz and Yildiz 2017). Irradiating plant tissues with gamma radiation induces the formation of ions, which produce certain chemical reactions and impairment of chromosomal DNA and, ultimately, genetic mutations in the plant genome. The spectrum and frequency of radiation-based mutations are directly correlated with the type and dose of radiation, the linear energy transfer (LET), and the type and condition of the irradiated tissue (Hase et al. 2018). Thus, investigating the in-depth mutagenic effects of different radiations is crucial for successful breeding programs and the related functional genomics study. If the objective of a study is to produce a high rate of mutations to knock out different genes, the irradiating mutagens causing frequent but small deletions are more suitable. Targeting induced local lesions in genomes (TILLING) can easily detect these small deletions or SNPs in the genome (Taheri et al. 2017). Contrarily, if the objective of the study is to knock out duplicated genes in tandem simultaneously, the radiations causing large-scale mutations can be an option. De-TILLING analysis can detect these larger deletions by specifically amplifying the large deletion of mutant DNA fragment (Rogers et al. 2009). Biological damage to the irradiated cells depends upon the amount of energy received by certain cells in terms of LET. The correlation between the gamma-ray dosage and cell survival rate is expressed in terms of the mean lethal dose (LD_{50}). This gamma-ray dosage effect varies with the plant species (Chang et al. 2020). The more advanced high energy cyclotrons, heavy ions and fast neutrons have multi-fold LET as compared to low LET gamma rays.

Non-particle-based (gamma, X-ray) and particle-based (fast neutron, heavy ion) irradiations produce a range of deletions, i.e., a few to few million bases. Particle-based fast neutron-mediated mutagenesis is gaining more popularity to study the loss or gain-of functions of certain genes in plants. The reason is the production of larger (>1 Mb) deletions, non-repairable double lesions and chromosomal rearrangements in the plant genome (Gilchrist and Haughn 2010). There is an increasing trend to use a particle-based radiation source with high LET for induced mutation. Unstable inheritance of large deletions created by gamma rays is another reason to replace them with high LET particulate radiation (Li and Zhang 2002). Fast neutrons generate double-stranded breaks (DSB) with high frequency as compared to gamma rays and hence have higher relative biological effectiveness (RBE). The fast neutrons technique is very effective in disrupting small genes or a tandem array of genes but requires expensive instrumentation, a specialized lab set-up and a large

sample size. In addition, such mutations are not genetically stable, despite a high mutation rate. Moreover, physical mutagens cause large segmental aberrations in chromosomes, uncontrolled disruption of linked genes and thus not suitable for genetic mapping (Ram et al. 2019).

7.3.3 Insertional Mutagenesis

Insertional mutations involve the disruption of DNA sequences and subsequently help to identify and isolate genes. Mutagenesis is an indispensable approach to investigate the biological systems of crop plants. A range of mutagenesis approaches has been successfully employed to classify and characterize the functional regulations of novel genes involved in certain biological mechanisms in plants. Gene inactivation is a successful approach to determine the exact function of an unknown gene in plant species. The inactivation of the targeted endogenous gene is accomplished using sense and anti-sense copies of the target gene. The insertional mutagenesis is the most suitable approach for gene inactivation, which is accomplished by adding exogenous DNA base pairs as the mutagens (Ram et al. 2019). It is performed using a range of molecular tags such as T-DNA, activator/dissociation (Ac/Ds), transposons, retrotransposons and tagged DNA. The extra nucleotides or DNA fragment is inserted in the middle of the coding sequence of a gene. The tagged gene can be investigated well by studying the mutated locus and the gene tag works as an identifier. This insertional mutagenesis approach has been successfully used in the improvement of rice crops using T-DNA and transposon mutants to accelerate functional genomic studies (Ram et al. 2019). Another version of insertional mutagenesis is the site-selected insertion (SSI), which is a PCR-based approach to detect the insertional mutation in the known gene. Briefly, in this technique two specific primer pairs are synthesized for the target gene and the transposon. The PCR amplicons are investigated for the genes with an insertional mutation (Chaudhary et al. 2019). Many studies are available to perform insertional mutagenesis for morphological and functional studies using transposon and T-DNAs (Kim et al. 2018). The major limiting factors in insertional mutagenesis to generate large mutation population are the stringent plant transformation methods and low mutation frequency. However, with the efficient transformation protocols and advancement of vector repertoire, insertional mutagenesis could be a significant genetic tool for the crops with particular genetic context.

7.3.4 Somaclonal Variation

Plants grown under an in vitro regeneration system or tissue culture-based transformations may experience a different phenotype from the mother plants due to random mutations in the genome (Fig. 7.2). Such mutations are caused by the direct exposure to the exogenously applied hormones during in vitro regenerations, which ultimately can cause somaclonal variations (Krishna et al. 2016). Such changes can be either related to phenotypic or genotypic variations (Al-Khateeb et al. 2020). It is difficult to pinpoint exactly the causes of somaclonal variations; however, the rearrangements in chromosomes, stimulation of endogenous transposable elements, epigenetic alterations in the nuclear DNA and re-programmed DNA-methylations can collectively cause these mutations (Azizi et al. 2020). The appearance of mutants through somaclonal variations can be of the same frequency as the insertion of T-DNA and thus, these untagged mutants can be misleading during in vitro regeneration experiments. Thus, it may be difficult to discriminate the insertional gene mutations and somaclonal variants, particularly if these are interlinked. Somaclonal variations can be avoided by using semi-developed or embryogenic calli for transformations or inserting DNA directly into the germline cells such as meristems or microspores. However, somaclonal variants are not always undesirable; somaclonal variations can be a potential source of variation for crop genetic improvements (Azizi et al. 2020; Chadipiralla et al. 2020). The induction of biotic

Fig. 7.2 Somaclonal variation in multicarpel fruits of date palm (Photos courtesy of Nasser S. Al-Khalifah, Riyadh, Saudi Arabia)

and abiotic stress tolerance has been successfully exploited in date palm (Al-Khateeb et al. 2019a, b; El Hadrami and El Hadrami 2009).

7.3.5 Targeted or Site-Directed Mutagenesis

Despite the effective role of physical and chemical mutagens in accelerating the genetic improvements in crop plants, these approaches are not preferable due to random mutagenesis, laborious workouts and prolonged time-consuming screenings. These non-targeted mutagenesis approaches are thus not effective for woody crops. Nevertheless, modern nuclease-based GE tools are a revolutionary step in precise and site-specific mutation in the plant genome. Among these approaches, ZFN is one of the prime technologies, which has enabled researchers to perform site-specific mutagenesis in plants (Hilioti et al. 2016). However, ZFN was soon replaced by another contemporary technique, TALEN, which has been exploited for a large number of plant species as a powerful GE tool (Sprink et al. 2015). However, currently, ZFNs and TALEN approaches have the least preference due to another groundbreaking GE tool CRISPR-Cas9 (Table 7.1). This technique is based on RNA-guided nucleases, which are engineered and applied in various crop models due to simple, effective and versatile utility. The use of CRISPR-Cas9-based precise GE tool would be fascinating to investigate the integrated genetic networks and platforms in date palm to further explore the potential of genomic discoveries (Sattar et al. 2017). The detailed discussion is provided Sect. 7.4 of this chapter.

7.4 Mutation: A Source of Genetic Variability

Mutagenesis has inevitably played a role in producing cultivars with superior phenotypic and genotypic traits (Ahloowalia et al. 2004). Induced mutation provides the raw material for selecting better traits in crop plants and is a primary driving force for evolution. For example, ~3000 crop cultivars of 170 species have been released as mutants in more than 60 countries. Among them, 50 cultivars belong to 20 different fruit crops species (Jankowicz-Cieslak et al. 2017). Although spontaneous mutations have an important role in fruit crop breeding, these are less frequent than induced mutations. Contrarily, induced physical or chemical mutagenesis have high mutation frequency and genetic variability. Many useful traits have been introduced into fruit crops through mutation breeding such as alteration in flowering time, fruit ripening, fruit color, breaking self-incompatibility, resistance to biotic and abiotic stresses, breaking undesirable genetic linkages, enhancing genetic variation, fertility enrichment in sterile hybrids, producing autotetraploid, induction of dwarfism and fruits with better taste and aroma. Mutation breeding has been successfully employed in various fruit crops such as almond, apple, banana, grapes, papaya, peach, pear, plum, rough lemon, sour/sweet cherry and many others.

Table 7.1 Different genome editing tools (ZFNs, TALENs, CRISPR-Cas) and their specificities

Characteristics	ZFNs	TALENs	CRISPR-Cas9
Length of recognized DNA target	9–18 bp	30–40 bp	Variable, usually 20 bp + PAM sequence
Targeting	Poor	Good	Good
Feasibility	Difficult	Difficult	Easy
Mechanism of target DNA recognition	DNA-multimeric protein interaction	DNA-protein interaction	DNA-RNA interaction via Watson-Crick base pairing
Mechanism of DNA cleavage and repair	ds DNA break induced	ds DNA break	ss or ds DNA break
Recognition site	Zinc-finger protein	RVD tandem repeat region of TALE protein	ss gRNA
Modification pattern	Fok1 nuclease	Fok1 nuclease	Cas nuclease
Specificity	Can tolerate a small number of positional mismatches	Can tolerate a small number of positional mismatches	Tolerating positional/multiple consecutive mismatches
Targeting limitations	Difficult to target non-G-rich sites	5′ targeted base must be a T for each TALEN monomer	Targeted site must precede a PAM sequence
Design	Challenging. Available libraries of zinc-finger motifs with pre-defined target specificity, but zinc-finger motifs assembled in arrays can affect the specificity of neighboring zinc-finger motifs, making the design challenging	Easy TALE motifs with target specificities are well defined	Easy SgRNA design based on complementarity with the target DNA
Criteria for target site	Preferential binding sequence for zinc-finger proteins or Cys2His2 fingers	TALE binding sites should start with a T with the space between two TALEN arms better 15–21 bp	gRNA target site should be 20 bp long starting with a G for U6-directed transcription and GG for T7-directed transcription; PAM sequence (NGG) is indispensable for Cas9 nuclease activity
Cloning	Requires engineering linkages between zinc-finger motifs	TALEs do not require linkages. Cloning of separate TALE motifs can be done using Golden Gate assembly	Expression vectors for Cas9 available. SgRNA can be delivered to cells as a DNA expression vector or directly as an RNA molecule or pre-loaded Cas9-RNA complex

7.5 Mutation Induction in Crop Improvement

Limited genetic diversity creates a barrier to genetic improvement in certain crop plants. Under such circumstances, induced mutations are an essential tool to create new alleles for the genetic improvement of crop plants. Induced mutations have been extensively employed, not only to generate genetic variations, but also to identify the critical genes and molecular mechanisms involved in economically significant traits for crop genetic improvement (Kozgar et al. 2012). Induced mutation is thus a propitious approach for the development of novel cultivars with higher potential to cope with biotic and abiotic stresses and for biofortification. Induced mutations have been used to improve important agronomic traits in major fruit trees for disease resistance (apple, banana, Japanese pear, peach), seedless fruits (citrus, guava), short stature (papaya, pomegranate), earliness (apricot, banana, jujube, plum) (Atay et al. 2018). Among various physical and chemical mutagens, the EMS-based induced mutations have been commonly utilized in breeding programs to enrich the genetic background of existing crop cultivars. Three stay-green rice mutants were successfully established through EMS-induced mutation for better harvesting index under drought stress (Ramkumar et al. 2019). Besides crop plants, induced mutagenesis has also been applied for genetic improvements in medicinal plants. For example, black cumin, *Nigella sativa*, mutants with improved thymoquinone contents were produced using a combination of EMS and gamma-ray mutagens (Asif et al. 2019). During the study, key proteins involved in seed yield, plant morphology and active secondary metabolism were also elucidated and thus, can be effectively explored for genetic improvement of medicinally important plants. Likewise, EMS-induced mutagenesis developed resistant tomato lines against two potyviruses; potato virus Y (PVY) and tobacco etch virus (TEV), by mutating a translation initiation factor eukaryotic initiation factor 4E (elF4E), respectively (Piron et al. 2010). In recent years, the use of fast neutrons has also been popularized for impacted mutations in crop plants. The fast neutron-mediated mutagenesis can create a few bases to more than 1 Mb random deletions. Fast neutron mutagenesis has been empirically practiced in many crops such as *Arabidopsis*, lotus, *Medicago*, peanuts, rice and soybean (Kumawat et al. 2019). The utility of NGS to characterize the mutant lines and mapping induced mutation through fast neutron can be very effective in the application of forward and reverse genetics approaches in crop improvement.

Recent NGS-based mutant screening techniques have a wide range of applications, cost-effectiveness and robust use to identify the genes of interest within the shortest possible time. The fast neutron approach is a relatively innovative approach and has never been explored for date palm mutagenesis.

7.6 In Vitro Mutagenesis and Selection

Biotic and abiotic stresses cause substantial damage to the date palm, leading to a severe loss of yield around the globe. Both types of stresses, either alone or in concert, have a significant effect on plant physiology, metabolism, growth and development, which ultimately result in compromised yields. To boost fruit yield, various strategies ranging from classical to contemporary biotechnological techniques including cultural, mechanical, chemical and mutational breeding are being practiced in different regions of the world. Among these techniques, the production of resistant date palm stock has been found promising since the early decades of the nineteenth century (Selvanarayanan et al. 2020).

Advancements in breeding and biotechnological techniques via somatic variation in date palm have successfully produced varieties tolerant to various abiotic and biotic stressors. Somatic variation has enabled researchers to produce tolerant and resistant cultivars against many biotic and abiotic stresses (El Hadrami and

El Hadrami 2009) and in combination with various, in vitro culture techniques, it represents the simplest, fastest and most efficient method of crop improvement.

In vitro selection is a promising biotechnological technique that is commonly practiced in plant breeding to achieve tolerance against different stresses. Depending on the selective agent, in vitro selection is usually executed at the early stages of regeneration such as the protoplast, cell suspension or callus stages. However, it can also be performed at later stages of root and shoot regeneration. During in vitro selection, test plants or developing embryos are exposed to physical or chemical mutagens and subsequently, selection for the desired trait is made (Suprasanna et al. 2015). As date palm has a reliable plant regeneration system via somatic embryogenesis, so mutation induction can be a promising option for plant genetic improvement. The development of somatic embryos is a desirable method owing to the fact that somatic embryos are developed from a single cell, preventing the formation of chimeras. Additionally, direct plantlets are generated from mutant somatic embryos in just one step, thus avoiding the tedious step of rooting (Jain 2012).

7.7 Selection Against Biotic Stress

Biotic stresses include primary fungal, bacterial and viral diseases or damage caused by insect pests, animals, nematodes and weeds. In vitro mutagenesis techniques have been successfully implemented in crop plants to improve disease resistance; however, little or almost no success stories have been reported for insects. Induced mutations can change the gene-for-gene interplay between the pathogen and the host plant by disrupting the infection mechanism. A number of mutants have been reported showing resistance to various viral, bacterial and fungal pathogens in crop plants. For example, in most of the mutagenesis studies in barley, the ml-o locus on chromosome 4H has been targeted to induce mutations against powdery mildew and barley yellow mosaic virus (BYMV) (Hoseinzadeh et al. 2020).

7.7.1 Fungal Diseases

Date palm is a widely grown plant commodity in harsh desert ecosystems and has to constantly interact with various heterotrophic microbes invading the surrounding niche. This might be the reason that date palm is susceptible to a variety of pathogenic diseases. Among them, bayoud disease or *Fusarium* wilt (caused by *Fusarium oxysporum* f. sp. *albedinis*) and black scorch disease or rhizosis (caused by *Thielaviopsis punctulata*) are of economic importance. Many other secondary fungal diseases such as foliar decay, root rots and false smut are caused by *Omphalia pigmentata*, *O. tralucida*, *T. paradoxa*, *Mycosphaerella tassiana* and *Graphiola phoenicis*. Some of the related fungal pathogens include *Ceratocystis paradoxa*, *C. radicicola*, *Aspergillus flavus*, *Alternaria* sp., *A. niger*, *Trichoderma* sp. and *Penicillium* sp. (Nishad and Ahmed 2020).

Bayoud is one of the deadliest diseases of date palm, which after its initial report in Morocco, has spread across North Africa with an estimated loss of 12 million date palms. In 2008, a joint venture was initiated by the International Atomic Energy Agency (IAEA) with Algeria, Morocco and Tunisia on the production of the mutant and resistant date palm to bayoud disease by gamma irradiations (Jain 2005). In this experiment, gamma radiation was used to induce mutants followed by culturing them on toxins extracted for *Fusarium oxysporum* (Sedra 2011). Research led by INRA and the IAEA has used an innovative approach based on the use of *F. oxysporum* toxin in in vitro selection to yield rapid bayoud resistance plants (Sedra 2011). Resistant mutants against *Phytophthora* spp. are known from many crop plants through induced mutagenesis (Kumari et al. 2019). Similarly, mutant tomato lines produced via exposure of nystatin have been found effective against *Phytophthora* spp. (Zhang et al. 2000). Many molecular markers are available for screening of bayoud resistant date palm cultivars. Nevertheless, the genetic marker assisted selection of resistant date palm cultivars has not always been effective against bayoud disease due to complex genetic

inheritance of resistance traits (Cohen 2020). Besides, Saleh et al. (2017) suggested a detailed protocol to use mitochondrial markers for the identification of genetic resistance in date palm. However, further investigations are needed to expand more scientific insights into the resistance against fungal pathogens in date palm. The use of ionizing radiations like X-rays and/or fast neutrons to induce mutation and developing disease-resistant lines can be helpful, as these mutagens have been effectively used to produce many disease-resistant cultivars (Kumawat et al. 2019; Rezk et al. 2019).

Besides, a number of studies revealed that *Trichoderma* spp. (a biocontrol agent) after gamma irradiation attained the ability to control soil-borne fungal pathogens (Abbasi et al. 2016). Induced mutations in such antifungal biocontrol agents have been found to promote sporulation and enhance colonization rate, which ultimately improves the antagonistic abilities against soil-borne fungal pathogens.

7.7.2 Insect Pests

The major date palm infesting pests are red palm weevil (*Rhynchophorous ferrugineus*), old-world date mite (*Oligonychus afrasiaticus*), lesser date moth (*Batrachedra amydraula*), dubas date bug (*Ommatissus binotatus*), green pit scale (*Palmapsis phoenicis*), carob moth (*Ectomyelois ceratoniae*), longhorn date palm stem borer (*Jebusaea hammerschmidti*), rhinoceros beetle (*Oryctes agamemnon*), fruit stalk borer (*Oryctes elegans*) and almond moth (*Cadra cautella*).

Red palm weevil (RPW) is the most devastating insect pest in date palm growing regions including Saudi Arabia, United Arab Emirates, Iran and Egypt (Dembilio and Jaques 2015). First observed in the Middle East in 1985 it has caused huge economic losses to the date palms in these countries. Control of RPW is mainly achieved by the application of chemical insecticides directly injected into the trunk of the date palm tree or by fumigation. Pheromone traps are also commonly used to control RPW, but these control measures require more comprehensive and effective pest management strategies. Entomopathogenic viruses can also be a management option for RPW, especially engineered viruses with enhanced virulence, reporter genes (like luciferase gene) and stress tolerance (El-Mergawy and Al-Ajlan 2011).

Unlike plant diseases, the interaction between host plants and insect pests has limited utility because one insect pest may infest other plant species or genera. This limited host-pest interactions may be a reason that there are almost negligible examples of mutant varieties against insect pests. Three major insect resistance mechanisms are known including non-preference, antibiosis and tolerance. It is therefore difficult to generate new genes through mutagenesis, and induced mutation may not be preferable for mutation breeding against insect pests. Under these circumstances, transgenic breeding can be more successful for pest resistance such as the introduction of the BT toxin gene in many crop plants against chewing insects. Likewise, in date palm, no mutant line has yet been established for insect pest resistance/tolerance. However, various physical and chemical mutagens have been successfully used to produce resistant mutants against various lepidopteran and coleopteran insect pests (Saravanaraman et al. 2015). EMS has also been found promising for producing resistant lines in groundnut against *Spodoptera* spp. (Rajendraprasad et al. 2000). Major insect pests of date palm also belong to the lepidoptera and coleopteran orders. Therefore, induced mutagenesis can be employed to control major insect pests of economic value in date palm.

Although no studies are available of date palm plants to yield RPW-resistant plants after exposure to any type of radiation, RPW has nevertheless been exposed to gamma radiation as a control tool and to assess the effects on its sterility, histology and morphology (Al-Ayedh and Rasool 2010; El Naggar et al. 2010; El-Sabah and Fetoh 2011).

7.8 Selection Against Abiotic Stress

Major abiotic stressors, which limit the productivity of date palm, include heat, waterlogging, salinity and/or drought conditions (Bishnoi et al. 2017). To develop resistance against abiotic stresses, various studies have been conducted to evaluate the date palm lines through in vitro culturing (Al-Khayri and Ibraheem 2014; Al-Khayri et al. 2018; Bekheet 2015).

In a recent study, the role of a metallothionein (*PdMT2A*) date palm gene was investigated, which confers high tolerance to elevated salt, drought and oxidative stress in a salt-sensitive yeast mutant (Patankar et al. 2019). In the same study, *Arabidopsis* mutants expressing the *PdMT2A* gene showed comparatively lower Na^+ accumulation as compared to wild-type plants. In another study, yeast mutants were produced expressing date palm aquaporin *PdPIP1-2* gene with high salt and drought tolerance (Patankar et al. 2019). Similarly, a sodium hydrogen antiporter gene (*PdNHX6*) was recently used to engineer yeast and *Arabidopsis* mutants and it conferred high salinity tolerance, respectively (Al-Harrasi et al. 2020). These recent studies overwhelmingly unraveled the basis of genetic variation in date palm against abiotic stresses. Nevertheless, the genetic basis of such responses is not fully explored and there are no candidate genes or mutants available to pinpoint the major control of these variable traits in date palm. Various mutagenic approaches coupled with genetic mapping can be employed to study stress tolerance traits in date palm, which can be helpful to know the candidate genes and mutations that are responsible for genetic variations among different date palm cultivars. Genetic linkage mapping has been successfully applied in many forest tree and fruit crop species to map genetic variants produced by somatic cell mutagenesis (Jain 2012; Khan and Korban 2012). Linkage mapping is quite applicable in date palm because hundreds to thousands of seedlings can be generated through controlled crosses to generated full-sib or half-sib progenies, which are prerequisites for standard linkage mapping or pedigree-based mapping, respectively (Hazzouri et al. 2020).

Alternatively, the reverse genetic approaches (site-directed mutagenesis or recombinant DNA-technique) are a good option to advocate abiotic stress tolerance in date palm (Yaish and Kumar 2015). Thus, the candidate genes identified through these approaches can be further assessed through gene editing techniques in the abiotic stress-sensitive commercial date palm cultivars.

7.9 In Vitro Screening and Evaluation of Date Palm Mutants

The production of disease-resistant plants necessarily requires in vitro screening of somaclonal variants and mutants. However, over the past three decades of efforts only a few disease-resistant trees were generated through these approaches because these are very time-consuming and require more work. On the other hand, in vitro culturing and selection have a pivotal role in generating mutant trees to study diseases and pests. One of the reasons is that performing experiments for defense response of a tree species requires readily available plant material, which is simply possible through in vitro culturing. Thus, incorporation of basic in vitro techniques into such experiments can facilitate rapid propagation of valuable plant lines for their experimental or breeding prospects (Fenning 2019).

Validation of results through experimental trials at nursery or field level are important to evaluate the mutant plants against disease-causing organisms (Fig. 7.3). Thus, in vitro mutagenesis for disease-resistant plants followed by proper field trial replications has proven more successful for stable resistance. Most in vitro mutagenesis studies, despite expending great time and effort, are only pilot-scale experiments or the results have not been validated through field trials.

Fig. 7.3 Screening of date palm mutant plants against bayoud disease using *Fusarium* spore treatment. *Left*: highly susceptible plant to *Fusarium* spore (control): *Right*: survival of date palm mutant after the *Fusarium* infection (Photos by S. M. Jain)

In vitro screening against a pathogen is usually commenced by using pathogen toxins or metabolites. Such toxins can be effectively exploited for pre-selection of mutant date palm plants against bayoud disease (Fig. 7.4). These toxins may include mycotoxins, phytotoxins or host specific or non-host specific toxins (Sedra and Lazrek 2011). These toxins and/or metabolites can be standardized by incorporating them into the in vitro culturing media to grow date palm cells. However, in countries free of bayoud disease the bioecological surveys of such mycotoxins are very important prior to introducing them into the experimental procedures.

7.10 Molecular Mutagenesis

Mutations have profound effects on a plant genome, particularly in evolution and on the development of a variety or trait. Since the advent of molecular breeding techniques, a strong correlation between mutagenesis and introgression of the desired traits has been the driving force behind varietal development. However, such mutations were an aberrant type with a stochastic nature that makes it difficult to observe and study. Indeed, decades have been spent developing increasingly sophisticated assays and methods to study these low-frequency genetic errors, to understand their mode of action and to provide guidance to prevent undue genetic exposure. Contemporary and emerging new tools, in the form of enhanced NGS platforms and protocols, are abruptly transforming this paradigm. Various tools and anticipated technologies, such as TILLING-NGS, high-resolution melting (HRM) genotyping, key point technology, exome capturing, and single-cell analysis are discussed in the following sections.

7.11 CRISPR-Cas-Based Targeted Mutations

In the past, the generation of new plant varieties with desirable or improved traits has relied mainly on laborious and time-consuming breeding techniques. However, GE technologies (especially CRISPR-Cas) have laid the foundation stone of a new era of genome engineering, enabling efficient, accurate and rapid engineering of plant genomes (Wada et al. 2020). Cas protein can be programmed to target any DNA sequence, the specificity of the target sequence being determined by the sgRNAs. Based on the sequence homology, the Cas protein generates a site-specific blunt-ended double-strand break (DSB) (Jinek et al. 2012). The created DSB is then repaired by cell endogenous DNA repair machines, either by error-prone non-homologous end junction (NHEJ) (Lieber 2010), leading to the insertion or deletion of small random DNA fragments or nucleotides at the cleavage site, or by high fidelity homology-directed repair (HDR), leading to a precise modification of the genome (Filippo et al. 2008). DSBs are crucial events in GE and can lead to genome instability and unpredictable DNA repair effects. To circumvent

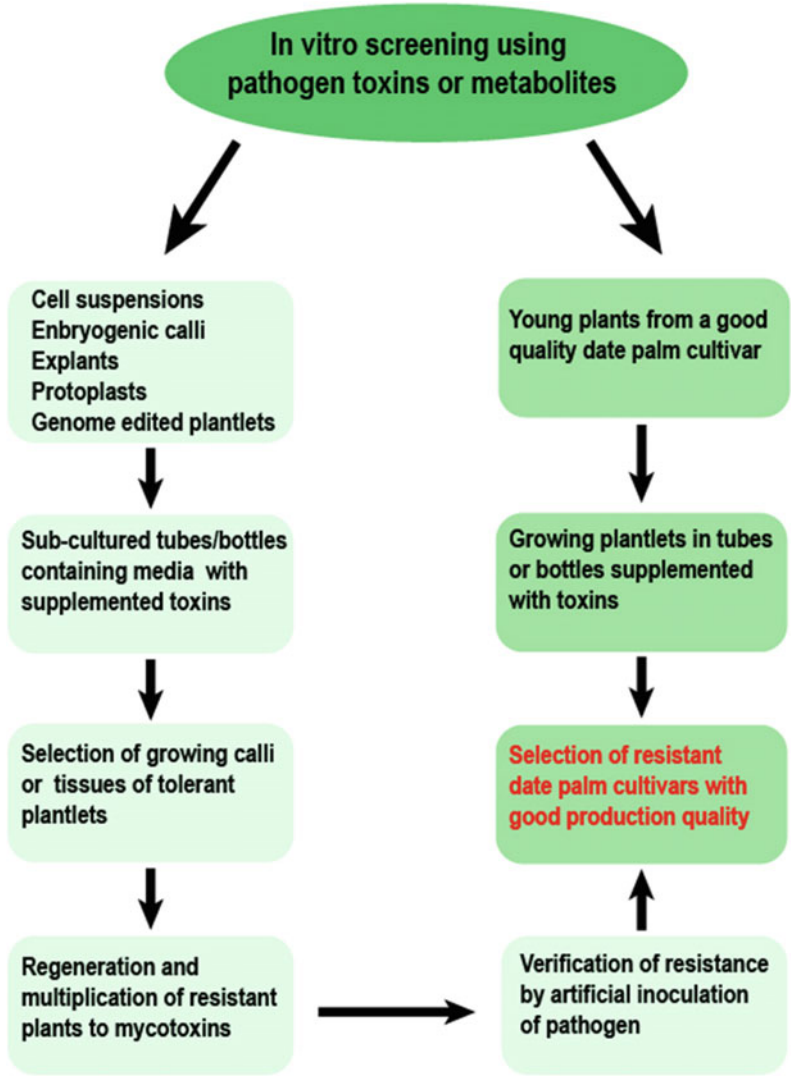

Fig. 7.4 Screening and evaluation procedure for resistant date palm cultivars generated through mutagenesis or conventional breeding against bayoud disease using pathogen toxins and other metabolites (Figure constructed by M.N. Sattar using Adobe Illustrator 2019)

this problem, modified approaches have been explored to target DNA or gene expression without inducing DSBs with a higher rate of accuracy and precision. The targeted mutagenesis using CRISPR-Cas9 based approaches has been applied in many crop plants against plant pathogens (Iqbal et al. 2016; Sattar et al. 2019a, b). Two very recently developed approaches including base editing and prime editing technique are becoming front liners in precise GE.

7.11.1 Base Editing

Base editing (BE) is a contemporary addition to precision GE and includes single-base substitution/alteration for accelerating crop improvement. Contrary to standard CRISPR-Cas-mediated GE techniques, BEs do not induce DSB, as a result the genome remains free from indels and hence BEs deliver precise GE with much-reduced on- and off-target indels

(Komor et al. 2017). In BEs, all four transition mutations (C-T, G-A, A-G, T-C) can be executed at the target genome with the CRISPR-Cas system. The adenine base editor (ABE) can substitute A-to-G, while the cytosine base editor (CBE) can substitute C-to-T. In RNA, A can also be converted to Inosine (I). Both these BEs have been implicated for precise BE of different plant genome including thale cress (*Arabidopsis thaliana* (L.) Heynh.), wheat, maize, rice, tomato and potato (Ishizu et al. 2017; Jaganathan et al. 2018; Jin et al. 2019; Li et al. 2017a, 2018; Veillet et al. 2019; Zhang et al. 2019).

A CBE system contains two additional components: a cytosine deaminase for C-to-U conversion and an uracil glycosylase inhibitor (UGI) to subvert the cellular U base excision repair (BER) pathway. Two types of cytosine deaminases have been exploited; in the first type, rat cytosine deaminase (rAPOBEC1) is fused at the N terminus of dCas9 via an XTEN linker while UGI at the C-terminus (Komor et al. 2016). In the second type, sea lamprey deaminase (PmCDA1) along with UGI was linked to the C-terminus of dCas9 (Nishida et al. 2016). Target-AID, a type of CBE, utilizes pmCDA1 deaminase, while BE1-BE4 utilizes aAPOBEC1 deaminase. By employing the BE3 and target-AID systems up to 75 and 96.1% editing efficiency was achieved, respectively (Komor et al. 2016; Nishida et al. 2016). Via the CBE system, the deamination of C is catalyzed to U, which subsequently converted to T via DNA repair or replication pathway. Initially, the CBE system was investigated in rice for three selected targets: first target in *OsPDS* (P2), which encodes phytoene desaturase, and two other targets in *OsSBEIIb* (S3 and S5), which encode starch branching enzyme IIb. A precise point mutation was achieved successfully in each target gene and the efficiency of the desired mutations remained 19.2, 10.5 and 1.0% at the S5, S3 and P2 targets, respectively, and these disruptions of the intron-exon boundary resulted in high amylose contents in rice (Li et al. 2017a). In another study, rice plants with stable NRT1.1B and SLR1 base modifications were obtained and the observed base editing efficiencies were 2.7 and 13.3%, respectively (Lu and Zhu 2017). BE system was used to develop herbicide (sulfonylurea-, imidazolinone- and aryloxyphenoxy propionate-type) tolerance in the wheat plant by editing the acetolactate synthase (ALS) and acetyl-coenzyme A carboxylase genes. The results revealed that base ALS Pro-174 codon (TaALS-P174) endowed sufficient resistance to nicosulfuron herbicide in the MS growth medium. When TaALS-P174 editors were paired with editors for other targets of interest, co-editing occurred in nicosulfuron-resistant plants, and selection for resistance in growth medium increased the frequency of coupled targets by several times (Zhang et al. 2019). The dCas9 protein fused with epigenetic modifier was used to introduce heritable epigenetic (methylation and demethylation) modification in *Arabidopsis thaliana* plants (Gallego-Bartolomé et al. 2018). The proof-of-concept was verified by choosing an established example of stable epiallele in plants is FLOWERING WAGENINGEN (FWA), which get up-regulated and induce heritable late-flowering phenotype upon the loss of DNA cytosine methylation (5mC) in the promoter region. The CRISPR-dCas9-based demethylation system using the TET1cd and SunTag-modified systems targeted the demethylation and activate gene expression when directed to the FWA or CACTA1 loci. Higher specificity minimal off-target effects were observed at the target region.

To develop an ABE, *Escherichia coli* tRNA adenosine deaminase (ecTadA) was engineered and subjected to mutations to yield TadA* that can efficiently (53%) deaminate adenine in DNA (Gaudelli et al. 2017). Four classes of ABEs (ABE6.3, ABE7.8, ABE7.9, ABE7.10) were developed after using heterodimeric TadA (wtTadA–TadA*) and then fusing them with nCas9 (D10A). Of the four ABE classes, the most efficient is ABE7.10. ABE systems catalyze the deamination of A to Inosine (I), which is considered as G by the DNA replication machinery, thus ultimately leading to A-to-G substitution. A fluorescence-tracking ABE (rBE14) with nCas9 (D10A)-guided TadA: TadA7.10 heterodimer was employed for the

conversion of A·T to G·C in *OsMPK6*, *OsSERK2* and *OsWRKY45* rice genes and a higher level of editing efficiency at 16.7, 32.1 and 62.3%, respectively, were observed (Yan et al. 2018). By using engineered SpCas9 variants in the ABE system, two rice genes (*OsSPL14*, *OsSPL17*), were targeted at 25 and 45% editing efficiency, respectively (Hua et al. 2019). Besides these successful examples, both ABE and CBE have been executed concurrently in rice for adenine and cytosine base editing (Hua et al. 2019).

RNA BE (RBE) is another form of precise GE that allows protein modification without inducing any change in the genome. RBE substitutes the A-to-I at the transcript level, earlier to translation. RNA editing for the programmable A-to-I replacement (REPAIR) system has recently been devised by combining dCas13 with ADAR2 deaminase to correct G-A mutations (Cox et al. 2017). REPAIR system can modify up to 28% disease-relevant mutations. Two versions of the REPAIR system (v1, v2) have been developed; the latter is an improved version with up to 900 times reduction in off-targets. REPAIRv1 can target virtually all RNAs, as Cas13 did not require any PAM.

7.11.2 Prime Editing

Recently, a new GE technology, without inducing DSBs or requiring a donor DNA template, was developed and referred to as *prime editing* (PE) (Anzalone et al. 2019). In this system, Cas9 nickase is fused to reverse transcriptase (RT) to drive an engineered primary editing guide RNA (pegRNA), which shares three unique characteristics; (a) primer binding site (PBS), (b) the desired edited sequence and (c) a sequence that recognizes the target DNA. PegRNA is driven by the Cas9 nickase to the target DNA sequence, and nicks are created. The 3′ end of the nicked DNA strand hybridizes to the PBS of pegRNA, priming the reverse transcription of the desired edited sequence on the pegRNA by RT. Hybridization between the target DNA and the reverse transcription product produces a 3′ overhang bearing edited sequences or 5′ overhang bearing unmodified sequence. The 3′ overhang is ligated to the DNA strand while the 5′ overhang is cleaved by the endonuclease. Eventually, newly formed heteroduplex DNA is repaired by an endogenous DNA repair process leading to the integration of modified sequence at the target genome. Via PE insertions (up to 44 bp), deletion (up to 80 bp) and different types of point mutations have been achieved efficiently, precisely and with much reduced off-targets compared to other Cas proteins. Moreover, PE showed more advantages than BE in cases where multiple cytosines or adenines were present in the base-editing window because PE enables precise single-nucleotide replacement. The target scope is also extended in PE because, unlike BE, the need for a PAM sequence is not limited to an appropriate distance from the target nucleotides.

The game-changer PE system was recently developed and opted for in rice and wheat plants (Lin et al. 2020). Three different PE systems (pPPE2, pPE3, pPPE3b), were compared to each other after codon-optimization and expressed them using the Ubiquitin-1 (Ubi-1) maize promoter. To drive pegRNA and nicking sgRNA transcripts, plants promoter TaU6 (or OsU3) and TaU3 were used, respectively. To verify proof-of-concept, PE systems were used to convert blue fluorescent protein (BFP) to green fluorescent protein (GFP) in rice protoplast, which can be achieved by just modifying the codon 66 from CAC (histidine) to TAC (tyrosine). The results demonstrated that PPE3b could introduce the desired targeted mutation in plants with 6.6% efficiency. Subsequently, the editing of 6 endogenous genes in each rice and wheat was achieved by building 21 pegRNAs to test the pPPE2 and PPE3 (or PPE3b) systems in the protoplasts. The editing efficiency was measured by deep amplicon sequencing and results showed that these PE systems induced comparable editing efficiencies in rice and wheat plants. In rice, 6-bp deletions at a frequency of 8.2% in OsCDC48-T1, 3-bp deletions at a frequency of 2% at OsCDC48-T2, and the six types of single-nucleotide substitutions, including C-to-T, G-to-T, A-to-G, G-to-A, T-to-A, and C-to-A, at

frequencies up to 5.7% were noted. In wheat, single-nucleotide substitution frequencies, including A-to-T, C-to-G, G-to-C, T-to-G, and C-to-A, reached 1.4% (Lin et al. 2020).

Xu et al. (2020) developed different types of PE systems and their editing efficiencies were appraised in rice plants. Initially, a plant prime editor 2 (pPE2) system was developed to target HPT^{-ATG} reporter gene in rice and the pPE2PPE system-induced modification at 0–31.3% efficiency at different genome sites in T0 plants. Subsequently, another PE system (pPE3) was generated, after following the strategies of PE3 and PE3b in human cells, to yield better editing efficiency but ends up at comparable or even lower editing frequencies. Besides, a third PE system was developed, a surrogate pPE2 system, by incorporating the HPT^{-ATG} reporter to enrich the prime-edited cells. Results revealed that nucleotide editing was easily detected in the resistant calli, probably due to enhanced screening efficiency of the modified cells.

Thus far, the developed plant PE systems are less efficient than BE systems for transition point mutations, but PEs provide induced changes that cannot be accomplished with other GE systems. Nonetheless, in the future, improved versions could be generated with improved efficiency.

7.12 High-Throughput Mutation Screening Techniques

Genetic markers are heritable biological characteristics, which are present in allelic forms or genetic loci and can therefore be exploited as experimental tags or probes to report/document the cellular or tissue-specific genes. Genetic markers are classified into two categories: classical markers and DNA markers. DNA markers can demonstrate mutation/variation in the genome to reveal polymorphism (deletion, insertion and/or substitution) between different genotypes in a population. The assessment of polymorphism in date palm and other plant species is extremely important for the improvement of genetic resources. DNA markers have developed into three main categories based on different polymorphic detection techniques: hybridization-based (Southern Blotting, RFLP and fingerprinting), PCR-based (RAPD, AFLP, SSR, SCAR etc.), and DNA sequence-based (SNP).

Restriction fragment length polymorphism (RFLP) was initially used in date palm to differentiate five elite cultivars (Barhee, Deglet Noor, Khalassa, Medjool) on the basis of the presence of a unique restriction site *Eco*RI and the employed cDNA1 probe was found to be highly polymorphic (Corniquel and Mercier 1994), subsequently the same group of researchers executed the same approach to differentiate five other date palm cultivars (Kenessy, Lulu, Nabtha Saïf, Sheshi), Corniquel and Mercier (1997). The PCR-RFLP-based approach was exploited to decipher polymorphism between two haplotypes after amplification and RFLP analysis of the 15 regions of plastid genomes, the results demonstrated that both date palm accessions shared a common genetic background (Hela et al. 2003). A sex-linked region was identified in date palm (Al-Dous et al. 2011) and later the same region was used to determine sex in date palm by employing PCR-RFLP-based approach (Al-Mahmoud et al. 2011).

AFLP analysis using 13 key combinations revealed minor genetic differences in the tissue-derived from date palm offshoots of different cultivars. The percentage of polymorphism in the cvs. Bertamoda, Gandila and Sakkoty was found to be 2.6, 0.79 and 1%, respectively (Saker et al. 2006). Results from an investigation using 4 AFLP primers genetic variation in 23 Medjool and 33 Deglet Noor date palm accessions in California demonstrated the presence of a huge number of intravarietal variations in Medjool, but almost none in Deglet Noor (Devanand and Chao 2003). To detect polymorphism in the offshoots and tissue culture of developed elite date palm cvs. Barhee and Medjool, AFLP results revealed low levels of genetic variations in Barhee plants, either developed from offshoots or tissue culture. However, a significant level of genetic variation was observed among Medjool plants produced from tissue culture (Gurevich et al. 2005). AFLP-mediated genetic variations in tissue culture-derived (through embryogenesis or

organogenesis) of 10 date palm varieties cultivated in the United Arab Emirates were readily distinguishable and a low incidence of somaclonal variations was observed in the plants developed through organogenesis as compared to plants developed through embryogenesis. (Al Kaabi et al. 2007).

RAPD and SSR markers have been extensively employed to demonstrate genetic variations in tissue culture-derived (via embryogenesis or organogenesis) date palm plants (Ahmed et al. 2009; Al-Khalifah and Askari 2007; Kumar et al. 2010; Mirani et al. 2020; Saker et al. 2000). In addition, RAPD markers were used to compare different date palm plants developed after inducing floral buds with auxins and cytokinins in somatic embryogenesis (Solangi et al. 2020). RAPD markers have recently been executed to assess somaclonal variations in salt-adapted and non-adapted date palm plants (Al-Khateeb et al. 2019b, 2020).

Next-generation sequencing (NGS) platforms for the identification of genetic variations have been widely used recently and this technique seems ideal, straightforward and comprehensive to precisely map the mutation to unravel the phenotype of interest. However, due to the numerous unrelated polymorphisms segregating with the causative mutation in a mutagenized population, a very low signal is obtained and the cost of identifying potential variants from an enormous amount of sequence data is a major constraint. To overcome such limitations, NGS has been coupled with other techniques. Mapping by sequencing through NGS has accelerated the identification of occasional mutations at the SNP level (Schneeberger and Weigel 2011). NGS mapping has been performed in different plant species, as reviewed by Taheri et al. (2017). SHOREmap is an integrated approach employing simultaneous mapping and mutation identification via NGS and is based on the principle of mutant allele frequency (MAF) estimation. This approach has been successfully implemented to map the causative mutation using the Illumina sequence of a population of 500 pooled *Arabidopsis thaliana* F2 lines (Schneeberger et al. 2009). Subsequently, another DNA-based-mapping-by-sequencing approach was developed, referred to as *next-generation EMS mutation mapping* (NGM), based on the principle of homozygosity mapping. This approach easily mapped the candidate causative mutations even in small F2 *Arabidopsis* populations (Austin et al. 2011). To minimize the number of causal candidate mutations, Hartwig et al. (2012) developed a deep candidate resequencing (dCARE) approach. In this technique, mapping was achieved by resequencing coupled with the use of Ion Torrent genome sequencing to identify causative mutations for suppression of proteins such as heterochromatin1, a gene involved in chromatin-mediated gene repression. Even so, this method exploits the backcross principle and the SHORE pipeline for mapping analysis and the need for targeted deep sequencing of candidates to isolate exact causative SNP (Hartwig et al. 2012). HRM analysis was also coupled with NGS for cost-effective and rapid validation of genetic variations such as SNPs, insertions or deletions (InDels) and simple sequence repetitions (SSRs).

Although NGS-based assays to identify genetic variations share high throughput and reliability, these approaches are still not cost-effective and unambiguously labor-intensive. Recently, a method based on the sequencing of only coding sequences (exons) comprising only ca. 2% of the eukaryotic genome was developed to identify the genetic variations (Cosart et al. 2011; Hodges et al. 2007). This approach has been used to map SNPs within parents of the wheat population to build high-density map (Winfield et al. 2012), identification of induced mutations in wheat (Hussain et al. 2018), the discovery of large-scale mutations in rice (Henry et al. 2014) and detection of a unique mutation responsible for the reduction of cuticle wax in rice plants (Kim and Tai 2019).

Targeting induced local lesion in genomes (TILLING) is a high-throughput technique for the identification of candidate mutants in the gene of interest. TILLING is a robust, non-transgenic, low-cost and reverse genetic-based approach capable of identifying even a single-nucleotide change, small deletions and mutant

lines in a population (McCallum et al. 2000), this technique applies to all organisms, but is highly suited to plants. TILLING involves traditional mutagenesis using the optimum type and concentration of mutagens, the development of a non-chemical population, the extraction and pooling of DNA, the detection of mutations via PCR and the validation of results. The most advantageous mutation detection method in plants is the use of TILLING coupled with celery nuclease, CEL I (Gottwald et al. 2009). This technique has been largely superseded by the development of more sensitive and higher throughput TILLING-high-resolution melt (HRM) techniques. With HRM, mutations in target genes are detected by PCR, followed by denaturing and re-annealing, during re-annealing DNA-binding fluorescent dye helps to monitor the double-stranded DNA product. The observed fluorescence is directly related to the re-annealing (Reed et al. 2007). Although TILLING and TILLING-CEL1 are robust and high-throughput platforms for the detection of genetic variations in plants, such approaches are labor-intensive, challenged by pooling more than eight samples, limited to screen a single target at a time and sequence is required to characterize mutations. TILLING-NGS technology demonstrates great potential for mutation detection. Some examples of the use of Illumina sequences in TILLING (Granier et al. 2015; Tsai et al. 2011) is the implementation of a flexible and effective combination of TILLING-NGS methods to detect mutations in targeted loci in rice and wheat. This technology has been used to detect genetic mutations in peanut for biotic and abiotic stress resistance (Guo et al. 2015). This approach shared substantial advantages such as high-performance, reliable and multidimensional pooling, high probability threshold, identification of mutations with the associated base change, allows a flexible choice of pooling methods and is scalable according to experimental combinations (Taheri et al. 2017). The sensitivity of the aforementioned screening approaches for variant detection can be extended to date palm mutation breeding programs for efficient mutant detection. The Tilling-NGS-based approach can also open unparalleled utilities to decipher the genetic variations between/among different date palm cultivars grown in the Arabian Peninsula.

7.13 Variations in DNA Copy Number During Random Mutations

In addition to SNPs, InDels and SSRs, copy number variants (CNVs) are another major form of genetic variations arising after the gains or losses of a DNA segment(s) in animal and plant genomes. CNVs arise naturally after the imbalanced DNA changes that led to variations at the particular DNA sequence and cover relatively large segments of DNA (ranging from 1 kb to several Mbs) (Alkan et al. 2011). Identification of CNVs in the genomes of living organisms has demonstrated the rationale to consider genomes as dynamic entities. The exploration of CNVs' role in plants is still at a beginning phase (Francia et al. 2015). Only a few studies have encompassed the role of CNVs and have shown that they are widespread in plant genomes, have significantly impacted the evolution and contributed to the genetic diversity.

As CNVs mainly affect members of large families of functionally redundant genes, so the generation of CNVs is important for implementation in mutation breeding programs of plant species. Maize is the first plant species that was thoroughly studied for the presence of CNVs (Springer et al. 2009; Żmieńko et al. 2014), and many reported CNVs, such as 19 CNVs in inbred maize lines and 14 in teosinte accessions, have a role in the domestication (Chia et al. 2012; Swanson-Wagner et al. 2010). The presence of different copies of a gene may affect the cumulative effect of the gene that can lead to a change in phenotype. For example, Palmer amaranth (*Amaranthus palmeri* S. Wats.), a major weed in the USA, swiftly developed resistance to glyphosate and investigation revealed that resistance was driven by the increase in 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) copy number (Gaines et al. 2010; Sammons and Gaines 2014). Some studies have

deciphered the phenotype linked CNVs having a role in plant stress tolerance. The CNV of *Bot1*, boron efflux carrier, gene has a substantial role in conferring boron tolerance in barley plants (Sutton et al. 2007), the CNVs of three soybean genes have a role in nematode resistance, and a CNV in the Fr-A2 locus of durum wheat induces frost tolerance (Sieber et al. 2016). CNVs have been found associated with nucleotide-binding leucine-rich repetition (NB-LRR) genes and receptor-like kinase (RLK) genes, known to be involved in plant defense-related mechanisms (Saxena et al. 2014). A number of disease resistance genes in rice in the CNV regions have also been identified (Yu et al. 2011). Several other examples of CNVs have been reviewed (Dolatabadian et al. 2017; Żmieńko et al. 2014).

Fast neutron radiation has been used in plants to develop extensive mutant collections. Genome-wide structural variants of soybean plants have been achieved after exposure to fast neutron radiation. These structural variants were found to be associated with quantitative changes in seed composition and short petiole mutant phenotypes. These results demonstrated the successful utilities of fast neutron-irradiated mutants as a source of novel genetic losses and gains (Bolon et al. 2014). An optimized array comparative genomic hybridization (aCGH) platform was developed to characterize barrelclover (*Medicago truncatula* Gaertn.) mutants after irradiating to fast neutron, and a CNV database associated with *M. truncatula* mutant lines was subsequently generated using this platform (https://medicago-mutant.noble.org/mutant/FNB.php) analysis of one of the mutated *M. trunculata* line FN6191 revealed a hyper-nodulation phenotype and showed a 22 kb deletion of chromosome 4 involving the SUNN gene (Au-Chen et al. 2017).

Gamma radiation has been used for decades to produce genetic mutants, including CNVs, in many crop species. Interspecific poplar hybrids were generated when *Populus nigra* L. pollen was exposed to gamma radiation to pollinate *Populus deltoides* W. Bartram ex Marshall (Henry et al. 2015). Whole-genome sequencing was performed to detect mutations in Red-1 rice derived from gamma-irradiated *Oryza sativa* L. species (Cheng et al. 2014). Compared to the *O. sativa*, 9.19% of the Red-1 rice genome sequence was mutated and the types of mutations were indels, CNVs and SNPs. These mutations were associated with cellular components, binding function, catalytic activity and metabolic processes that were susceptible to radiation. In another study, four consecutive generations of *O. sativa* were irradiated and WGS revealed the presence of scattered mutations across all the 12 chromosomes and types of mutations were indels, SNPs, CNVs, single-base substitution and structural variations (Li et al. 2016b). CNVs were identified in two dwarf rice mutants, isolated from the descendants of gamma-irradiated rice plants. All the detected CNVs were found to be less than 10 kb in size.

Novaria is a commercial banana cultivar, produced through mutagenesis using gamma rays. A low-coverage WGS-approach was used to map CNVs in Novaria, which demonstrated that it contains multiple large deletions, ranging from 0.3 to 3.8 Mbp, spanning 189 coding regions (Datta et al. 2018). A guideline for gamma irradiation mutagenesis and CNV screening in cv. Cavendish bananas was then developed using cv. Williams and the results showed that deletion events were predominant in the new cultivar.

In date palm, CNVs have been detected via sequencing-based platforms. More than 10,000 CNVs have been detected in cvs. Deglet Noor and Medjool after comparing them with the reference genome (cv. Khalas) and identifying regions where the observed number of matching sequences in the genome is significantly different (up or down) from the expected number. The CNV-SEQ 28 program predicted several imbalanced sequence counts, but these identified sequences were referred to as *imbalanced sequence count regions* (ISCRs) (Al-Dous et al. 2011). A sliding window analysis was used to scan the fruit sugar composition of the GWAS region for the detection of genetic variations, including CNVs. Two polymorphic deletions were found, the first of which was ~40 kb (at ca. 2.467–2.507 Mb), which included the 5′ half

of CWINV1 (including the first two coding exons and their promoter region), the region ~5 kb downstream of CWINV3 and the putative pseudogene of CWINV2. The extent of this deletion makes CWINV1 non-functional. A second polymorphic deletion of ~5 kb is located near the center of the GWAS peak (at ca. 3.088 and 3.093 Mb) in the non-coding sequence between the 5′ end of A/N-INV1 and the adjacent histone deacetylase (HDA) gene. Coverage depth data analysis revealed that CWINV1/3 and A/N-INV1 deletion regions in varieties (enriched with sucrose) such as Soukar Iraqi are more homozygous than reducing-type varieties such as Khalas (Hazzouri et al. 2019).

7.14 Off-Target Mutations and Strategies to Reduce Off-Target Mutations During Site-Directed Mutagenesis

Site-specific GE technologies such as TALENS, ZFNs and, in particular, the contemporary CRISPR-Cas system have certain unique advantages, such as high efficiency, cost-effectiveness, robustness, simplicity and multiplexing capacity. However, one of the key weaknesses and crucial concern of these systems is unwanted mutations at off-site locations of the target genome. Various strategies have been developed to mitigate off-targets, such as GC content, gRNA length, truncated gRNA, sgRNA and Cas protein concentrations, transformation methods, Cas variants and aptazymes. The mitigation of off-targets in mutation breeding of plant species, especially in date palm mutation breeding, is crucial to yield desired traits without disturbing the genetic background of elite cultivars (Fig. 7.5).

7.14.1 GC Content of SgRNA

The structural sequence of gRNA, particularly its GC contents, has an impact on the target activity of the CRISPR-Cas system. Ideally, GC contents between 40–60% in the gRNA sequence form stable DNA:RNA duplex but destabilize the off-target binding and eventually enhance the on-target activity (Wang et al. 2014). In addition, purine residues at the end of four nucleotides in gRNAs, in particular guanine at the twentieth position and cytosine at the sixteenth position, improve editing efficiency (Doench et al. 2014; Wong et al. 2015). A positive correlation between PAM-proximate GC% of sgRNAs has been identified through mutagenesis, and these parameters help in the design of gRNAs with reduced off-target activities.

7.14.2 GRNA Length and Mismatches

The length of gRNA can profoundly affect the efficiency of GE and can lead to off-target/unwanted mutations. The length of gRNA and mismatches to the target genome sequence determine the efficiency and off-target activity. For example, a study appraised 16–20 nucleotides long gRNA effect on GE efficiency and off-target mutations in liverwort, *Marchantia polymorpha* L., and gRNA length up to 17 nucleotides revealed a higher GE efficiency as compared to its length (18–20 bp); however, 20 bp long gRNA did not exhibit any unwanted mutations (Fu et al. 2014; Sugano et al. 2018). Dead RNA off-target suppression (dOTS) is the new strategy in which dead truncated gRNA, which guides Cas9 but suppresses cleavage, has resulted in reduced off-targets and increased 40-fold on-target activity (Rose et al. 2020). Furthermore, a chimeric gRNA with a partial DNA substitution approach has been executed to minimize off-targets in human cells and a partial DNA substitution at 5' end substantially reduced off-targets due to a lower tolerance of DNA-DNA mismatches compared to DNA-RNA duplexes (Yin et al. 2018). Multiplexing of CRISPR-Cas systems has also led to an increase in off-targets in rice and tomato plants (Brooks et al. 2014; Xie et al. 2015; Yin et al. 2018). General guidelines to circumvent off-targets have been formulated as: a) more than three mismatches within the first 7–10 bp of the PAM and b) gRNA bulges within the first 12 bp of the

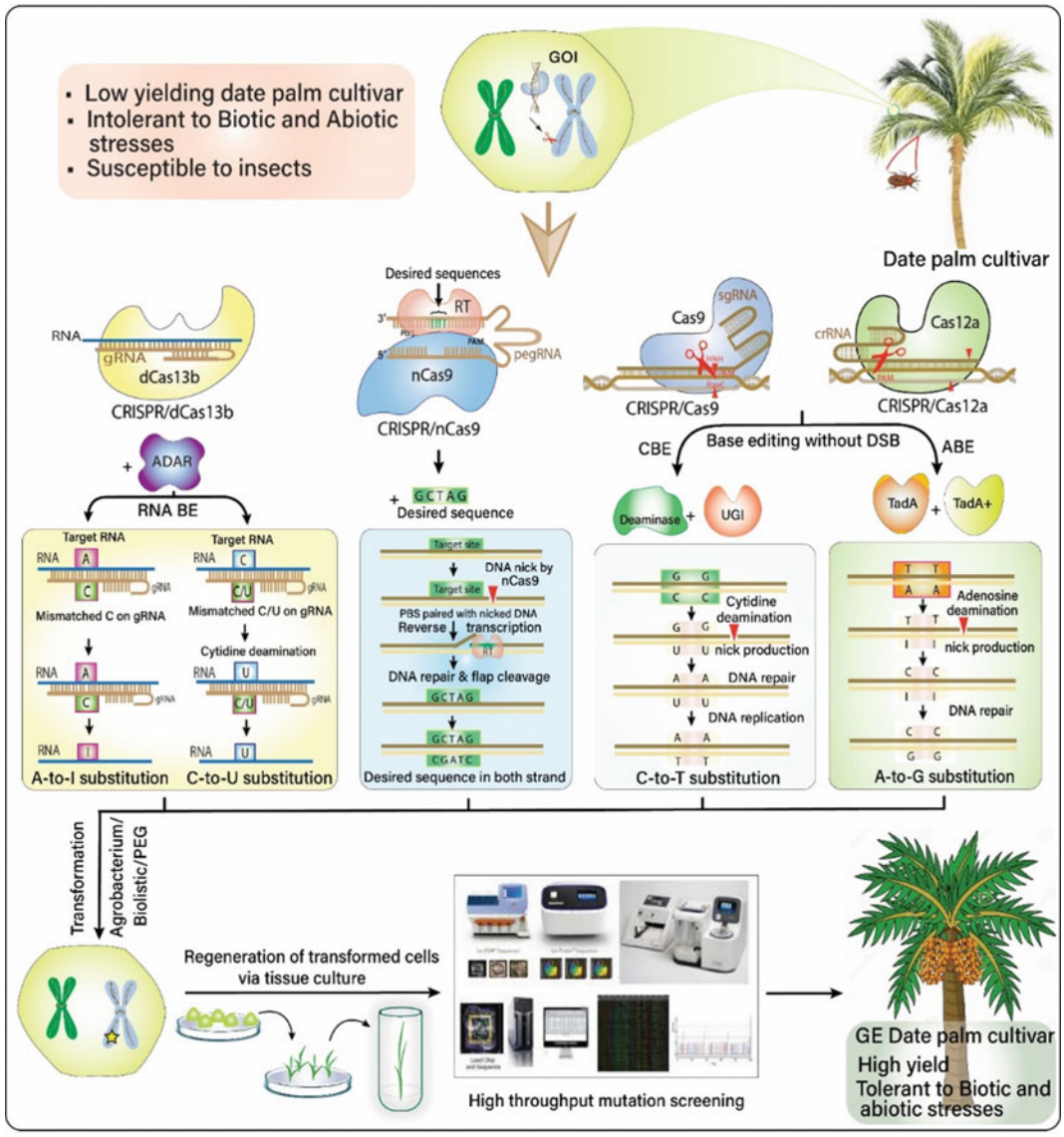

Fig. 7.5 A schematic diagram for the application of CRISPR-Cas in date palm targeted mutation breeding (Figure constructed by Z. Iqbal using Adobe Illustrator 2019)

PAM, should be avoided (Zhang et al. 2017). Li et al. (2016) analyzed off-targeted mutations in rice plants using four sgRNAs and, based on their findings, the highest undesired changes (67.5%) were observed when two mismatched bases occurred between sgRNA and its off-target site, while the lowest off-target rate (2.5%) was found with six mismatches for sgRNA. Therefore, the use of sgRNAs having more than two mismatches to the target sequence within an off-target site can prevent unintended changes in rice (Li et al. 2016a).

7.14.3 Chemical Modification of GRNA

The gRNA chemical alteration approach can increase on-targets GE efficiency. A 40–120-fold reduction in off-targets was witnessed after incorporating a 20-O-methyl-30-phosphono acetate into the gRNA ribose-phosphate backbone (Ryan et al. 2017). Specificity improved by ~ 25-fold on-target by integrating bridged and locked nucleic acids into guide sequences through the formation of dynamic RNA-DNA duplex (Cromwell et al. 2018). In addition, the modification of the hairpin structure at 50 bp upstream of gRNA enhances the specificity of Cas proteins (especially Cas9 and Cas12) by reducing up to 55-fold off-target effects (Kocak et al. 2019).

7.14.4 Concentration of Cas Protein/GRNA

Using low expressions of Cas protein/gRNA is another potential way of minimizing off-target effects. This can be opted for after careful selection of the promoters to drive the expression of Cas protein. In *Arabidopsis* plants, the expression of Cas9 was appraised under the control of CaMV35S (a constitutive) promoter and an egg-cell (EC; inducible) promoter. The CaMV35S promoter revealed a low editing frequency compared to the EC promoter (Begemann et al. 2017). Likewise, the use of embryo-specific promoters (YAO) yielded better and more efficient GE in orange, *Citrus sinensis* L., after specifically expressing the Cas9 and gRNA at the plant reproduction stage (Zhang et al. 2017). Observations implied that a higher and constitutive expression of Cas proteins and a higher gRNA level had a low editing potential in tomato and *Arabidopsis thaliana* (Pan et al. 2016). Several studies encompassed the expression of Cas9 protein in monocots and the results of all studies unanimously revealed that the use of plant endogenous promoters led to higher on-target mutations than a constitutive CaMV35S promoter (Liang et al. 2014; Miao et al. 2013; Shan et al. 2013; Xu et al. 2014). The efficiency of soybean promoter (U6-10) and *Arabidopsis* Ubi (AtUbi) promoter was investigated and compared in *Glycine max* (L.) Merr., a 2-4-fold improvement in on-target efficiency was achieved by endogenous U6-10 promoter compared to AtUbi promoter (Sun et al. 2015b). Nonetheless, AtUbi and CaMV35S promoter induced comparable on-target GE efficiency in tomato plants (Pan et al. 2016).

The impacts of different endogenous promoters on the heritability of mutations were investigated in different plant species and results revealed that endogenous and specific promoters yielded better heritability and on-target efficiency as compared to other promoters (Hyun et al. 2015; Li et al. 2013; Malnoy et al. 2016; Wang et al. 2015). However, the selection of the promoter depends on the objectives of the research, targeted gene and the target plant species. A comprehensive GE approach in date palm providing all the important parameters of the gRNA design, evaluation of the off-targets, selection of a suitable approach to assess the off-targets has been investigated (Sattar et al. 2017).

7.14.5 Cas Protein Variants

The development and deployment of Cas variants have made significant contributions in the reduction of off-target effects. Two of the most exploited Cas proteins for plant GE are Cas9 and Cas12a (Cpf1), both differ substantially from each other in recognizing PAM sequences. Cas12a recognizes TTTV (V = A, C or G) while Cas9 recognize NGG PAMs. A comparative study of these Cas proteins showed that Cas9 mechanism is more efficient (90–100%) and accurate than Cpf1 (0–60%) to promote on-target mutations in maize plants (Lee et al. 2019). Several variants of both of these Cas proteins have been engineered to minimize the off-targets and to enhance the on-target efficiency. Two natural variants of Cas9, SaCas9 (*Staphylococcus aureus*) and StCas9 (*Streptococcus thermophilus*), recognize longer PAM sequences, including NNGRRT and NNAGAAW,

respectively, that can ultimately enhance their on-target efficiency. Nonetheless, SaCas9 demonstrated higher on-target efficiency compared to SpCas9 in *Arabidopsis thaliana* (Wolter et al. 2018). Two engineered versions of SpCas9, referred to as SpCas9-VQR and SpCas9-EQR, were investigated to identify atypical targets in *A. thaliana* (Yamamoto et al. 2019) and results showed that SpCas9 variants are more efficient in GE than the conventional SpCas9 and could be effective in recognizing atypical targets.

A simple but robust approach to suppress off-target effects in plants uses a Cas9-paired nickase mutation in one of the nuclease domains (HNH or RuvC-like), although Cas9-paired nickase produces a 3'-overhang with a lower frequency of on-target mutation than a 5'-overhang structure. However, the main advantage of the Cas9-paired nickase compared to Cas9 is its potential to reduce unwanted mutations. Other variants of Cas proteins referred to as deactivated/dead variants (dCas9 and dCas12) have been developed by inducing mutations in the nuclease domain, and these deactivated variants have been widely used in the CRISPR GE systems to prevent off-target mutations (Brocken et al. 2017). The dCas protein variants bind to the target sequence and block the transcription elongation (Bikard et al. 2013). In addition, these variants were added to the cytidine deaminase enzyme and to the transcription regulators. Former versions have the ability to convert C-to-T and G-to-A in the target plant gene (Komor et al. 2016; Lu and Zhu 2017), while later on, they have the ability to regulate gene expression in plants (Lowder et al. 2015).

7.14.6 CRISPR Delivery Techniques and Vectors

The delivery of the CRISPR-Cas system is one of the vital factors to produce better on-target and least off-target mutations. There are several transformation methods to deliver the designed CRISPR-Cas-sgRNA cassettes into a plant genome: PEG-treated transformation *Agrobacterium*-mediated transformation, biolistic method, ribonuclease protein (RNP) complex, lipid and polymer-mediated transformation and delivery via viral vectors.

Polyethylene glycol (PEG)-mediated transformation of the CRISPR-Cas system was initially achieved in maize plants (Liang et al. 2014). Since that first execution, it has been successfully achieved in protoplasts of different plants, including *Arabidopsis thaliana*, apple, maize, petunia, rice, soybean, tobacco and wheat (Sandhya et al. 2020). To generate PEG-mediated transformation in date palm plants, the cells need to be subcultured to yield protoplasts. Date palm protoplasts can be achieved by treatment with different enzymes, such as driselase, cellulases and pectinases. The protoplasts concentration of 2×10^6–2×10^7 may result in better transformation efficiency; the count can be performed by microscope or hemocytometer. Similarly, the concentration of CRISPR-Cas plasmids (~20–30 ug) can produce better efficiency. After mixing the plasmid, the PEG is added to induce transformation and the successful transformants are further grown on the selection media. The PEG-mediated transformation is simple, efficient, allows multiple samples to be processed simultaneously and produces a transformed cell population with high survival and division rates. The main challenges and limitations of the PEG-mediated delivery system are the establishment of suspension cells and protoplasts isolation.

The most employed method of CRISPR-Cas system delivery into plants is *Agrobacterium*-mediated transformation. The utility of this system is not limited to callus, leaf and floral organs can also be used. Several plant species have been efficiently engineered/edited after employing this delivery technique. The transformation efficiency of the *Agrobacterium*-mediated transformation is more than particle bombardment and ranged from 40 to 100%, depending on the plant species (Li et al. 2017b; Naim et al. 2018; Yu et al. 2010). *Agrobacterium*-mediated transformation via the floral dip technique has also been used in plants for successful GE and has been employed in *Arabidopsis thaliana* (Castel et al. 2019), *Brassica rapa* L., flax, tomato, radish, *Setaria*

viridis (L.) P. Beauv. and wheat (Bastaki and Cullis 2014; Curtis and Nam 2001; Sharada et al. 2017; Zale et al. 2009). The transformation efficiency of 50–60% was reported in flax, higher than those reported for *A. thaliana* using the floral dip approach (Bastaki and Cullis 2014).

Biolistic delivery is widely used for the delivery of the CRISPR-Cas system into plants after coating the CRISPR-Cas components onto certain metal (Cu, Hg, Au) particles. This approach has been executed in many plant species, including soybean (Li et al. 2015), maize (Svitashev et al. 2015), potato (Andersson et al. 2018), wheat (Wang et al. 2014) and brassica (Murovec et al. 2018). However, the regeneration of transformed tissues, optimization of selection pressure, time-consumption, less cost-effective and low transformation efficiency are the major challenges associated with this technique. A very low GE efficiency is achieved in maize between 2.4 and 9.7% (Svitashev et al. 2015). Likewise, delivery of the sgRNA and nuclease as RNP complexes via electroporation into plant protoplasts has demonstrated a low frequency of off-target mutations. Recently, Cas9 with a cytidine base editor along with gRNA was transcribed in vitro and used to deliver wheat and rice protoplasts for base editing (Li et al. 2018).

Recently RNP has been used as a technique for achieving GE plants including apple (Malnoy et al. 2016), rice (Foster et al. 2018), potato (Andersson et al. 2018), lettuce (Park et al. 2019) and brassica (Murovec et al. 2018). In RNP complex, Cas9-sgRNA cleaves the target site and gets degraded rapidly in the cells leading to less off-target cleavage DNA-free GE plant cells, which can mitigate genetic modifications raising ethical concerns. The transgene-free GE of six polyphenol oxidase genes of mushrooms was achieved using the RNP technique. The GE mushroom exhibited a 30% reduction in the enzyme activity responsible for browning and also avoids US regulations on GE plants (Waltz 2016). Moreover, this technique is also useful for vegetatively propagated crops such as date palm, where removing a transgene from the GE plants via backcrosses is not possible (Sattar et al. 2017).

Efficient and self-replicating virus-based vectors have been demonstrated to convoy the CRISPR-Cas system into plants. Both DNA viruses (bean yellow dwarf virus [BeYDV] (Baltes et al. 2014; Cermak et al. 2015); wheat dwarf virus [WDV] (Gil-Humanes et al. 2017); cabbage leaf curl virus [CabLCuV] (Yin et al. 2015) and RNA Virus (tobacco rattle virus [TRV] (Ali et al. 2015) have shown effective gene targeting rates in *Nicotiana benthamiana* and potato, tomato, rice and wheat The BeYDV-based vector produced a 12-fold increase in on-target efficiency compared to *Agrobacterium*-mediated transformation, WDV-based vectors led to a 10-fold increase in on-target efficiency in wheat and 19.4% higher efficiency in rice. The use of TRV-based vectors led to a 15% reduction in off-targets, which may likely be due to a lack of mutation inheritance.

The total expression amount of Cas9 and sgRNA cannot be well controlled by vector-based CRISPR delivery methods, compared to the direct use of RNP complexes. Carriers are therefore urgently needed to protect Cas9 and sgRNA from the destructive physiological environment and convey them simultaneously to the nuclei. Several delivery methods have been reported for this purpose, such as cell-penetrating peptides (Ramakrishna et al. 2014), DNA nanoclews (Sun et al. 2015a), Cas9En-arginine nano-assemblies (Mout et al. 2017) and poly (ethylene imine)(PEI)-based nanocarrier (Gori et al. 2015). Above all, lipids and polymers are increasingly important for the delivery of Cas9 protein/sgRNA, Cas9 pDNA and mRNA. Delivery methods based on lipids and polymers will shape CRISPR-Cas technology in the coming years.

The general strategy of these methods is to stabilize on-target binding stability and destabilize off-target activity. The first type of method uses strategies to strengthen on-target stability (Guilinger et al. 2014; Ran et al. 2013; Tsai et al. 2014). These strategies effectively implement double target identification checkpoints by increasing the number of matched base pairs for target sequences. These approaches are highly successful and can lead to a substantial reduction

(50–1000-fold) in off-target frequency (Ran et al. 2013). However, these approaches are not free from drawbacks such as the requirement for multiple CRISPR-Cas9 system components, which thus pose challenges in concurrent delivery of multiple gRNAs.

7.15 Conclusions and Prospects

Physical and chemical mutagens have made a significant contribution to boosting the worldwide agricultural productivity. Nonetheless, the problems associated with the use of these mutagens, such as the unwanted load of genetic mutations and their laborious screening, have induced researchers to explore alternative approaches. The development of site-specific nucleases responds to needs and can lead modern agriculture to producing sustainable crop production and nutrition security. The potential of CRISPR-Cas-based GE tools to precisely edit/mutate the plant genome and to produce transgene-free plants has a clear advantage over the previous random induced mutations. CRISPR-Cas tool provides a simple and flexible approach for targeted mutagenesis to knock out or knock in genes, single-base editing, prime editing, replacing the entire gene, regulation of indigenous transcriptional events and many other site-specific genetic mutations in crop plants. However, with the continuous developments of new GE tools, it will be critical to explore the alleles with better agronomic and commercial traits. Additionally, state-of-the-art GE tools must be sought out for transversion mutations in the future. Successful execution of CRISPR-Cas-based GE tools to accomplish site-directed mutagenesis in many woody trees has paved the way, established its hierarchy and opened a myriad of applications in date palm genome engineering. The prospects of CRISPR-Cas-based GE will be helpful to develop date palm plants resistance against different biotic and abiotic stresses. In addition, date palm breeding cycles will be shortened and help to achieve sustainability in the production of date palm fruit.

Appendix I: List of Some Research Institutes Relevant to Date Palm

Institute name	Specialization research activities	Contact information and Web site
Date Palm Research Center of Excellence	Biotic and abiotic stresses through conventional and modern techniques	King Faisal University, Eastern Province—Al-Ahsa City PO Box 380 Postal Code 31982. Saudi Arabia https://www.kfu.edu.sa/en/Centers/palms/Pages/Home-new.aspx
Date palm research group	Biotic and abiotic stresses	King Saud University, College of Food and Agricultural Sciences, Riyad, Saudi Arabia http://cfas.ksu.edu.sa/en/content/date-palm-research-group
Center for Desert Agriculture Research	Date palm genomics and molecular breeding	King Abdullah University of Science and Technology, Thuwal 23955-6900 Saudi Arabia https://www.kaust.edu.sa/en/
Date palm Research Institute,	Micropropagation and varietal development	Shah Abdul Latif University, Khairpur Mirs, Sindh, Pakistan www.salu.edu.pk
Date Palm Research and Development Unit	Varietal development, quantity and quality enhancement through modern biotech approaches	United Arab Emirates University, P.O. Box 15551, Al Ain, Abu Dhabi, United Arab Emirates https://www.uaeu.ac.ae/en/dvcrgs/research/centers/dpdrud/
National Center for Palm and Dates	Develop dates sector by concentration on production efficiency (cost reduction), product quality, effective marketing programs	7345 Prince Turky bin Abdulaziz Alawal—Hittin—13512-2141. Saudi Arabia https://ncpd.org.sa/en/about

(continued)

Date Palm Research Unit, University of Baghdad	To develop technologies for production, protection and post harvest technologies for date palm	University of Baghdad, Baghdad, Iraq https://www.dateresearchinstitute.com/
Date palm Research Station, Mundra	To develop technologies for production, protection and post harvest technologies for date palm	Sardar krushi nagar —385506. Dist.Banaskantha. Gujarat, India http://www.sdau.edu.in/detail/728914/date-palm-research-station-mundra
Date Palm Research Center	Quality and quantity enhancement in date palm	University of Basrah, Iraq http://uobasrah.academia.edu/Departments/Date_Palm_Reaserch_Center/
Palm Desert Center	Conservation and development of elite cultivars using modern approaches	75080 Frank Sinatra Drive Palm Desert, CA 92211 University of California, Riverside, USA https://palmdesert.ucr.edu/research

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Date Palm Quantitative Trait Loci

8

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Abstract

Most agronomic traits in plants are quantitatively inherited. Quantitative trait loci (QTL) are chromosome regions associated with a particular phenotypic trait. QTL mapping is used to gain insight into the genetic architecture of complex quantitative traits in plants. Genome-wide association study (GWAS) has become a routine strategy to understand the genetic basis of quantitative traits. QTL mapping and GWAS study requires genotypic and phenotypic data that have been scored across a large number of individuals within a population. Utilization of statistical methods is a crucial step for identifying QTLs or causal genes associated with traits to explain the genetic basis of complex traits. In this chapter, we review current advances and strategies in the QTL mapping such as the creation of various populations, application of different types of DNA markers, collection of phenotypic data, and selection of appropriate statistical software to ensure the execution of association mapping. Association studies between DNA regions and traits can provide an optimistic assessment of the prospects of marker-assisted selection (MAS) employed in plant breeding programs. Candidate genes associated with complex traits can be discovered and cloned after validation of QTLs through the functional study by gene editing or the combination of QTL mapping with RNA-seq.

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8.1 Introduction

The date palm (*Phoenix dactylifera* L.) is a diploid (2n = 2x = 36), perennial and monocotyledonous plant, which is one of the earliest (~7000 years ago) domesticated tree crops in the Near or Middle East (Flowers et al. 2019). It belongs to the family of Arecaceae (palms) including important African commercial trees such as the African oil palm (*Elaeis guineensis*

J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_8

Jacq.) and coconut palm (*Cocos nucifera* L.) (Beal 1937). The *Phoenix* genus contains 14 species that are found in hot and arid regions from North Africa to the Middle East and South Asia (Barrow 1998; Mathew et al. 2014, 2015; Tengberg 2012). Date palm germplasm contains two highly differentiated gene pools. One is the eastern population, including cultivars from the Middle East and the Arabian Peninsula to northwest India and Pakistan and another gene pool is western population consisting of germplasm from North Africa and Sub-Saharan Africa (Sallon et al. 2020). Date palm accessions display high genetic diversity and a relatively low level of heterozygosity, as observed in the analyzed simple sequence repeat (SSR) loci (Chaluvadi et al. 2019). The genetic diversity in important traits such as yield, biotic and abiotic stress resistance, fruit quality and longer shelf life contribute great value to modern date palm breeding (Chaluvadi et al. 2019).

Plant breeding relies on genetic variation and uses selection to improve traits of interest. One of the important genetic variations is quantitative variation resulting from the combined action of many segregating genes and environmental factors (Asins 2002). To unravel the segregation pattern of such polygenic inheritance requires statistical methodologies. By joint analysis of genotypic variation along with phenotypic variation patterns, it is possible to detect the association between genotype and phenotype values and locate the quantitative trait loci (QTL) influencing such traits (Jamil et al. 2016). Once the regions of QTLs are identified on chromosomes, these regions can be analyzed for identification of key genes controlling traits using bioinformatics tools. The identified putative genes need to be validated, and then they can be used as tools in molecular breeding.

The main objective of this chapter is to describe QTL mapping in various mapping populations, including family-based and natural population-based mapping, and the utilization of statistical methods for QTL analysis and genome-wide association study (GWAS) to detect the DNA regions influencing quantitative traits. A genotypic profile obtained by screening a population with polymorphic molecular markers and the phenotypic profile through measurement with modern approaches are prerequisites for a precise QTL analysis; the process is discussed in this chapter. Sophisticated statistical tools are essential to handle extensive genotypic and phenotypic data. Some popular software for QTL analyses in various populations are listed in this chapter.

8.2 Quantitative Trait Loci

Date palm is an important fruit crop due not only to its food value, but also as a valuable source of vitamins, mineral fibers and carbohydrates (El Hadrami and Al-Khayri 2012). Recently, several reports addressed nutritional and medicinal properties of date palm, such as antioxidant activity (Al-Harrasi et al. 2014); reduction of Alzheimer's disease (Subash et al. 2015); cardio protective agent (Alhaider et al. 2017) and therapeutic value (Al-Alawi et al. 2017). To better understand and utilize these value-added traits, along with agricultural traits of date palm, researchers are focusing on the determination of their genetic basis.

Many important agricultural traits such as fruit quality and yield, resistance to pathogens and tolerance to abiotic stresses are controlled by polygenes of small effect, or by a few genes of large effect. The cumulative action of these genes and their interaction with the environment results in continuous distribution of phenotypes that are measurable and called a *quantitative trait*. The genetic variation of a quantitative trait is associated with the collective effects of genes, which are located on the same or different chromosomal regions. These regions of the chromosomes that underlie continuous traits are known as quantitative trait loci (QTLs). The genetic architecture of such a trait may be composed of the number of QTLs and their interactions among loci and environments. Because it is impossible to identify QTLs based only on conventional phenotypic evaluation, QTL mapping can be applied to identify genomic regions containing causal genes associated with complex traits.

8.3 Quantitative Trait Loci Mapping

QTL mapping requires a diverse population and QTL analysis to bridge the gap between genes and phenotypic traits. The diverse population can be a segregating population derived from a family within which genetic recombination occurs in progenies. In addition to segregated populations, the natural population is another useful tool for QTL mapping that depends upon historic recombination accumulated over many generations (Xu et al. 2017). Various molecular markers are used to detect genetic variation formed in both populations and generate genotypic data. QTL analysis uses statistical methods to identify an association between the genotypic data and measurable phenotypic data in an attempt to explain the genetic basis of complex traits. Accordingly, once an association is detected, such genetic data can provide useful tools for marker-assisted selection (MAS) of the traits of interest in a tree-breeding program, such as the date palm.

8.3.1 Family-Based Mapping

A family-based population, such as a biparental population, is often used for QTL mapping to identify QTLs associated with phenotypes. The selection of parental varieties that differ for traits of interest is the first step to create a segregating population, such as F_1, F_2, backcross and recombinant inbred lines (RIL) (Fig. 8.1). Since the date palm is dioecious, the F_1 population itself can be used as a segregating population for QTL mapping. Before genotyping a segregating population, molecular markers showing polymorphism between two parents are chosen for generating genotypic data in the population. In date palm, several molecular markers including simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) have been developed during the past decade (Al-Faifi et al. 2017; Hazzouri et al. 2015; He et al. 2017; Khierallah et al. 2017; Maryam et al. 2016; Mathew et al. 2014; Mokhtar et al. 2016; Thareja et al. 2018; Zhao et al. 2013). These markers and their sources are listed in Table 8.1. Figure 8.2 shows that the genetic polymorphism is detected by three expressed sequence tag-simple sequence repeat (EST-SSR) markers among 12 date palm genotypes. The availability of date palm genome sequence provides an excellent opportunity to develop genome-wide markers (Al-Dous et al. 2011; Flowers et al. 2019; Hazzouri et al. 2015). Phenotypic data can be measured and obtained from the population. Suitable statistical methods are used to identify an association between genotypic and phenotypic data, i.e. to determine

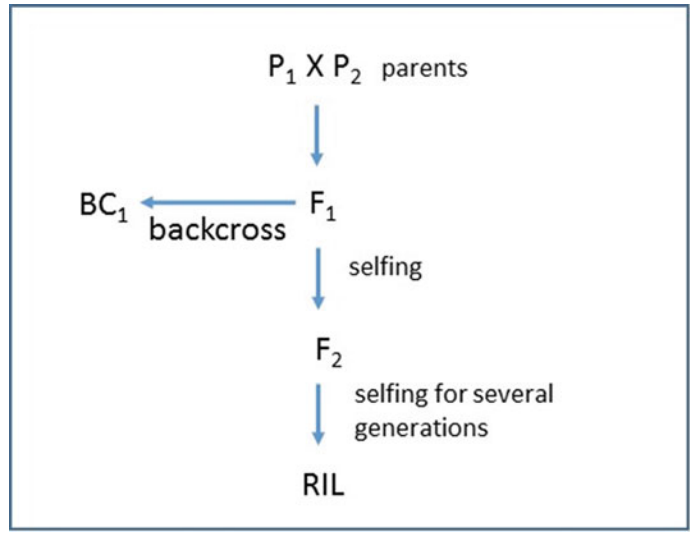

Fig. 8.1 Flow chart of biparent populations for QTL mapping. Parent P_1 is crossed with Parent P_2 to generate F_1 population. The F_1 population is either backcrossed to develop BC_1 population or selfed for several generations to develop the recombinant inbred line (RIL)

Table 8.1 Molecular markers developed in date palm

Marker type	References
EST-SSR	Zhao et al. (2013)
RAPD and ISSR	Al-Khalifah and Shanavaskhan (2017), El Sharabasy and Soliman (2017), Purayil et al. (2018)
SCAR	Al-Qurainy et al. (2018), Kharb and Mitra (2017)
AFLP, SCoT	Atia et al. (2017), Sabir et al. (2014), Saboori et al. (2020)
SSR	Al-Faifi et al. (2017), Maryam et al. (2016), Khierallah et al. (2017), Mokhtar et al. (2016)
SNP	Flowers et al. (2019), Hazzouri et al. (2015), He et al. (2017), Mathew et al. (2014), Thareja et al. (2018)

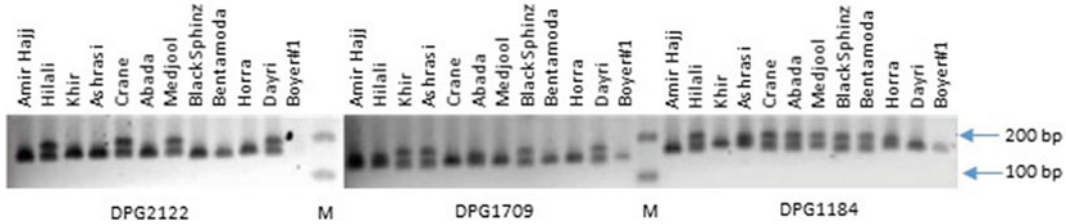

Fig. 8.2 Polymorphism detected by three EST-SSR markers among 12 date palm genotypes; gel electrophoresis was cited from Zhao et al. (2013)

the locations of QTLs on chromosomes that have effects on complex traits. The limitations of using biparental populations is a relatively narrow genetic base resulted from combining only two genomes of parents and only a few recombination events occur and the resolution is low for QTL detection due to the lack of wider allelic diversity (Bandillo et al. 2013; Jannink 2007). The power of detection of QTL in biparental populations is also affected by the phenotypic diversity of the two parents, which may represent only a small proportion of the genetic variation (Xu et al. 2017).

The multiparent population is another type of family-based population. Nested association mapping (NAM) and multiparent advanced generation intercrosses (MAGIC) are examples of multiparent populations that can be used for QTL mapping. The NAM population (Fig. 8.3) is designed to identify and dissect the genetic architecture of complex traits (Buckler et al. 2009; Yu et al. 2008). Yu et al. (2008) selected 25 diverse maize lines as the parental lines and each parental line was crossed to the B73 maize inbred, followed by selfing, to create 25 segregating F_2 populations. At the end of selfing each F_2 population to the F_6 generation, 200 RILs were selected as the NAM population. The NAM population was used to identify QTLs associated with the genetic architecture of maize flowering time (Buckler et al. 2009). Using statistical methods, they successfully identified 39 QTLs explaining 89% of the variance in days to silking and days to anthesis and 29 QTLs explaining 64% of the variance in the silking-anthesis interval. The NAM population increases resolution and power to detect QTL compared to the biparental population. The first genetic map of date palm was constructed in the NAM of 4 populations with the same female parent but different male parents and each population contained 29, 24, 17, and 15 F_1 individuals, respectively (Mathew et al. 2014). The map contained ~4000 SNP markers from the total of 64,783 SNPs generated by genotyping by sequencing (GBS) and spanned a total of 1293 cM. The date palm sex-determination region was identified at the telomere of the

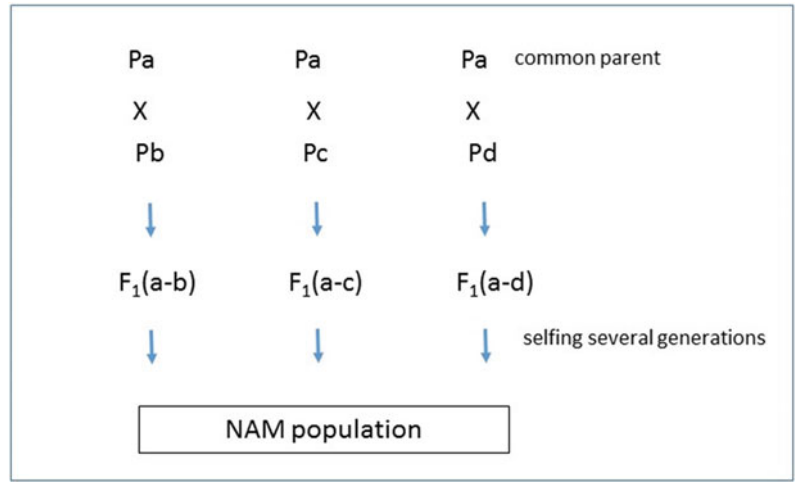

Fig. 8.3 Creation of a NAM population. Common parent Pa is crossed with parent Pb, Pc, and Pd, respectively, to generate F_1 (a–b), F_1 (a–c) and F_1 (a–d). Through several generation selfing, the NAM population is created

linkage group 12 because of the cosegregation between the region and gender.

A MAGIC population is created by intercrossing multiple varieties, as founders, over several generations (Fig. 8.4). To generate greater diversity in a MAGIC population, the founders must be chosen based on genetic and/or phenotypic diversity and geographical adaptation (Lincoln et al. 2018). Because multiple diverse founders contribute more allelic diversity, a MAGIC population is used to interrogate multiple alleles and to provide increased recombination and mapping resolution (Bandillo et al. 2013). The first MAGIC population, consisting of 527 lines, was developed using 19 intermated accessions in *Arabidopsis thaliana* (L.) Heynh. (Kover et al. 2009). A MAGIC population is a balanced, evenly differentiated mosaic of the

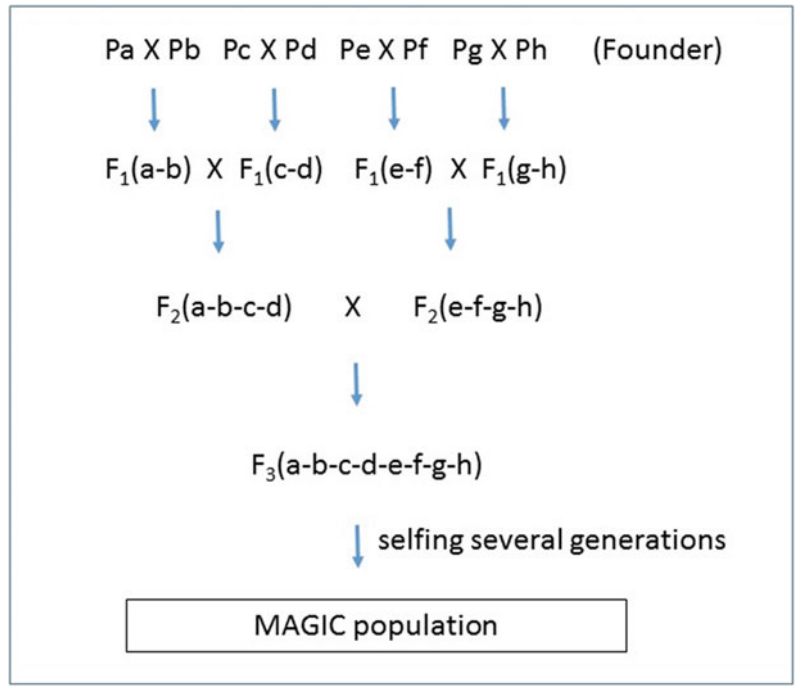

Fig. 8.4 Development of a MAGIC population. Several founders are intercrossed to generate the F_1 population F_1(a–b), F_1(c–d), F_1(e–f) and F_1(g–h). The F1 population is intercrossed to generate the F2 population (F2(a–d) and F2(e–h)) which is then crossed to create the F3(a–h), then followed by selfing several generations to create a MAGIC population

founders, with mapping power and its resolution strengthened by high minor allele frequencies and fast decay of linkage disequilibrium (Dell'Acqua et al. 2015).

8.3.2 Natural Population-Based Mapping

Natural population-based mapping has become a powerful tool to detect the natural variation underlying complex traits, because it provides high resolution, high allelic variation, and avoids the tedious development of a mapping population (Xu et al. 2017). It is especially useful for QTL mapping in tree species that are difficult and costly to develop as family-based populations due to their long juvenile periods and having to wait for the physiological maturity of the plant to assess the trait, such as in fruit characteristics (Iwata et al. 2016). With the advent of high-throughput sequencing technology, it is possible to carry out association studies on genome-wide regions of genetic variation. This approach is known as genome-wide association study (GWAS) and has become a routine strategy for revealing genotype-phenotype associations in a natural population or a collection of diverse individuals (Liu and Yan 2019). The emergence of GWAS provides an opportunity to discover genes or regions associated with the trait of interest at a relatively high resolution and unbiased in broad-based and diverse populations. Xu et al. (2017) depicted the main steps for natural population-based mapping using the GWAS approach: first, collection of diverse genotypes; second, genotyping and phenotyping traits; third, quantification of the linkage disequilibrium (LD) extent; fourth, identification of the influence of population structure and kinship and fifth, testing the associations between genotypes and phenotypes using appropriate statistical methods (Fig. 8.5).

With high genetic diversity in a natural population, it is ideal if the rapid decay of LD and minimal genetic structure exist. LD is fundamentally important for GWAS when genotyping does not capture all sequence variation in a genome (Du et al. 2018). The length of a genome segment that can be covered by a marker depends on the decay of LD in the population. With a slow LD decay, genotyping of the population requires a small number of markers to scan the genome, while with a quick LD decay, numerous markers are needed to cover the genome. In a population with a low extent of LD, once an

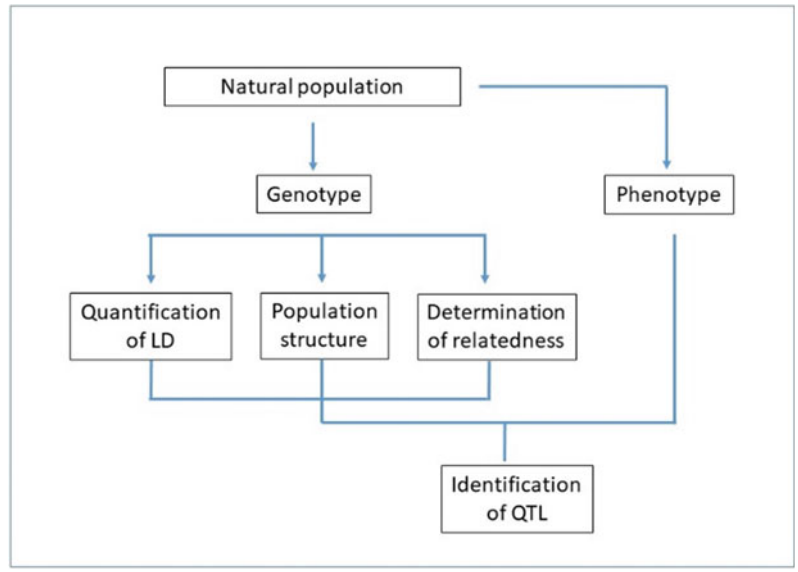

Fig. 8.5 QTL analysis in a natural population. Flow chart modified from Xu et al. (2017)

association is detected, the marker could be physically close to the causal gene (Du et al. 2018; Ingvarsson et al. 2016).

Many neutral markers are significantly correlated with trait differences among subpopulations and lead to false positives. As the number of subpopulations and the probability of an individual belonging to each subpopulation increases, the population structure needs to be estimated to avoid a spurious association. Due to genetic background, related individuals may share both causal and noncausal alleles. Therefore, relatedness between pairs of individuals presents a portion of the phenotypic variation and results in a false association. These spurious associations can be corrected using statistical approaches.

8.3.3 Genotyping

A few studies have reported QTL analysis using GWAS in tree species due to insufficient genome-wide markers to cover the genome. However, as sequencing cost continues to decline, GBS becomes more popular for genotyping (Flowers et al. 2019; Thareja et al. 2018). High-density markers throughout a genome facilitate the detection of major QTLs with GWAS. In African oil palm, large numbers of SNPs were identified from 108 F_2 progeny using the GBS approach (Pootakham et al. 2015). Among these identified SNPs, 1085 were distributed on 17 linkage groups and pinpointed 3 QTLs associated with trunk height and a 1 QTL with fruit bunch weight. Similarly, using GBS, 2413 SNPs were mapped on a linkage map of biparental population and 2 QTLs were identified in association with leaf area in oil palm (Bai et al. 2018). GWAS was performed for oil-to-dry-mesocarp content (O/DM) in a 2045 oil palm population using 200,000 SNP markers by whole-genome resequencing and identified 80 QTLs significantly associate with O/DM (Teh et al. 2016). They also found the LD of the oil palm populations decays considerably faster as compared to cereal species due to its heterogeneous character. With a diversity panel of 200 oil palm genotypes, 274 SNP markers were significantly association with fatty acid compositions by GWAS using 1,261,501 SNP markers (Xia et al. 2018). GWAS with 55,000 SNP markers in a collection of 172 apple accessions led to the finding of QTL underlying resistance to a major fungal pathogen, apple scab (McClure et al. 2018). By comparison of GWAS in a natural population and linkage mapping in the biparental population, they found that the superior resolution in natural population resulted in identifying a novel candidate gene underlying a well-studied, major QTL involved in apple firmness. The sex determination locus was identified through the GWAS approach using dense SNP markers from whole-genome resequencing of a diverse set of male and female varieties (n = 157) in date palm (Hazzouri et al. 2019) (Fig. 8.6).

8.3.4 Phenotyping

QTL analysis requires high-quality phenotypic data besides genotypic data. However, most phenotypes are affected by interactions between genotype and environment resulting in a challenge to precise phenotyping. Traditional phenotyping methods in the plant often depend on manual measurements and visual scoring, which are time-consuming and labor-intensive, and cause a variation of scoring by different persons who may have different views. In recent years, observations in support of modem technologies, such as automated recording and screening of phenotypes by various imaging techniques, provide a new platform to ensure the collection of precise phenotypes. For instance, 2D and 3D images were used for the measurement of morphological and architectural traits (Topp et al. 2013); fluorescence microscopy images of leaflet cross-sections utilized to differentiate date palm tree varieties (Arinkin et al. 2014); fluorescence imaging for traits such as photosynthetic fingerprint of disease-infected leaves in tree (Cen et al. 2017) and digital imaging for traits such as height, shoot biomass, yield traits, and plant-pathogen interaction (Fordyce et al. 2018; Yang et al. 2014). High-throughput phenotyping has

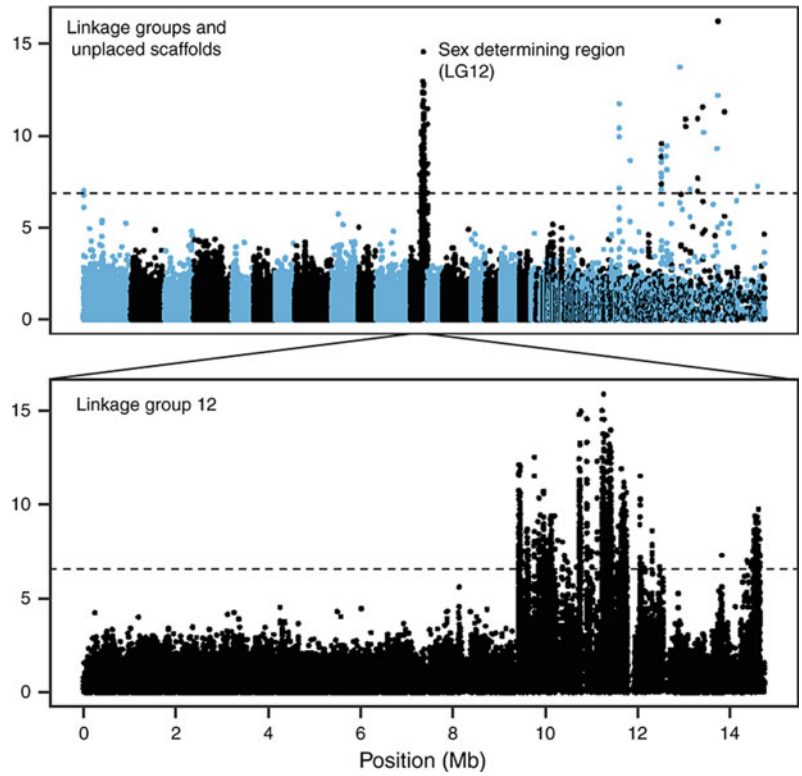

Fig. 8.6 GWAS analysis of the sex determination region of date palm. Top: Manhattan plot using the randomly downsampled SNP set (392,948 SNPs) for all linkage groups and unplaced scaffolds, below: Manhattan plot using the full SNP set on LG 12. *Source* Hazzouri et al. (2019)

gained greater attention for phenotyping in tree species.

With high-throughput phenotyping techniques, it is possible to monitor the dynamic growth of certain traits and analyze the developmental process, such as root architecture, without destroying the plant (Honsdorf et al. 2014). Multiple measurements of phenotypes during plant development using automatic platforms have introduced time as an extra dimension to the phenotypic data (Li and Sillanpaa 2015). Dynamic QTLs influenced by temporal and/or spatial environmental factors facilitate the systematic study of the interaction of genotype and environment. Besides, dynamic QTL analysis allows detection of QTLs associated with the entire developmental process of the traits, leading to an understanding of functional QTLs. This can be especially useful in tree species that have a long generation time.

The advent of next-generation and RNA-sequencings has generated numerous transcript sequences. Transcript abundance relates to the measure of the number of transcript copies derived from gene expression. Because transcript abundances for 40–70% of genes are heritable (Liang et al. 2013), these transcripts can be employed as heritable quantitative traits, i.e. expression quantitative trait loci (eQTL) (Druka et al. 2010). Transcripts are produced by expression of different sets of genes, even if they have the same DNA, and are controlled by gene regulation. Phenotype variation will occur due to different proteins being generated, if there are changes in the regulation of the gene expression. Depending upon the proximity to the gene being regulated, eQTL can be classified into two groups: *cis*-eQTL coincides with the location of the underlying gene and *trans*-eQTL represents the location of a transcription factor at a different position from the gene (Druka et al. 2010; Kliebenstein 2009; Ranjan et al. 2016). The *cis*-eQTLs have a significant effect on local expression levels, whereas *trans*-eQTLs often have global influences on gene regulation (DeCook et al. 2006). Thus, using eQTL analysis, gene

expression that influences phenotypes through changes in gene regulation can be measured (Fagny et al. 2017). Biological pathways are regulated by different groups of genes with different levels of effects. Some genes with a high level of expression may contribute large effects on pathways and others may have small effects. For instance, the plant defense system is under the regulation of a limited number of loci with strong effects, while photosynthesis and leaf development are regulated by many loci scattered throughout the genome, with weaker effects in tomato (Ranjan et al. 2016). By analysis of 25,660 eQTLs for 17,311 genes, putative *trans*-eQTLs were identified as regulators for some metabolic pathways, such as transcription factor R1 and hexokinase HEX9 for flavonoid biosynthesis and glycolysis, respectively (Wang et al. 2018). The eQTL analyses have the potential to reveal a genome-wide view of the complex genetic architecture of gene expression regulation and the underlying gene regulatory networks, and may also identify master transcriptional regulators. Such large omics data generated from genomics, transcriptomics and proteinomics coupled with bioinformatics support a better understanding of the biological system (Manzoni et al. 2018). The eQTL analysis is especially useful in the study of omics data for date palm.

8.3.5 Statistical Methods for QTL Mapping

After collecting genotypic and phenotypic data on all the individuals of a population, statistical analysis is a crucial step to sort out linked loci and their strength of linkage with the phenotypic variation. With statistical significance between genotypic data and phenotypic traits, it is feasible to identify QTLs or chromosome regions containing causal genes associated with phenotypes (Jamil et al. 2016). Appropriate statistical methods should be chosen based on the population used and the power of mapping.

8.3.5.1 Statistical Methods for Family-Based Mapping

A single-marker approach is used for initial QTL mapping in biparental populations. The identification of QTLs using this method depends on the difference between the average phenotypes of different genotype groups. The major limitation is that the likelihood of QTL detection significantly decreases as the distance between the marker and QTL increases (Mulualem and Bekeko 2016). To improve the power of mapping, single interval mapping (SIM) was proposed based on maximum-likelihood parameter estimation (Lander and Botstein 1989). SIM hypothesizes that only one QTL affects a quantitative trait. Therefore, SIM cannot consider genetic variation caused by other QTLs. Analysis of multiple QTLs simultaneously provides more accurate QTL mapping. By combining regression and interval mapping, composite interval mapping (CIM) was developed to select a subset of markers as covariates for the effects of linked QTLs (Zeng 1993). A more powerful and precise interval mapping, multiple interval mapping (MIM), addresses the simultaneous estimation of multiple QTLs and interactions between QTLs (Kao et al. 1999). Many software packages are available to map biparental populations, such as QTL Cartographer and R/qtl.

The methods developed for biparental mapping cannot be directly used for multiparent mapping because the parental origin of alleles cannot be inferred from the observed marker information (Xu et al. 2017). The first interval mapping approach for multiparent populations were developed by Xu (1996). An R package for QTL analysis in multiparent populations, based on the linear model, can analyze any type of multiparental populations like NAM populations, diallels or factorial designs (Garin et al. 2017). A random model approach for MAGIC populations was developed to detect QTLs in multiparental populations (Wei and Xu 2016). These approaches are useful in multiparent mapping to deal with the uncertainty of founder allele inheritance.

8.3.5.2 Statistical Methods for Natural Population-Based Mapping

Because population structure and relatedness usually exists in natural populations and leads to false-positive associations, several statistical models have been applied to natural population-based mapping to reduce the number of false-positive associations. A model-based clustering method to infer population structure and assign individuals to subpopulations was described by Pritchard (2000). A mixed linear model (MLM) suggested by Yu et al. (2006) first corrects for population structure and relatedness to control the false positive rate. Under the framework of the MLM model, several improved models have been developed for the power of QTL identification. The efficient mixed-model association (EMMA) was used to control population structure in GWAS (Kang et al. 2008). A compressed MLM was generated to decrease the effective sample size of large datasets (Zhang et al. 2010). The genome-wide efficient mixed model (GEMMA) was performed to improve computational speed (Zhou and Stephens 2012). A high-performance web server was provided for the analyses of large-scale GWAS (Kim et al. 2019). An R package for network-based genome-wide association studies was designed based on undirected graphical models to accomplish linkage map construction and reconstruction of linkage disequilibrium networks (Behrouzi et al. 2019). These improved methods successfully reduce the rate of false association.

8.3.5.3 Statistical Methods for Various Populations

There are numerous software packages available in the public domain. Several popular methods are listed in Table 8.2, which were selected for their use in different populations.

8.4 Validation of QTLs

False-positive associations will still occur because of statistical inferences, low-accuracy genotyping for some loci and small population size (Liu and Yan 2019). An independent validation of QTLs is needed to confirm the association detected from GWAS. Liu and Yan (2019) proposed two methodologies: validating QTLs in different populations and validating QTLs using laboratory experiments, such as gene knock-out, overexpression or genetic complementation. Several studies have demonstrated the cross-population validation to confirm the reliability of the association (Buckler et al. 2009; Li et al. 2013; Wang et al. 2015). The emerging genome-editing technologies could speed up accurately the validation of QTLs in laboratory experiments. The CRISPR/Cas9 technique is a reliable tool for gene function study, which is effective and with high throughput to identify the functional gene within each GWAS peak (Lu et al. 2017; Meng et al. 2017).

Recently, integration of QTL analysis with RNA-seq technique represents a promising approach to identify and clone genes associated with the quantitative trait. Identification of candidate causative genes associated with various biological traits was demonstrated by integrating QTL analysis and the eQTL mapping result, derived from the RNA-seq (Carrasco-Valenzuela et al. 2019; Li et al. 2018). Candidate genes can be rapidly identified within major QTLs for a complex trait to replace the fine-mapping process by the combination of conventional QTL mapping and RNA-seq using deep-sequencing technologies (Derakhshani et al. 2020; Wen et al. 2019). From a RNA-seq profile, differentially expressed genes (DEGs) detected in the QTL interval facilitate the validation of QTLs and functional study of genes (Jian et al. 2019).

8.5 Conclusions and Prospects

In this chapter, we summarized quantitative traits and identification of loci controlling traits using QTL analysis. Currently, dissection of the quantitative trait of interest has been more reliable to understand the genetic basis of a complicated trait as next-generation sequencing platforms have been improved and the cost reduced. Genotyping platforms, such as

Table 8.2 Source of statistical methods for QTL analysis in various populations

Population Type	Source	Links
Biparental population	Windows QTL Cartographer V2.5, Wang et al. (2012)	http://statgen.ncsu.edu/qtlcart/WQTLCart.htm
	R/qtl, Broman et al. (2003)	https://github.com/kbroman/qtl
NAM population	mppR: an R package for QTL analysis in multiparent populations, Garin et al. (2017)	https://github.com/vincentgarin/mppR
MAGIC Population	A random-model approach to QTL mapping in multiparent advanced generation intercross (MAGIC) populations, Wei and Xu (2016)	http://www.genetics.org/lookup/suppl/10.1534/genetics.115.179945/-/DC1/FileS3.gz
Natural population	Structure, Pritchard et al. (2000)	https://web.stanford.edu/group/pritchardlab/structure.html
	EMMA: an R package for correct for population structure and genetic relatedness, Kang et al. (2008)	http://mouse.cs.ucla.edu/emma
	GEMMA: genome-wide efficient mixed-model analysis for association studies, Zhou and Stephens (2012)	http://stephenslab.uchicago.edu/software.html.
	TASSEL, Bradbury et al. (2007)	http://www.maizegenetics.net/tassel
	GWASpro: web server for analysis of large-scale molecular genetic data, Kim et al. (2019)	https://bioinfo.noble.org/GWASPRO
	R/qtl2, Broman et al. (2019)	https://kbroman.org/qtl2
	netgwas: an R package for network based GWAS, Behrouzi et al. (2019)	https://cran.r-project.org/web/packages/netgwas

microarray, genotyping by sequencing, and RNA-seq, are promising approaches to construct highly dense maps and provide a detailed genetic variation to better analyze the QTL responsible for the trait of interest. When the reference genome of a given crop is available, it is easier to characterize genome-wide genetic variation. The phenotyping with modern phenotyping techniques, such as high throughput 2D and 3D image analyses can truly measure physical and biochemical traits that respond to genetic diversity and environmental effects in a plant (Jamil et al. 2016). It is also possible to perform multitrait analyses to reveal pleiotropy and genes influencing traits with the advanced genotyping and phenotyping platforms (Alonso-Blanco and Méndez-Vigo 2014).

When RNA-seq technology is integrated into QTL mapping, gene discovery and gene function study are promoted. Furthermore, the combination of QTL analysis and CRISPR/Cas9 technology can help in the identification and validation of functional genes. Although the reference genome sequences and numerous DNA markers, such as SNPs by GBS, are available in date palm, limited genes have been discovered and cloned. In particular, these approaches are useful and important in future date-palm research.

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CRISPR-Cas Based Precision Breeding in Date Palm: Future Applications

9

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Abstract

Both biotic and abiotic stresses impact date palm cultivation in harsh and dry desert environments around the world. There is a dire need to improve the adaptability, productivity, and robustness of date palms cultivated under stressful environments. The modern—omics tools coupled with next-generation sequencing (NGS) have progressed in date palm genomics during the current decade. However, date palm has long been an ignored crop for the application of modern approaches. Mutation breeding through physical and chemical mutagens has long been in use to bring about genetic modifications for improved yield and quality-related traits in crop plants. Nevertheless, these mutations are random in their impact and precise genetic modifications have long been a standing question for plant researchers. Modern genome editing (GE) tools have advanced from biomedical sciences to modern agriculture in this post-genomic era. Under these circumstances, CRISPR-Cas-based GE tool can pave the way towards innovations in date palm breeding. It has emerged itself as a state-of-the-art technology due to its robustness, sophistication, cost-effectiveness, versatility, precision, and efficiency. This technique is versatile in single base editing (BE), multiplexing to target gene families, gene knockout or knockin, gene transfer/replacement, epigenetic modifications, DNA-barcoding, genotyping, pathogen profiling and many other expanding applications beyond GE.

9.1 Introduction

Among the earliest cultivated fruit trees, date palm (*Phoenix dactylifera* L.) has been a primary source of sustainable biodiversity in oasis agriculture (Albishi et al. 2019). Date palm is a perennial, monocotyledonous and dioecious (2n = 2x = 36) fruit tree, which belongs to Arecaceae (Palmae) family (Mahmoudi et al. 2008). It has a rich history spanning some 5000 years of extensive cultivation in the tropical and temperate regions. There is no authenticated source of origin of date palm. Some reports considered the Arabian Peninsula, while others posit multiple origins simultaneously (Flowers et al. 2019). However, the earliest evidence of date palm cultivation was

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J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_9

found ~3700 BC in the Euphrates and Nile rivers civilizations. The dioecious mode of reproduction may increase the chances of cross-species hybridizations with other *Phoenix* species (Flowers et al. 2019). Over time, date palm has been extensively dispersed from Africa and the Arabian Peninsula into Asian countries. Until now, hundreds of cultivars have gained regional popularity depending upon the specific physiological, morphological, and nutritional profiles. Nevertheless, the cultivation of regional date palm cultivars have promoted monocultural cropping, which has caused severe genetic depression of the biodiversity of many potential cultivars (Maina et al. 2019). Advanced biotechnological techniques can assist traditional breeding techniques in date palm by using next-generation sequencing (NGS), marker-assisted selection, -omics approaches and more advanced genome editing (GE) tools (Sattar et al. 2017).

The essential food, feed and fuel consumables of humans are dependent upon continuous genetic improvements in crop plants. Historically, targeted and precise genomic modifications have been a challenging task in many animal and plant species. The rapidly growing human population coupled with multiple factors (conferring direct or indirect yield losses to crops) necessitates innovative breeding techniques to accelerate agriculture production. Among others, breeding through crossing, random mutations and transgenes play a vital role in crop improvement, but share certain drawbacks as crossbreeding takes a long-time span to introduce the desirable alleles to enhance the genetic variability (Scheben and Edwards 2018). Nevertheless, breeding and genetic variations in crop plants through irradiation and physical or chemical mutagens has fixed the larger parts of the genomes of major crops and reduced genetic variability. However, the stochastic nature of mutation breeding, generation and screening of large numbers of mutants make mutation breeding a time-consuming, laborious and inefficient technique (Pacher and Puchta 2017). The introduction of transgenes to bring desirable traits into the genome of crop plants with elite genetic background isolation may be a good choice. However, the strict regulatory framework and general public concerns about genetically-modified organisms (GMOs) limit the commercialization of GM crops.

Various tools have been introduced for targeted genetic changes in crop plants. Such genetic changes require the generation of double-stranded breaks (DSBs) using sequence-specific nucleases (SSNs) at a specific locus in an organism's genome. These DSBs are predominantly repaired through the non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways in the higher eukaryotes. Earlier, zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (ZFNs) were used as successful SSNs (Bibikova et al. 2002; Christian et al. 2013). However, these precise genome editors require protein-based DNA-protein interactions for their specificity and thus, involve difficult genome engineering and multiplexing. Genome editing (GE) was revolutionized in 2012 when the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated (Cas) based approach was first employed for programmable in vitro GE (Jinek et al. 2012). The CRISPR-Cas system is a bacterial and archaea immune system against invading bacteriophages and consists of short sequences repeats, which are separated by spacer sequences. The diversity of various CRISPR systems is actually based upon the specific Cas proteins, which are encoded by small gene clusters around the CRISPR arrays. Precise GE has the ability to produce specific alterations in an organism's genome, which may include specific base changes, DSBs or single-stranded breaks (SSBs) or nicks. These specific base changes activate an endogenous repair mechanism, which causes an alteration in the targeted genome (Broeders et al. 2020).

Since then, CRISPR-Cas based GE approach has been extensively studied and used for targeted modifications in microbial, animal, and plant genomes. In plant genomes, precise GE using the CRISPR-Cas-based approach was demonstrated in 2013 for crop improvement (Nekrasov et al. 2013). To date, several CRISPR-Cas-based genetic tools have been developed for targeted

mutations, BE, HDR and transcription-oriented regulations (Chen et al. 2019). In total, CRISPR-Cas based GE has been employed in 11 crop species (Table 9.1). In this chapter, we described the leading-edge developments in CRISPR-Cas-based technology, various CRISPR-Cas variants, multiplexing expression system, diverse frontiers of GE and futuristic achievements of GE in date palm. Moreover, we also highlight specific considerations in date palm GE such as traits related to yield, quality, plant height, fruit shape, fruit color, transgene-free GE, biotic and abiotic stresses. This chapter presents a comprehensive guide towards the current developments and future directions to use CRISPR-Cas based technology to accelerate date palm breeding.

Table 9.1 List of targeted traits and related genes edited through CRISPR-Cas based approaches in various crops

Common name	Scientific name	Genes targeted	Phenotypic traits	References
Banana	*Musa* spp. L.	*eBSV*	Resistance against banana streak virus (BSV)	Tripathi et al. (2019)
		RAS-PDS1; *RAS-PDS2*	Carotenoid biosynthesis	Kaur et al. (2018)
		PDS	Carotenoid biosynthesis	Naim et al. (2018)
Kiwifruit	*Actinidia lindl* Lindl.	*PDS*	Carotenoid biosynthesis	Wang et al. (2018)
Grape	*Vitis vinifera* L.	*VvPDS*	Carotenoid biosynthesis	Ren et al. (2019)
		IdnDH	Biosynthesis of tartaric acid	Osakabe et al. (2018)
		VvWRKY52	Resistance to *Botrytis cinerea*	Wang et al. (2018)
		VvPDS	Carotenoid biosynthesis	Nakajima et al. (2017)
		MLO-7	Resistance to powdery mildew	Malnoy et al. (2016)
		IdnDH	Biosynthesis of tartaric acid	Ren et al. (2016)
Orange	*Citrus sinensis* (L.) Osbeck	*DMR6*	Resistance to huanglongbin disease	Zhang et al. (2018)
		PDS	Carotenoid biosynthesis	Wang et al. (2018)
		PDS	Carotenoid biosynthesis	Zhang et al. (2017)
		CsLOB1	Resistance to citrus canker disease	Peng et al. (2017)
		CsPDS	Carotenoid biosynthesis	Jia and Wang (2014)
Grapefruit	*Citrus paradise* Macfadyn	*CsPDS*, *Cs2g12470*, *Cs7g03360*	Carotenoid biosynthesis, leaf development	Jia et al. (2017b)
		CsLOB1	Resistance to citrus canker disease	Jia et al. (2017a)
Watermelon	*Citrullus lanatus* Thunberg	*ALS*	Resistance to herbicides	Tian et al. (2018)
		PDS	Carotenoid biosynthesis	Tian et al. (2017)
Apple	*Malus pumila* Dieck	*PDS*	Carotenoid biosynthesis	Charrier et al. (2019), Nishitani et al. (2016), Osakabe et al. (2018)

(continued)

Table 9.1 (continued)

Common name	Scientific name	Genes targeted	Phenotypic traits	References
		DIPM-1, *DIPM-2*, *DIPM-3*	Resistance to fire blight disease	Malnoy et al. (2016)
		udiA	Reporter gene	Peer et al. (2015)
Cassava	*Manihot esculenta* Crantz	*elF4E*	Resistance to cassava streak disease	Gomez et al. (2019)
Poplar tree	*Populus tomentosa* Carrière	*PDS*	Carotenoid biosynthesis	Fan et al. (2015)
Pear	*Pyrus communis* L.	*TFL1.1*	Early flowering	Charrier et al. (2019)
Cocoa	*Theobroma cacoa* L.	*NPR3*	Defense response to salicylic acid	Fister et al. (2018)

Abbreviations used are *Citrus sinensis* (cs), DspE-interacting proteins from Malus (DIPM), elongation factor 4 (elF4), idonate 5-dehydrogenase (ldnDH), lateral organ boundaries (LoB), Mildew resistance Locus O (MLO), non-expressor of PR gene 3 (NPR3), phytoene desaturase (PDS) and terminal flower 1 (TFL1)

9.2 Current Status of Genetic Improvement in Date Palm

Date palm (*Phoenix dactylifera*) is cultivated as an important fruit crop in arid to tropical areas spanning Africa, Asia, the EU, Middle-East and the United States. About 90% of the world's collective date palm production is produced in Gulf Cooperative Council (GCC) countries. Date palm micropropagation is an alternative to conventional somatic embryogenesis or organogenesis methods to achieve true-to-type and elite date palm plants (Fig. 9.1). During micropropagation, somaclonal variations arise, which can serve as a valuable tool in date palm breeding technologies to develop novel traits. The genetic transformation of date palm has been achieved through particle bombardment or *Agrobacterium*-based infiltration methods to achieve germplasm improvement and sustainable development (Aslam et al. 2015; Mousavi et al. 2014). However, the applications of modern biotechnological tools require efficient and well-established tissue culturing protocols in date palm. Improvements in various available tissue culture steps such as explant selection, induction, multiplication, and elongation of shoot and roots are very important. However, these techniques can only be used to support and accelerate the application of modern genetic engineering tools. Recently, CRISPR-Cas has been reviewed as a potential technique for synthetic biology and domestication of food crops (Zhang et al. 2020).

Over the course of a long life span, date palms under cultivation must cope with extreme salinity, high drought regimes and heat stress as primary abiotic stresses. Depending upon the cultivar, the date palm has the ability to adapt to a gradual increase in salt and drought conditions (Al-Khateeb et al. 2019a, b; Al-Khateeb et al. 2020). However, the underlying molecular mechanisms/pathways to cope with abiotic stresses in date palm are not fully understood. A proteomic study explored 47 differentially-expressed proteins during salinity and drought stress in date palm (El Rabey et al. 2015). In another study, the genes related to cadmium stress was investigated through transcriptomic analysis of date palm cultivar Deglet Noor (Rekik et al. 2019). In addition to abiotic stresses, biotic stresses are also a limiting factor in low

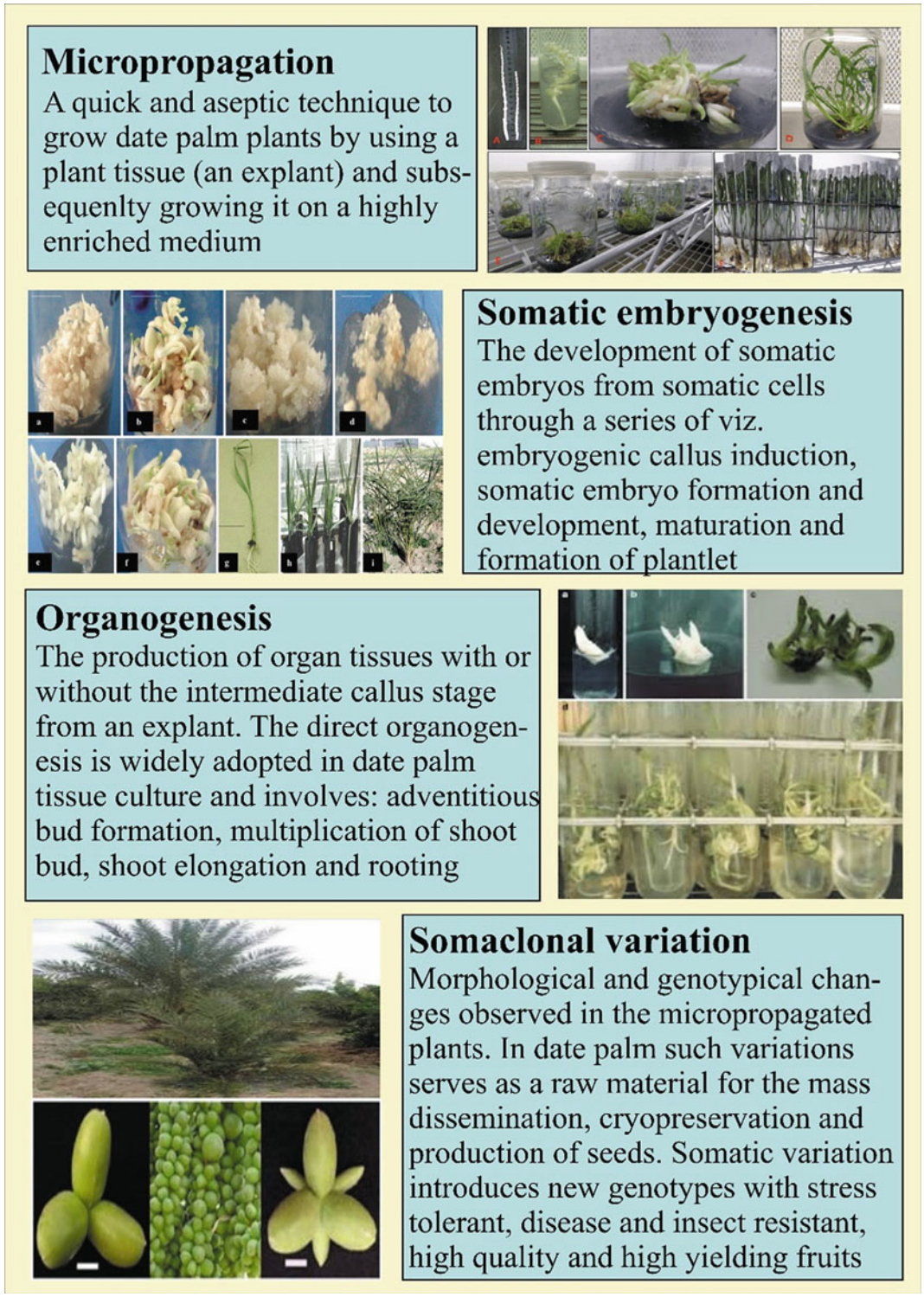

Fig. 9.1 Common tissue culturing techniques used in date palm micropropagation (Figure produced by Z. Iqbal in Adobe Illustrator 2019)

date palm production. These may include bayoud disease in North Africa and the red palm weevil in the Asia, Middle-East, and Mediterranean countries. Moreover, date palm roots have also been found infected with diverse fungal and *Oomyces* spp. in many countries (Alam et al. 2019). These limiting factors are better addressed with the use of modern genetic, genomics and GE approaches to develop multifaceted, broad-term, and sustainable genetic resistance in elite date palm cultivars (Yarra et al. 2020).

Despite the fact that date palm has a rich domestication and agronomic history, the application of high-throughput techniques only began in the last decade (Sattar et al. 2017). Overall, the members of the family Arecaceae have always been overlooked for genetic improvement through modern biotechnological approaches; Mathew et al. (2015) developed the first genetic map of Khalas cultivar after the chloroplast (Sabir et al. 2014), mitochondrial (Fang et al. 2012) and whole nuclear genome of date palm was sequenced completely (Al-Mssallem et al. 2013). In parallel, Hazzouri et al. (2015) cataloged the molecular variations in the date palm genome (Hazzouri et al. 2015). Other related studies also received a boost after the whole date palm genome was sequenced. These included transcriptomic analysis (Yin et al. 2012), miRNAs profiling (Xin et al. 2015), application of genetic modeling, comparative genomics and application of single-nucleotide polymorphism (SNPs) to assess genetic diversity in date palm (Mathew et al. 2015). In the following years, the application of—omics approaches coupled with bioinformatics tools in date palm were carried out to establish a global database for the development of molecular markers (Mokhtar et al. 2016). Recently, Rekik et al. (2015) characterized and developed in silico molecular structuring of the *DnMRE11* gene in the date palm cultivar Deglet Noor. The characterization of *DnMRE11* is a significant development for the application of modern CRISPR-Cas based tools in date palm because this gene has been known to induce DSBs repair in date palm. Other in silico studies unravel the role of miRNAs to induce various genes involved in salt tolerance/adaptation, fruit development and evolution (Xiao et al. 2013; Xin et al. 2015; Yaish et al. 2015). However, recent genetic annotations and in silico analysis could not provide an authenticated validation for the involvement of those genes in various agronomical, physiological, and developmental processes in date palm. Thus, experimental validations and empirical confirmations are necessary to establish the expression and biological profiling of the annotated genes (Sattar et al. 2017). Moreover, most of the genetic information is available for just in a few date palm cultivars. Various NGS and SNPs approaches can be a good solution to assess the inter- and intra-varietal genetic diversity in various date palm cultivars; however, exploring the intrinsic molecular mechanisms and functional genomics studies are vital in date palm. Modern GE tools such as CRISPR-Cas can be the best solution to overcome the genetic barriers in cultivar development and genomic improvement in genetically isolated cultivars in date palm. Besides, the biotic stresses including insects, pests and other pathogens (fungi, phytoplasma, bacteria) can also be targeted by CRISPR-Cas-based approaches by directly targeting their invasions through a *gene drive* approach (Fig. 9.2).

9.3 The Diverse CRISPR-Cas Toolbox to Engineer Date Palm Genome

CRISPR-mediated GE has sparked researchers' interest to explore various versions of the CRISPR-Cas system with improved and effective GE efficiency. New CRISPR systems have been developed to address the shortcomings of earlier versions. Presently, the CRISPR-Cas toolbox is well-equipped with diverse arrays of Cas proteins and it is still expanding for targeted mutagenesis in woody plants, especially invaluable commodities like date palm. Depending on the effector module, CRISPR-Cas systems are classified into two main distinct classes: 1 and 2. If a single multifunctional protein accomplishes both the processing and the interference, then it is categorized as class 2, otherwise, it is categorized as class 1 (Makarova et al. 2015). Primarily, Cas proteins are responsible for the classification of

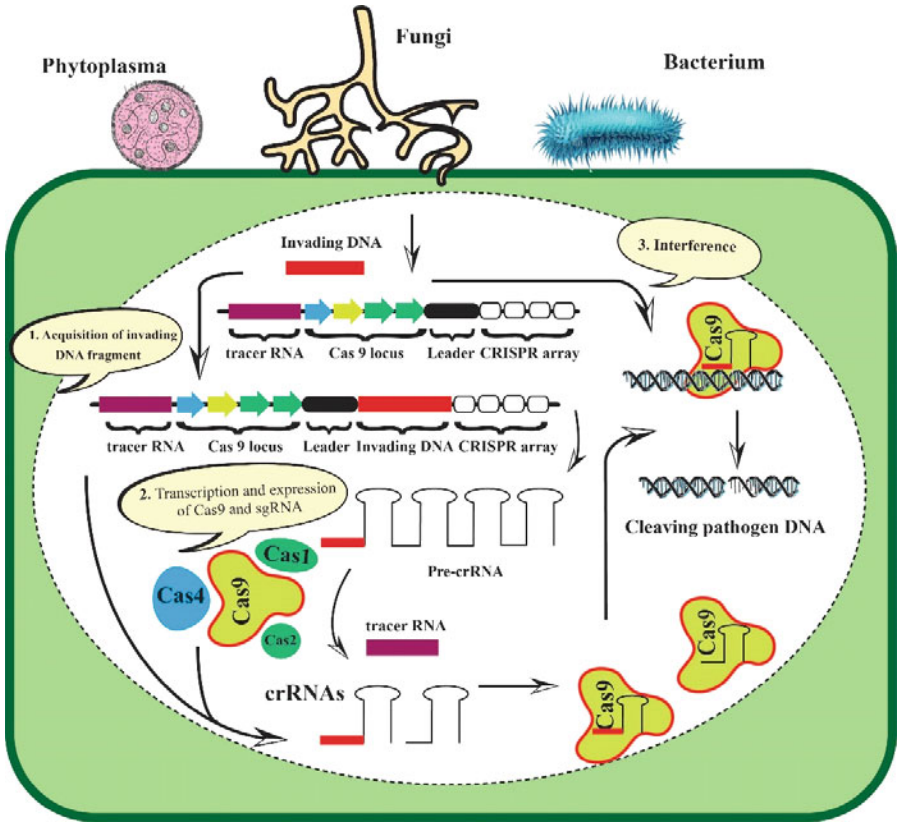

Fig. 9.2 A three-step CRISPR-Cas based resistance model against biotic stresses in date palm. **1** acquisition of the invading genetic material, which is integrated and then duplicated at the leader site of CRISPR locus, **2** transcription followed by the expression of different components of CRISPR system are matured using various Cas proteins, **3** interference involves the recognition and disruption of any foreign genetic material under the guidance of crRNA and Cas protein (Figure produced by M.N. Sattar in Adobe Illustrator 2019)

CRISPR systems and are based on the signature Cas protein: Cas 3, Cas 10, and CsfI belong to class 1 with type I, III, and IV, respectively. Whereas, Cas9, Cas12a–e, Cas12g, Cas13a–d, and Cas14a–c belong to class 2 (Burstein et al. 2017; Moon et al. 2019; Shmakov et al. 2017). In the following section, different versions of CRISPR-Cas with the potential to engineer date palm genome are discussed.

9.3.1 CRISPR-Cas9 System

The most extensively exploited Cas9 belongs to class 2, type II CRISPR system. Cas9 is guided by single-guide (sg) RNA to the target genome where Cas9 recognizes a very simple PAM sequence (5'NGG) to induce the DSBs, the recognition of simple PAM sequence makes the CRISPR-Cas9 system a widely chosen GE system. In addition, PAM-interacting (PI) domain of Cas9 have been engineered to increase the PAM recognition, three different variants of *Streptococcus pyorgenes* (sp) Cas9, VQR, EQR, VRER, and QQR1 have been developed which can robustly recognize NGA, NGAG, NGCG and NAAG PAMs, respectively (Table 9.2), Kleinstiver et al. (2015b). Furthermore, xCas9 has been developed which can identify NG, CAA and GAT PAM sequences (Hu et al. 2018). Alternatively, different orthologues of Cas9 from bacteria or archaea can recognize different PAM

sequences, reviewed by Zhang et al. (2019). Similarly, many other Cas9 variants have been engineered to expand the CRISPR-Cas9 applications. Codon optimized Cas9 has been employed widely for GE in various species, including plants, insects, humans and microbes (Ma et al. 2015; Sattar et al. 2019; Zhang et al. 2019). Several versions of Cas9 have also been generated through protein engineering to achieve desired traits/functions. A Cas9 nickase (nCas9) was developed to cleave only the single strand of the target DNA (Mikami et al. 2016). Similarly, dead Cas9 (dCas9) was produced by a double-point mutation that lacks endonuclease activity, was equipped with a transcriptional activator to achieve hyperexpression of the target genes (Piatek et al. 2015). Several other Cas9 orthologues have been developed to engineer the genomes of a variety of tree species (Table 9.2), which can also be executed in date palm genome engineering. Such Cas9 systems include *Streptococcus thermophilus* Cas9 (St1Cas9), *Brevibacillus laterosporus* Cas9 (BlatCas9) and *Staphylococcus aureus* (SaCas9) to engineer *Arabidopsis*, maize and citrus genomes, respectively. Moreover, SpCas9 has been employed in many plant species including rice, barley, tomato wheat, poplar, cocoa, and banana (Bewg et al. 2018; Jia et al. 2019b; Kleinstiver et al. 2016a; Nishimasu et al. 2018). The wide variety of Cas9 variants can help in avoiding most of the pitfalls in date palm GE.

9.3.2 CRISPR-Cas12a

Cas12a, formerly recognized as cpf1, belongs to the class 2 type V CRISPR system. It is a unique addition to the CRISPR toolbox, which can target PAM sequences with enriched T sequences (TTN) and requires a short crRNA of ~42 nucleotides (nt). The other valuable features of Cas12 are easy synthesis, engineering, multiplexing and it is considered as an alternative to Cas9 for GE (Zetsche et al. 2015). Besides endonuclease activity, it can also mediate cleavage of RNA, so potentially process a CRISPR array for multiplexed GE. Cas12a regulates processing, crRNA maturation, cleavage of the target DNA and cleaves the target genome distal to the PAM sequence, generates a DSB and is considered more specific than wild type Cas9 and SpCas9 (Kim et al. 2016; Kleinstiver et al. 2016b). All such outstanding utilities of Cas12a have made it an efficacious GE tool in date palm genome engineering. Leenay et al. (2016) showed that dCas12a yielded stronger repression of the target gene when GTTC and TTTN PAM sequences were used, in comparison to non-targeting RNA. Three different types of Cas12a, *Francisella novicida* U112 (FnCpf1), Lachnospiraceae bacterium ND2006 (LbCpf1) and *Acidaminococcus* spp. BV3L6 (AsCpf1) have been experimented with in *Arabidopsis*, cotton (*Gossypium hirsutum* L.), citrus, rice, tobacco (*Nicotiana benthamiana* and *N. tabacum* L.), tomato (*Solanum lycopersicum* L.) and soybean (Bernabé-Orts et al. 2019; Jia et al. 2019a; Kim et al. 2017; Li et al. 2019).

Different versions of dead Cas12a (dCas12a) such as dAsCas12a, DNase dead Cas12a (DDCas12a) to expand the utilities of Cas12a system have been engineered. The PI domain of two Cas12a, LbCas12a and FnCas12a, have been modified to yield RR and RVR variants to engineer plant (rice) genomes (Table 9.2). Recently, modified AsCas12a yielded improved GE activity at low temperature against a broad range of PAM sequences (Kleinstiver et al. 2019). Besides the known Cas12a variants, researchers are unearthing the new versions. A few more Cas12a variants from *M. bovoculi* Cas12a (Mb3Cas12a), *Thiomicrospira* sp. Cas12a (TsCas12a), *Moraxella bovoculi* 237 Cas12a (MbCas12a), *Helcococcus kunzii* Cas12a (HkCas12a), *Pseudobutyrivibrio xylanivorans* Cas12a (PxCas12a) and *Lachnospira pectinoschiza* Cas12a (LpCas12a) have been identified successfully so far. A few of those with the highest potential could potentially be employed to achieve date palm GE.

Table 9.2 A summary of Cas proteins variants, orthologues and their classification, PAM site, target nucleic acid, and types of ends generated along with the type of nucleic acid cleavage

Cas type	Organism	Size (amino acids)	Class/type	PAM site	Altered PAM	Types of end	Mutations	Plants	References
SpCas9	*Streptococcus pyogenes*	1368-1424	2/II	NGG	–	Blunt/ds or ss	–	Many plant species	Barrangou et al. (2007), Cong et al. (2013), Jinek et al. (2012), Makarova et al. (2015)
SpCas9 VQR	*S. pyogenes*	1372	2/II	NGA	Yes	Blunt/ds or ss	D1135V/R1335Q/T1337R	Rice	Kleinstiver et al. (2015b)
SpCas9 EQR	*S. pyogenes*	1372	2/II	NGAG	Yes	Blunt/ds or ss	D1135E/R1335Q/T1337R	–	Kleinstiver et al. (2015b)
SpCas9 VRER	*S. pyogenes*	1372	2/II	NGCG	Yes	Blunt/ds or ss	D1135V/G1218R/R1335E/T1337R	Rice	Kleinstiver et al. (2015b)
SpCas9 D1135E	*S. pyogenes*	1372	2/II	NAG/NGA	Yes	Blunt/ds or ss	D1135E	–	Cong et al. (2013)
SpCas9 QQR1	*S. pyogenes*	1372	2/II	NAAG	Yes	Blunt/ds or ss	G1218R/N1286Q/I1331F/D1332K/ R1333Q/R1335Q/T1337R	–	Anders et al. (2016)
SpCas9-NG	*S. pyogenes*	1372	2/II	NG	Yes	Blunt/ds or ss	R1335V/L1111R/D1135V/G1218R/ E1219F/A1322R/T1337R	*Arabidopsis* & Rice	Nishimasu et al. (2018)
SpCas9-HF1	*S. pyogenes*	1368	2-II	NGG	Enhanced specificity	Blunt/ds or ss	N497A/R661A/Q695A/Q926A	*Arabidopsis* & rice	Kleinstiver et al. (2016a)
eHF1-Cas9	*S. pyogenes*	1368	2-II	NGG	Enhanced specificity	Blunt/ds or ss	N497A/R661A/Q695A/K848A/ Q926A/K1003A/R1060A	Rice	Liang et al. (2018)
HiFi Cas9	*S. pyogenes*	1368	2-II	NGG			R691A	Rice	

(continued)

Table 9.2 (continued)

Cas type	Organism	Size (amino acids)	Class/type	PAM site	Altered PAM	Types of end	Mutations	Plants	References
					Enhanced specificity	Blunt/ds or ss			Vakulskas et al. (2018)
XCas9	*S. pyogenes*	1368	2-II	NG, GAA & GAT	Yes & Enhanced specificity	Blunt/ds or ss	A262T/R324L/S409I/E480K/ E543D/M694I/E1219V	Rice	Hu et al. (2018)
dCas9	*S. pyogenes*	1368	2-II	NGG	No	Blunt/ds or ss	D10A/H840A	*Arabidopsis* & *Oryza sativa*	Li et al. (2017), Piatek et al. (2015)
nCas9	*S. pyogenes*	1368	2-II	NG, GAA & GAT	Yes & Enhanced specificity	Blunt/ds or ss	D10A	Rice, tobacco	Hsu et al. (2019), Mikami et al. (2016)
SaCas9	*S. aureus*	1053	2-II	NNGRRT	–	Blunt/ds or ss	–	Nb, rice, *Arabidopsis* & citrus	Endo et al. (2019)
SaCas9-KKH	*S. aureus*	1053	2-II	NNNRRT	Enhanced specificity	Blunt/ds or ss	E782K/N968K/R1015H	Nb, rice, *Arabidopsis* & citrus	Kleinstiver et al. (2015a)
BlatCas9	*Brevibacillus laterosporus*	1092	2-II	NNNNCND		Staggered/ds	–	Maize	Karvelis et al. (2015)
FnCas9	*Francisella novicida*	1629	2B-II	NGG	–	Staggered/ds	–	*Arabidopsis*	Hirano et al. (2016)
Cpf1 (Cas12a)	*Prevotella* & *Franscisella*	1300	2-V	TTTN	–	Staggered/ds	–	Many plant species	Begemann et al. (2017), Endo and Toki (2018)
AsCas12a RR	*Acidaminococcus*	1307	2-V	TYCV & CCCC	Yes	Staggered/ds	S542R/K607R	–	Gao et al. (2017)

(continued)

Table 9.2 (continued)

Cas type	Organism	Size (amino acids)	Class/type	PAM site	Altered PAM	Types of end	Mutations	Plants	References
AsCas12a RVR	*Acidaminococcus*	1307	2-V	TATV	Yes	Staggered/ds	S542R/K548V/N552R	–	Gao et al. (2017), Zetsche et al. (2015)
LbCas12a	Lachnospiraceae bacterium	1228	2-V	TTTV	–	Staggered/ds	–	Many plant species	Zetsche et al. (2015)
LbCas12a RR	Lachnospiraceae bacterium	1228	2-V	TYCV & CCCC	Yes	Staggered/ds	G532R/K595R	Rice	Gao et al. (2017)
LbCas12a RVR	Lachnospiraceae bacterium	1228	2-V	TATV	Yes	Staggered/ds	G532R/K538V/Y542R	Rice	Gao et al. (2017)
FnCas12a	*Francisella novicida*	1300	2-V	TTV, TTTV & KYTV	–	Staggered/ds	–	Rice	Zetsche et al. (2015)
FnCas12a RR	*F. novicida*	1300	2-V	TYCV & TCTV	Yes	Staggered/ds	N607R/K671R	Rice	Tóth et al. (2018)
FnCas12a RVR	*F. novicida*	1300	2-V	TWTV	Yes	Staggered/ds	N607R/K613V/N617R	Rice	Tóth et al. (2018)
Cas12b	*Alicyclobacillus acidoterrestris*, *Bacillus thermoamylovorans*, *A. acidiphilus*	1100-1300	2-VB	TTTN	–	Staggered/ds	–	Many plant species	Shmakov et al. (2015), Yang et al. (2016)
Cas12X	Deltaproteobacteria	<1000	2-V	TTCN	–	Staggered/ds	–	–	Liu et al. (2019a), Yang and Patel (2019)

Abbreviations used are double-stranded (ds), single-stranded (ss), CRISPR-associated protein (Cas) and high-fidelity (HF)

9.3.3 CRISPR-Cas12b

Cas12b (formerly referred to as C2c1) belongs to the class 2 type V-B CRISPR system and is a dual-RNA-guide DNA endonuclease and generates staggered ends distal to PAM sequences. It resembles its siblings, Cas9 and Cas12a, in the RuvC domain but differs in the Nuc domain, which shares no sequence homology to the Nuc domain of Cas12a and the NHN domain of Cas9 (Strecker et al. 2019). The Nuc domain determines the target strand cleavage; any mutation in the Nuc domain of FnCas12b may lead to incomplete inactivation. Another remarkable difference is that Cas12b is smaller in size than Cas9 and Cas12a. It recognizes 5'-T-rich PAM sequence and generates 7 nts long DSB overhang at 5' end (Strecker et al. 2019). Initially characterized Cas12b variants from *Alicyclobacillus acidoterrestris* (AaCas12b) and *Bacillus thermoamylovorans* (BthCas12b), showed optimum activity at a high temperature (48–50 °C), which was not suitable for plant GE. Later, a few other orthologues were searched from *Alicyclobacillus acidiphilus* Cas12b (AaCas12b), which showed activity at a much broader temperature range. In date palm GE, such Cas12b variants with a wider temperature range can be applied to engineer the desired traits.

9.3.4 CRISPR-CasX System

CasX (also referred as Cas12e), a smaller Cas protein (ca 980 amino acids), was initially characterized through metagenomic analysis of uncultivated microbes. CasX is a dual-RNA-guided DNase that digests double-stranded DNA after recognizing a 5'TTCN PAM sequence (Table 9.2). It requires a 20 nts crRNA and a tracrRNA to cleave the target sequence with an overhang of approximately 10 nts (Liu et al. 2019a). It also exhibits nonspecific and PAM-independent cleavage of single-stranded (ss) DNA by binding to a crRNA-guide-complementary ssDNA (Burstein et al. 2017). No significant sequence similarity was observed between Cas12e and its sibling Cas12 proteins, except for a RuvC domain present at the C-terminus. Two variants of CasX, from *Deltaproteobacteria* (DpbCasX) and *Planctomycetes* (PlmCasX), have the potential to engineer the genomes of human and *Escherichia coli* cells (Liu et al. 2019a). The RuvC domain of CasX can be mutated to engineer the deactivated version of CasX. For date palm genome engineering, the smaller size of CasX renders it a genuinely feasible candidate to be used for any futuristic assays.

9.4 Workflow Model of CRISPR-Cas for Date Palm Genome Editing

The editing efficiency of the CRISPR-Cas based system principally depends on the DNA target, percentage of GC contents, type of Cas protein, sgRNA architecture, selection of promoter for Cas and sgRNA expression and others. Therefore, it is critical that the CRISPR-Cas system should be properly optimized and all the factors comprehensively considered before starting the experiments. A generalized workflow for the application of CRISPR-Cas in date palm is outlined in the following sections and in Fig. 9.3.

9.4.1 Data Mining, Designing and In Silico Analysis of SgRNAs

The selection of a suitable target region in the plant genome is the most important step for efficient GE in plants. In date palm, the target selection may be a challenging task due to genomic polymorphism, off-targets, alternative splicing of introns and high occurrence of SNPs. Besides, the design of sgRNA constructs is a vital step for precise GE through a CRISPR-Cas system. The selection and design of sgRNAs can be efficiently done through various available online web-based tools. These tools are easily accessible and permit the design and evaluation of the selected sgRNAs for plant GE and also help to identify the new targeted sites in the plant genome (Stemmer et al. 2015). Moreover, such

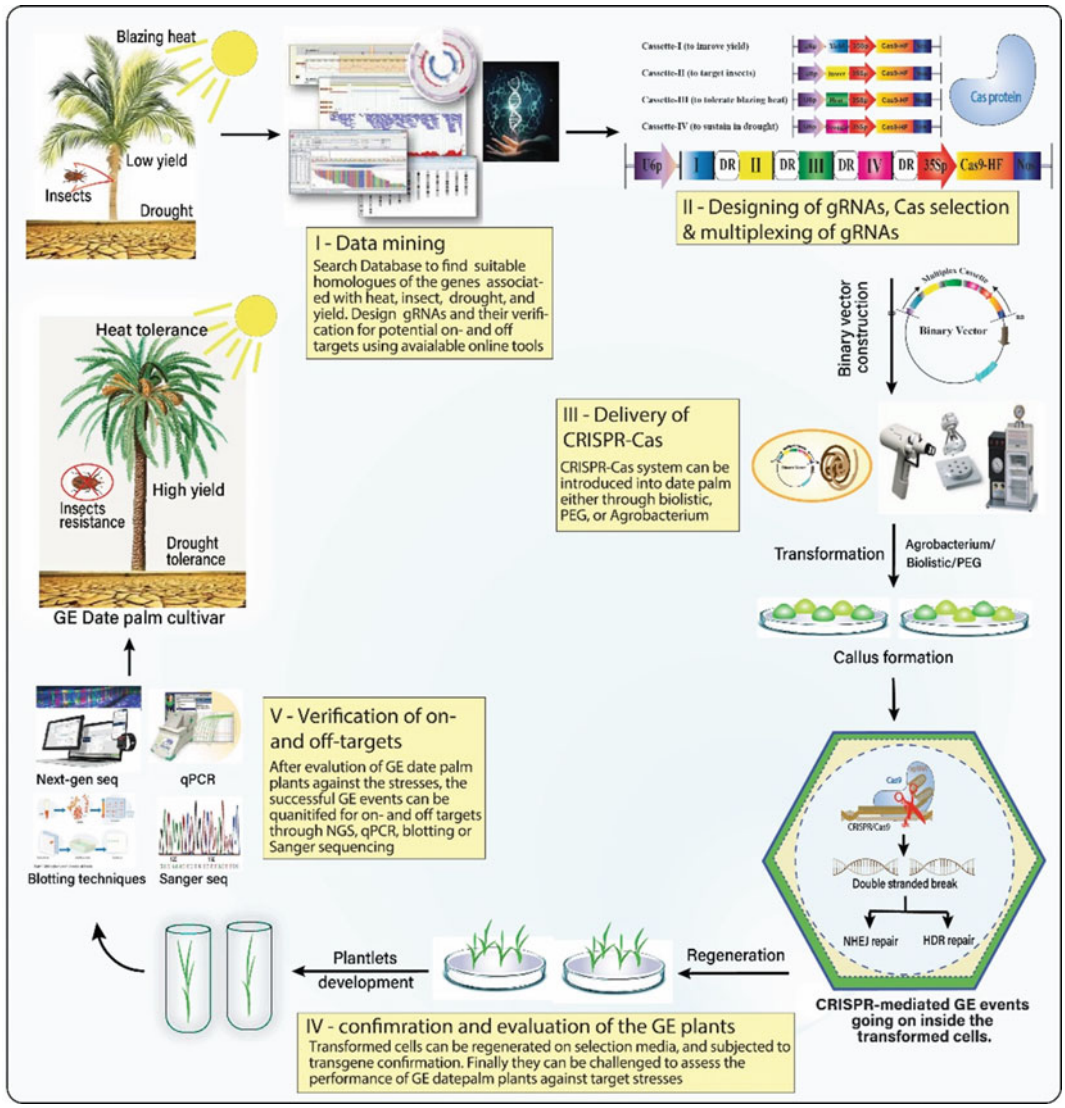

Fig. 9.3 Overview of basic workflow and step-wise strategy of CRISPR-Cas mediated genome editing in date palm. Abbreviations used in the figure are genome editing (GE), real-time PCR (qPCR), polyethylene glycol (PEG), next-generation sequencing (NGS), non-homologous end-joining (NHEJ), and homology dependent repair (HDR) (Figure produced by Z. Iqbal in Adobe Illustrator 2019)

online tools can do a pre-evaluation of the sgRNAs for any tentative off-targeted mutations inside the plant genome. Usually, these tools work in three major steps: a) identification of the appropriate target region and designing sgRNAs, b) off-targets verification of the designed sgRNAs, and c) assessment of cleavage rates of on- and off-targets (Lee et al. 2016).

9.4.2 SgRNAs, Cas, and CRISPR Cassettes Assembly

Initially, GE through the CRISPR-Cas system in plants was not very efficient. Over time, with advancements in technology, the efficiency of plant GE has been improved and progressed significantly. The discovery of new Cas variants,

cost-effective, and accurate screening for knockout or knockin mutants and more flexible vectors have made this technique more accurate, precise, and cost-effective.

The sgRNA expression cassettes guide the Cas/sgRNA complex to mediate GE. Therefore, it is crucial to design a unique sgRNA cassette. Usually, the size of sgRNA/promoter cassette is very small and thus, overlapping PCR and/or adaptors can be used for amplification and ligation, respectively. Ma et al. (2015) used the Gibson assembly or Golden Gate cloning method to directly clone sgRNA expression cassettes based upon PCR-technique (Ma et al. 2015). Similarly, Gao and Zhao (2014) transcribed pre-RNA using RNA Pol-II promoter to generate sgRNAs utilizing ribozyme mechanism and inducible or constitutive promoters (Gao et al. 2015).

In order to execute a GE event in plants, the successful expression of Cas protein in plant cells, codon optimization and use of an appropriate promoter are prerequisites. It has been noted that Cas protein joined with nuclear localization signals (NLS) can be more efficiently integrated into the plant genome (Belhaj et al. 2013). The list of appropriate plant promoters is limited and, therefore, it is crucial to select a suitable promoter for expression of sgRNAs and Cas protein inside the plant cell. In most of the cases, CaMV 35 s, CMV, EF1A, LTR and UBO promoters have been successfully employed in CRISPR-Cas-based studies. Moreover, RNA Pol-III promoters such as U3 and U6 have also been used quite efficiently (Belhaj et al. 2013). Usually, sgRNAs expressed significantly under endogenous promoters than the exogenous promoters. Moreover, sgRNAs expressed in monocots or dicot plants only work efficiently under the promotors originated from monocots or dicot plants, respectively.

9.4.3 Cassette Delivery and Transfection of Explants

The next step is the transformation of the whole cassette, including sgRNAs, CRISPR and Cas, into *Agrobacterium* and later their expression in the plant cells. Again, selection of a suitable plant expression vector is very important to deliver the whole cassette. Usually, CRISPR-Cas based GE is mediated either by a single-vector system or a binary-vector system. In a binary-vector system, multiple structural sgRNAs constructs can be coupled with numerous versions of Cas protein to obtain a unique gRNA-Cas nuclease. It provides a more accurate and user-friendly experimental design. However, a single-vector system containing sgRNA and Cas protein as separate expression cassettes are becoming more popular. In this system, generally sgRNAs are expressed by RNA pol-III driven promoters (such as U3 or U6) whereas, Cas gene is expressed through RNA Pol-II driven promoters (such as ubiquitin or CaMV35S). Recently, a single-vector system has been proficiently equipped with modified single Pol-II and dual Pol-II promoters. Single Pol-II vectors can be exploited to express sgRNAs and the Cas protein simultaneously under common promoter, whereas, with dual Pol-II vectors two promoters can drive the sgRNA and Cas proteins in a single cassette.

Another important step in GE through a CRISPR-Cas-based system is to express the sgRNA and Cas protein together at the targeted site through cargo-vector. Usually, plant transformation is either mediated through *Agrobacterium* or biolistic approaches. The efficiency of the plant transformation can be advanced with novel strategies such as ribonucleoprotein complexes, virus-based delivery systems, protoplast transformation, polyethylene glycol (PEG)-based technique, use of transient peptides and others (Ali et al. 2018). Protoplast transformations are usually used as a transient expression system to test the feasibility of the CRISPR-Cas toolkit. However, it is difficult to regenerate plants from protoplast cultures due to limitations in heritable mutations and thus, it is not equally applicable to all plant species. Particle bombardment and PEG-based transformation methods can directly deliver the sgRNA and Cas expression cassettes into the plant genome (Wolter and Puchta 2018). A stable genetic transformation of the CRISPR-

Cas based system can be effectively carried out in dicotyledonous and monocotyledonous plants (Zhang et al. 2018a).

Date palm tissue culturing is a difficult and challenging task and therefore, the selection of an appropriate plant expression vector is crucial in such woody plants. A DNA-free direct transformation of protoplast can be a good choice to transform the date palm genome. This strategy has been successfully exploited for vegetatively-propagated perennial plants (Woo et al. 2015).

9.4.4 Plant Regeneration, Screening and Verification for On- and Off-Target Mutations

Successful plant transformation of explants is followed by the regeneration of plantlets with successful GE events. The phenotypic and/or genotypic screening of the mutants requires a handy, cost-effective, and robust strategy, which can screen enormous numbers of mutants for successful GE events either off- and on-targets. Different techniques have been adopted to meet the challenges. These include high-resolution melting assay (HRMA), genotyping using polyacrylamide gel electrophoresis (PAGE), T7 endonucleases and annealing critical temperature-PCR (ACT-PCR), Sanger sequencing and NGS based high-throughput techniques. A tentative verification can be done with the use of reporter genes such as GFP, RFP, YFP, and GUS to mark the GE events in the mutants. Such reporter genes must use a frameshift mutation at the target site or may contain a duplication that can be reversed by the CRISPR-Cas system as a marker of a successful GE event of the desired trait (Shan et al. 2013). Each of these techniques has its own pros and cons for certain genotyping differences. Although, the phenotypic marker-based screening is only effective for those genes, making a connection between visual phenotype and the target gene is sometimes a challenging task. However, genotypic screening techniques are equally effective for all the mutants generated through CRISPR-Cas-based systems. For plant species edited through the DNA-free CRISPR-Cas based approach such as date palm, NGS is highly beneficial to detect the GE events.

9.5 Gene Multiplexing Using CRISPR-Cas-Based GE

Plants have the redundancy of several genes during fine-tuning of various cellular processes. It may be possible that editing one gene cannot produce the desired phenotype due to the compensation effect by another member of the same gene family. Under such circumstances, a multiplexed approach is feasible to achieve a laconic control by targeting more than one gene(s), simultaneously. The CRISPR-Cas-based systems offer outstanding utilities of multiplexing by designing multiple sgRNAs to target together one gene or multiple genes controlling the same trait. The CRISPR-Cas based system is quite flexible to harbor multiple sgRNAs expressed under single or multiple promoters in a single-vector system (Liu et al. 2017). Wang et al. (2017) found four katanin p80 subunits were successfully targeted in *Arabidopsis thaliana* through multiplexed GE (Wang et al. 2017). More extensive studies reported even eight genes targeted simultaneously by expressing multiple sgRNAs in a single cassette through the t-RNA based strategy in rice and maize (Qi et al. 2016; Xie et al. 2015). In another study, Tang et al. (2016) applied a self-cleaving ribozymes strategy for multiplexed GE by opting for a Pol-II promoter to drive multiple sgRNAs and Cas9 activity (Tang et al. 2016). After transcription, the sgRNAs and Cas9 were separated by ribozyme cleavage, which released functional sgRNAs and Cas9 separately. Furthermore, the CRISPR-Cpf1 system was employed for multiplexing in rice plants under a single promoter (Wang et al. 2017). A similar strategy proposed by Xie et al. (2015) can be adopted in date palm multiplexed GE (Xie et al. 2015). In this system, the endogenous tRNA processing machinery is hijacked to generate multiple sgRNAs using a single cassette. The constructs are cloned as polycistronic tRNA:sgRNA (PTG) form with repeated subunits of spacer and sgRNA scaffolds

separated by conserved tRNAs (Fig. 9.4). This PTG-based multiplexing technique has been successfully employed in allopolyploid rice (Wang et al. 2016) and maize (Qi et al. 2016). The implications of PTG-based multiplexing have also been extended to human GE where three histone deacetylase (HDAC) genes were edited successfully (Dong et al. 2017). Comparative genomic studies deciphered a match of 50% between the predicted date palm ORFs and rice ORFs (Al-Dous et al. 2011). Moreover, the proteomic comparison of date palm cultivar Khalas showed that ~8000 gene families are similar to both monocots and dicots (Al-Mssallem et al. 2013). Consequently, the universal tRNA-based multiplexed GE approach can be potentially effective in date palm to target multiple genes involved in abiotic and biotic stresses, particularly the two major challenges to date palm cultivation, red palm weevil (RPW) and bayoud disease.

Thus, multiplexed GE with a CRISPR-Cas-based system is a convenient approach to target multiple genes simultaneously with almost equal magnitude. It also helps to disrupt a desired gene in a family, which regulates multiple biological pathways. It is also an easy way to investigate epistatic associations among different genes in various genetic processes.

9.6 Gene Activation and Inactivation for Climate-Smart Traits

Availability of the date palm genome, its transcriptome profile and a diverse CRISPR-Cas toolbox have opened up unprecedented

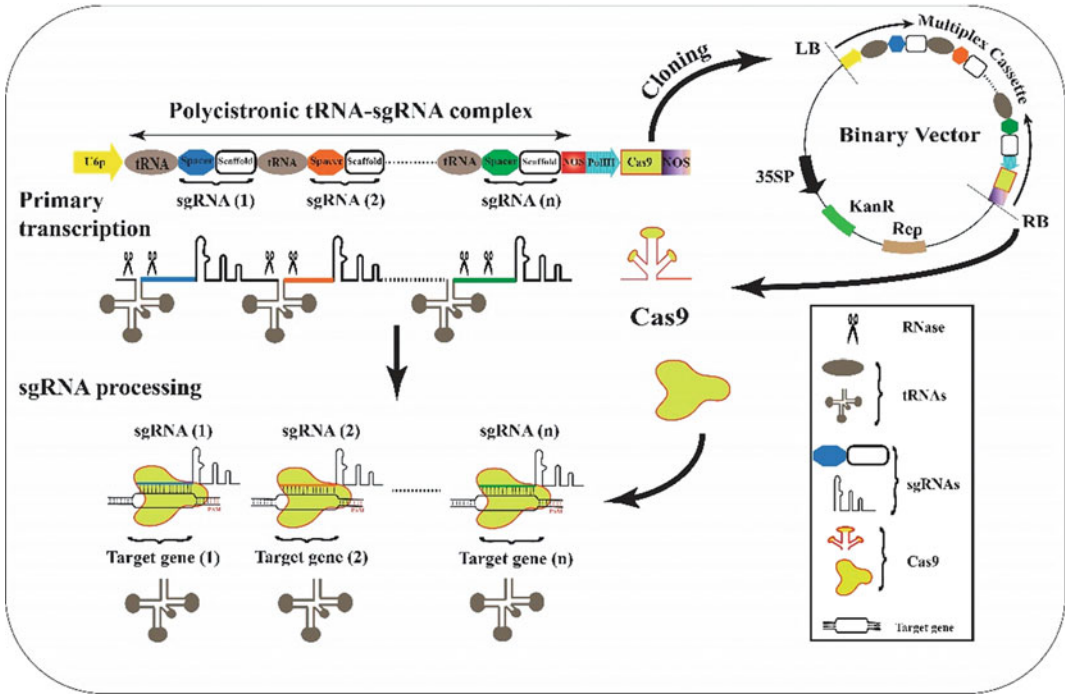

Fig. 9.4 Schematic diagram of CRISPR-Cas based multiplexing in date palm. Multiple sgRNA scaffolds targeting a single gene at multiple sites or multiple genes in a gene family can be assembled together with the spacer sequences with adjacent tRNAs. The whole assembly will follow a Nos terminator sequence at the downstream. The Cas protein can be expressed from a Pol-III promotor with a common Nos terminator sequence. A ubiquitin promotor (U6 or U3) can be used to drive all the sgRNAs together. The assembled cassette can be vectored through a common vector into the date palm genome where all the components in the multiplexed cassette would be transcribed and processed separately (Figure produced by M. N. Sattar in Adobe Illustrator 2019)

opportunities to engineer the date palm genome to meet the challenges of this century. Date palm GE holds substantial potential for the genetic improvements of elite cultivars to be engineered to introduce desired traits. Confronting extreme drought regimes, high salinity, blazing heat, susceptibility to insects, pests and fungal diseases and breeding for short stature, high yielding, and good fruit quality cultivars could be major areas of interest in date palm GE. The applications of CRISPR-Cas in date palm GE, such as CRISPR interference, CRISPR activation and precise BE, can help to address the aforementioned problems and can minimize the concerns of end-users due to its non-GM nature (Fig. 9.5).

9.6.1 CRISPR Knockin Mediated Date Palm Genome Engineering

The CRISPR-Cas9 system offers plausible opportunities to create knockin gene mutations in date palm. In CRISPR-mediated knockin, a typical gene can be substituted by a gene of interest (GOI) or a substitution of a single base can confer the desired trait or can lead to a change in gene expression at a precise locus. CRISPR-Cas9 induces DSB at the target loci during a usual cleavage procedure, thereby activating the cell repair mechanisms. Small insertions and deletions (indels) are used to repair the DSB, but the insertion of GOI of interest or exogenous DNA can also be achieved either using homology arms or randomly. In the homology arm, the insertion of GOI or any exogenous DNA is used as a donor template. Besides the homology arms, gene insertion or replacement can be performed either through canonical NHEJ or microhomology-mediated end-joining/HDR. Cas 9 and Cas12 have been successfully executed to generate DSBs and co-introduced with a repair mechanism for precise GE via HDR. A similar approach of GE has been discussed in date palm (Fig. 9.5). The ultimate advantages of knockin are speeding up the breeding program without generating allelic variants or linkage drag, and modification of multiple alleles by gene stacking in a single cultivar. Different Cas9 programmable nucleases have been identified in order to perform knockin genetic modifications and engineer in-frame insertions at the target locus. The hyperexpression of *Auxin-Regulated* Gene *Involved in Organ Size 8* (*ARGOS8*), a negative regulator of ethylene response, was achieved by knockingin the *GOS2* promoter with *ARGOS8* promoter to gain improved drought stress through enhanced*ARGOS8* transcripts level (Shi et al. 2017). A tomato with a long shelf life was generated by substituting/knockingin the *T317A* with an *ALC* gene (Yu et al. 2017). Similar genes or their homolog genes can be found and engineered to produce date palm fruit with extended shelf life and improved shape. A CRISPR knockin system based on geminivirus-replicon with efficient selection and reporter-free gene was developed in tomato plants to repair a *Cortiso* gene allele having a 281 base pairs deletion (Dahan-Meir et al. 2018). *Acetolactate synthase* (*ALS*) and *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSP*) genes were targeted to engineer herbicides-resistant maize, rice, and soybean using CRISPR-knockin based amino acid substitution. The successful execution of CRISPR-Cas9 knockin holds substantial advantages in date palm to create new genetic variability to accelerate date palm breeding, modify target genes, reduce the flowering latent periods, develop early maturing varieties, and to develop resistant cultivars against excessive heat, drought and salinity.

9.6.2 Knockout Mediated Date Palm Genome Engineering

The knocking out of negative genetic elements or undesirable traits is a promising strategy for genetic improvement. Thus, the CRISPR-Cas system was engineered to knockout mutant-inducing indels at the target site. Generating a CRISPR knockout is much easier than a knockin, therefore, a number of studies used this system to create GE in plants. Many published studies have described successful execution of CRISPR-mediated GE to achieve either a single gene or

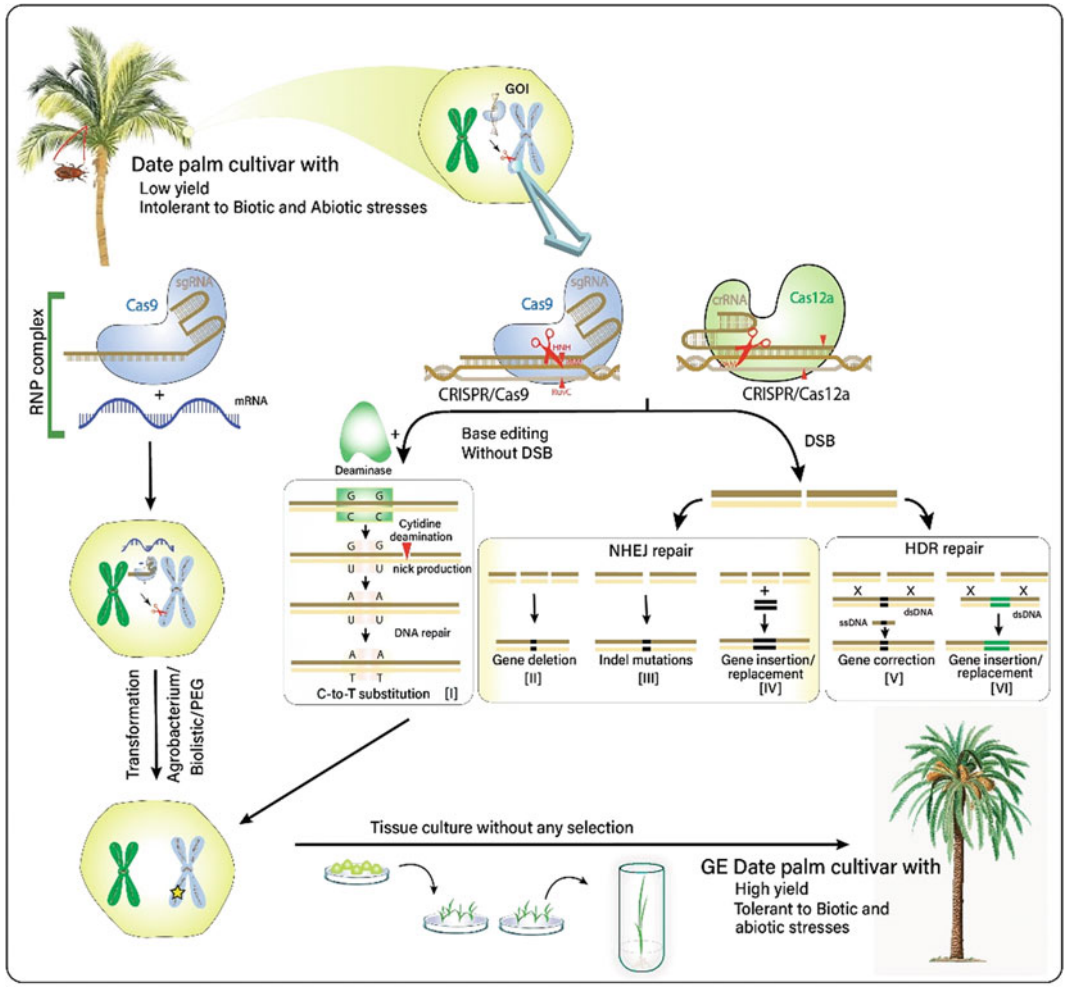

Fig. 9.5 CRISPR-Cas mediated genome editing, base editing/substitution and production of transgene-free/DNA-free GE date palm plants. Two Cas proteins (Cas9 and Cas12a) with higher GE potential in date palm are depicted. CRISPR-mediated GE can be executed either through DSB dependent pathways (either through homology arms [HDR] or randomly [NHEJ]) or DSB independent pathway (Base editing/substitution), which can be accomplished using a deaminase (C-to-T BE is shown). In addition, transgene-free/DNA-free GE date palm plants can be achieved via RNP-complex by transiently expressing CRISPR-Cas system in date palm (Figure produced by Z. Iqbal in Adobe Illustrator 2019)

multiple genes knockout mutants in various plant species to improve several traits related to abiotic and biotic stresses, fruit quality and yield traits. For sustainable food security, CRISPR-Cas based knockout mutants can be generated to achieve higher date palm yield. Yield is a complex trait controlled by multiple factors, nevertheless simultaneous knockout of multiple genes related to yield can lead to trait pyramiding, which can potentially increase yield. In rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.), several negative regulators of yield have been identified including grain size (*OsGS3*), grain number (*OsGn1a*), grain weight (*GW2*, *GW5*, *TaGW2*, *OsGW5*, *OsGLW2*, *TGW6*, *TaGASR7*) and tiller numbers (*OsAAP3*) (Xu et al. 2016; Zhang et al. 2018b). The homologue of such genes can be identified in date palm and their knockout mutants can be generated through CRISPR-Cas to increase yield. Recently, a

method was developed to identify the multiple genes that play a role in complex quantitative traits like yield, by integrating whole-genome sequencing, pedigree analysis and CRISPR-Cas technology (Huang et al. 2018). By selecting this method, 30 cultivars of the parents and their descendants of the Green Revolution's miracle rice varieties were analyzed, and more than 50 genes were identified to play a role in yield. The same working hypothesis can be executed in date palm to maximize yield and help in date palm molecular breeding.

Flowering in plants takes place due to a switch in a developmental phase; three important *Arabidopsis* genes, *SHORT VEGETATIVE PHASE* (*SVP*), *APETALA1* (*AP1*) and *TERMINAL FLOWER1* (*TFL1*), have a very important role to switch vegetative growth to reproductive state and flower formation (Blázquez 2005). The *AP1*, a transcriptional regulator, initiates flower growth, while *SVP*, a main floral repressor, suppress *TFL1* to determine the architecture of the inflorescence. *TFL1 is* chiefly responsible for the repression of floral initiation, inflorescence development regulation, and suppression of *AP1* to maintain the inflorescence meristem in a vegetative state (Baumann et al. 2015). Liu et al. (2019b) demonstrated the CRISPR-Cas system with RNA endoribonuclease (Csr4) to study the floral development in *Arabidopsis*. *AP1*, *TF1* and *SVP* were knocked out at multiple sites and all the mutant plants exhibited abnormal floral development. The *AP1* and *SVP* mutants have more plant branches, whereas *TF1* mutants showed a change from indeterminate to determinate inflorescence. In date palm, where the mechanism and the induction of flowering are still unknown, CRISPR-Cas mediated knockdown of these three candidate genes (or their homologs) could serve as a template to study the floral development.

Certain insects like RPW, excessive heat, and drought, as mentioned earlier, are leading constraints to date palm production. To combat this devastating insect in date palm, the homolog of *Oriza and sativa* gene, *OsCYP71A1*, can be used to knockout the serotonin biosynthesis and hyperproduction of salicylic acid by the CRISPR-Cas system, to confer resistance against stem borers and plant hoppers (Chen et al. 2019). A very insightful study revealed that the gene family of *OsPYL* abscisic acid receptor plays a role against certain biotic and abiotic stresses, thus CRISPR-Cas mediated triple knockout of *pyl1*, *pyl4* and *pyl6* were created in rice and compared to wild-type plants. Results revealed that the knockout mutants had enhanced grain yield and showed improved ability to tolerate high temperature (Miao et al. 2018). To engineer abiotic or biotic stress in date palm, structural genes can play a pivotal role, especially the genes involved in the production of reactive oxygen species (ROS); such genes initiate signals against many biotic and abiotic stresses. Nonetheless, a controlled regulation of ROS is very important as overproduction of ROS can lead to growth abnormalities, cell death, male sterility, and eventually to compromise yield (Hu et al. 2011). Several genes that play a role in the production of ROS are well studied and sometimes referred as *tolerance genes*, such as catalases, glutathione reductases, glutathione-S-transferases, peroxidases (POD), and superoxide dismutase. Several studies are available where these genes have been introduced, either through breeding approaches or genetic engineering, to achieve tolerant plants, for example, *Arabidopsis* plants engineered with *Papain like Cysteine proteases* from sweet potato (*SPCP2*) and wheat (*TaCP*) exhibited enhanced tolerance to drought stress (Liu et al. 2018). Similarly, melatonin has an antioxidant role and helps plants withstand reactive nitrogen species and ROS. The plants, engineered with melatonin biosynthetic genes, showed enhanced ability to tolerate different abiotic stresses (Antoniou et al. 2017).

The regulation of certain transcription factors (TFs) in response to regulation of *cis*-regulatory sequences are a well-known phenomenon. *Cis*-regulatory sequences are typically found in the upstream (promoter) region of open reading frames (ORFs) and have a strong influence on the expression of the genes. Different *cis*-sequences like the GCC box (AGCCGCC) and the W-box (TTGACC) act as a negative abiotic stresses regulator by providing a suitable binding site for

specific TFs such as *OsERF922* and *GhWRKY17*. In *Arabidopsis*, *ANAC069* TF by binding to *cis*-element inhibits the expression of many stress-responsive genes including *GST*, *POD*, *SOD* and *Pyrroline-5-carboxylate synthase*. The perturbation of any sequence in C[A/G]CG[T/G] sequence leads to the failure of gene regulation by ANAC069 thus, ultimately leading to stress tolerance (He et al. 2017). Notably, CRISPR-Cas based target mutagenesis of such *cis*-sequences in the promoters of several genes could potentially help improve complex traits like different abiotic stresses in date palm.

Another important, but ignored, class of regulatory genes is small non-coding RNAs and microRNAs (miRNAs). Until now, no CRISPR-Cas based study is available which directly targets the regulation of miRNAs to engineer desired traits in plants. The miRNAs execute their functions by regulating their target genes at the posttranscriptional level. Some characterized miRNAs have a role in the regulation of abiotic stress, such as *osa-MIR393* which mediates the expression of three key stress-responsive *O. sativa* genes (*LOC_Os05g41010*, *LOC_Os02g06260*, *LOC_Os05g05800*) to negatively regulate alkali and salt stress tolerance (Gao et al. 2011). Thus, identification and knocking out of such miRNAs in date palm through CRISPR-Cas may lead to enhanced tolerance to these stresses.

Besides creating mutants, either via knockin or knockout, CRISPR-Cas system can be employed in gene activation via CRISPR activation (CRISPRa) or gene repression via CRISPR interference (CRISPRi) after fusing inactive Cas (dCas) with a transcriptional activator or repressor motif (Bortesi and Fischer 2015). In addition to precise modification, precise control over gene regulation at a particular locus has added an unparalleled advantage over conventional GE methods, where the integration of transgene is random. Although CRISPRa and CRISPRi have been successfully applied in animal cells, work is still underway in plants.

9.6.3 Base Editing in Date Palm Genome Engineering

The CRISPR-Cas system with BE abilities has enhanced gene engineering technologies. Such CRISPR-Cas systems use facets of DNA modifying enzymes (like deaminases) to substitute a nt base. The nt base substitution is achieved without inducing DSB by the CRISPR-Cas system. So, the DNA repair mechanism of the cell is not invoked, resulting in considerably less on- and off-target indels at the target site. Different versions of CRISPR-Cas systems are available for all four transition mutations, such as C-to-T, G-to-A, A-to-G, and T-to-C. These BE systems hold great potential in date palm molecular breeding, as different agriculturally important traits can be conferred in the coding region of the genes (Fig. 9.5).

The notable superiority of BE over other GE systems is the outcome of a cleaner product with just a few off-targets or amounts of indels (Rees et al. 2017). Adenosine BEs (ABEs) yields a much cleaner product than Cytosine BEs (CBEs) and it has virtually no indels. Whole-genome sequencing of ABE edited wheat and rice plants yielded a ca. 99.9% cleaner genome with no off-target and undesired on-targets base editing (Hua et al. 2018; Li et al. 2018). Likewise, a unique *Gossypium hirsutum* base editor (GhBE3) was used in cotton to target two genes, *GhCLA* and *GhPEBP*, and the editing efficiency range of 26.67–57.78% with just 0.1% off-target activity (Qin et al. 2020). The great utility of the BEs system is to help understand the role of the conserved amino acid(s) having a crucial role in protein function. Employing the CBE approach, the function of four *Arabidopsis* genes were revalidated after modifying them either by constitutive splicing or impeding alternative splicing. The BEs could be employed to confer herbicide resistance and by using both BEs (ABEs, CBEs) system, herbicide-resistant rice varieties have been developed by targeting *Acetyl-coenzyme A carboxylase* (*ACCase*) gene and *Haloxyfop-P-*

methyl gene through CBE and ABE, respectively (Shimatani et al. 2017). Notably, several SNPs associated with important varietal or agronomic traits have been mapped in different crops; such SNPs can be engineered through BEs in elite cultivars to install the desired trait. In date palm cultivar Khalas, ca. 1,748,109 SNPs were identified in a genome size of ~381 Mb and ca. 49% of SNPs occur within 50 bp of the cultivar Deglet Noor, yielding a nonsynonymous-to-synonymous SNP ratio of 1.17 (Al-Dous et al. 2011). The frequent occurrence of SNPs in date palm genome renders it a valuable candidate plant to employ the BEs system. Many plant resistance genes have an allelic nature because of the difference of a single or just a few nts. So, if such alleles could be corrected by Bes, then resistance could be engineered against date palm diseases. Likewise, BEs can be employed to engineer the disease susceptibility (S) genes to generate alleles in the coding regions. Thus, in this case, S genes will still be able to perform the essential work. Nonetheless, such changes can lead to pleiotropic effects like reduction in yield, growth, or sensitivity to other stresses. So, instead of disrupting the coding sequence, the nts in the promoter region can be mutated through BEs to enhance resistance without pleiotropic effects. Another utility of BEs in date palm GE could be the regulation of RNA splicing pathways. The splicing of almost all the eukaryotic mRNA follows the canonical GU/AG rule. During splicing, introns bear a 5'GU (a splice donor) and AG3' (a splice acceptor) site. A BEs-mediated point mutation at these conserved sites can lead to mis-splicing or loss of a particular splice form. By following the same strategy, Xue et al. (2018) substituted G to A in the splice donor site to hamper the removal of an intron to gain hypersensitivity to abscisic acid. In a similar study, single null mutants of *Arabidopsis MTA* genes and double null mutants of rice genes *OsGL1* and *OsNAL1* were generated by mis-splicing (Li et al. 2019). Thus, CRISPR-Cas BEs holds remarkable potential for the creation of novel traits for speedy date palm GE.

9.7 CRISPR-Cas and GMO Regulatory Oversights

The available GE tools, either basic (like CRISPR-Cas systems) or their advanced version (like BEs), have exceptional potential for date palm improvement and to engineer desirable traits in elite cultivars. In comparison to conventional genetically modified (GM) plants, CRISPR-mediated GE technologies do not disturb the genetic makeup of the plant (Nekrasov et al. 2017). Nonetheless, the use of CRISPR-Cas system leaves some parts of the cargo vector in the plant genome either transformed through *Agrobacterium* or nonintegrating plasmids. To foster public acceptance, minimize the regulatory GM burden and mitigate the ecological challenges, it is of prime importance that CRISPR-edited final products be free of the transgene. However, this is not an easy task and poses unique challenges; one of the major concerns is the escape of CRISPR-Cas constructs to the environment through the spread of pollen. Another concern is the presence of Cas protein, which can provoke food safety worries.

Researchers believe that CRISPR-edited crops should not be considered GMOs and emphasize this to distinguish different types of GE processes; this subject was reviewed recently by Friedrichs et al. (2019). However, each process type has specific shortcomings pertaining to GM regulatory considerations. Recently, the Court of Justice of the European Union (ECJ) in Luxembourg ruled that CRISPR-edited crops are not exempt from GM regulations and will follow the same stringent regulation as that of other GM crops. Such legalities, unfortunately, provoke similar regulations, especially in other developed countries; however, US officials are not planning to impose similar regulations on GE crops. A similar tack has been opted for by Argentina, Australia, and Brazil. The US Department of Agriculture (USDA) has approved a CRISPR-Cas edited mushroom with a polyphenol oxidase mutated gene to increase shelf life by avoiding browning, and the approved mushroom cultivar

will follow the non-GMO legislation (Waltz 2016). In addition, two other GE crops are available in US markets: oil with high-oleic-acid contents extracted from GE soya beans and non-browning GE lettuce.

Researchers working on CRISPR-Cas systems describe those organisms that undergo simple and targeted GE through the CRISPR-Cas system as being as safe as crops developed through classical breeding approaches. From the scientific viewpoint, CRISPR-Cas edited crops are not comparable to GM crops and this is reflective of the fact that EU GMO regulations are outdated and not in line with recent scientific innovations. Such standing legislation will undermine the funding of CRISPR-Cas based research and, consequently, CRISPR-Cas based breeding could become less relevant, more expensive, and create an uncertain environment for large multinational companies attempting to set future priorities.

In the EU, draconian oversight of CRISPR-Cas edited crops is expected because the EU Court of Justice decision surprised and confused researchers and traders who were planning to promote GM crops. Many GE crops and their derived food products have already entered the markets of Asia, the EU, and certain other countries. To meet these challenges, a proposed solution is the inclusion of GE crops in a registry of GE crops developed in different countries (Eckerstorfer et al. 2019). In addition, the July 2019 conference, OECD Conference on Genome Editing: Applications in Agriculture—Implications for Health, Environment and Regulation was organized in the context of overarching agriculture and food policies. Over 200 participants from 35 countries took part, including leading academics, policymakers, risk assessors, private sector innovators, regulators, and other stakeholders were brought together to highlight the advancements in GE technology, its applications in the agricultural sector and to discuss the regulatory aspects of GE crops. The conference concluded that strenuous efforts are required from all stakeholders to facilitate the exchange of information regarding crop GE, in order to open avenues for the technology's beneficial products. Moreover, the estimation, assessment, evaluation, dissemination of health risks by both legislators and critics should be science- and fact-based, without overburdening the general public with abstruse details (Friedrichs et al. 2019). Such platforms should be organized regularly to comprehensively understand GE technologies, to respond to escalating information requirements, to mitigate risk tiering approaches, to modify/review legislation over GM and GE technologies and to mitigate the GM oversight of GE crops.

9.8 Regenerating Transgene-Free Plants Through Ribonucleoproteins (RNPs)

Unlike GM plants, the genetic makeup of GE plants resemble the naturally-mutated crops and remain the same except for just a few indels. Generally, the integrated CRISPR-Cas system cargo comprises of CRISPR array, Cas9, a marker gene (usually an antibiotic or herbicide resistance gene), T-DNA, and some other associated components. Markers are the key elements for in vitro selection of the transgene in the transformed cells, plant regeneration and selection of the next progeny. However, these pose a major risk of biosecurity. Although most of the CRISPR-Cas cargo can be eliminated through backcrossing and screening the segregating populations, this approach is not applicable to plants with a longer life span (such as date palm) and vegetatively-propagated plants. Creating transgene-free date palm plants is much trickier as they may require up to 5 years flowering, so it would take too long to get transgene-free seeds. Transient expression of the CRISPR-Cas system in plants has the ability to reduce transgene integration, but complete removal is almost impossible and resultant degraded DNA fragments will still be incorporated into the plant genome (Zhang et al. 2016). Development of transgene-free GE plants are extremely important, as it will persuade consumers, policymakers and legislators that GE plants have a comparable genome to a native plant. Then public acceptance

and commercialization of GE plant/crops will be much easier. Nevertheless, the complete removal of CRISPR cargo from the GE plants is neither simple nor short. In this regard, several techniques ranging from classical (self-crossing and backcrossing) to molecular biology (suicide transgene, protoplast transfection, viral vectors, transgene killer CRISPR technology) have been used (Ma et al. 2020; Sandhya et al. 2020; Sattar et al. 2019; Wada et al. 2020). Nonetheless, a recently described approach based on the use of ribonuclease protein (RNP) could help avoid all the pitfalls of DNA-based genome delivery methods (Liang et al. 2019).

In the RNP technique, preassembled RNPs comprised of purified Cas9 and in vitro transcribed or synthesized sgRNA are used, so the technique is completely devoid of DNA. Woo et al. (2015) first executed this technology in *Arabidopsis*, rice and tobacco. A RNA-guided endonuclease (RGEN) and RNP were delivered into protoplasts of the target plant species. A highly purified Cas9 protein was mixed with in vitro expressed gRNAs (1:10 molar ratio) to target four genes. The RGEN-RNPs were incubated with the protoplasts of plant species in the presence of PEG, and GE protoplast is then regenerated to a plant. The validation of GE events were achieved using T7 endonuclease-I assay and targeted deep sequencing; the results verified the presence of indels at 3'nts upstream of PAM (NGG) sequences. Furthermore, they cotransfected two gRNAs (having target site at a distance of 201 bp) in the *Arabidopsis* gene *Brassinosteroid Insensitive 1* (*BRI1*) to explore DSBs which were generated at the same target site. The results of Sanger sequencing revealed that a nucleotide at 223 positions was deleted in the protoplasts. No off-targets were detected in this study and GE efficiency showed a range of 8.4–44%. A detailed and comprehensive protocol of CRISPR-Cas mediated GE through RNPs in lettuce has also been discussed by Park et al. (2019). Later, a RNP-based technique was executed in apple, brassica, grape, petunia X hybrids, and soybean plants. Nonetheless, regeneration of GE plants from protoplasts is not an easy task, because many plant species are impossible to regenerate through protoplast, and/or tissue culturing can lead to somaclonal variation. Therefore, a biolistic inoculation (particle bombardment approach) has been developed for the transformation of Cas9 and RNPs in plant cells (Zhang et al. 2016). In addition, RNPs have been successfully delivered into plant zygotes, developed through in vitro fertilization, and later regenerated to achieve mature plants without any selection. As this technique is free of recombinant DNA, the resulting GE plants may be exempt from stringent GMO regulations and therefore pave the way for the use of CRISPR-Cas based GE in agriculture and plant biotechnology.

9.9 Conclusions and Prospects

At present, world agricultural sustainability is under threat due to abrupt climate changes, drought and exponential population growth. With the current pace of developing new cultivars it is challenging to produce safe, cost-effective, and high-quality agricultural products. Nonetheless, modern innovative technologies like the CRISPR-Cas system hold substantial potential to boost crop production and cope with the current challenges. In addition, advanced and sophisticated GE tools can improve the agronomic, physiological, nutritional, and physical traits in date palm to produce elite cultivars with superior traits. Moreover, these GE cultivars can be easily approved for specified locations without facing any strict regulatory framework. Over the last two decades, SSNs-based plant GE tools such as ZFNs and TALENs have maintained their role in modern plant breeding. However, present-day CRISPR-Cas versions have revolutionized the breeding and functional genomics in various crop plants. In numerous experiments, mutation breeding through chemical and physical mutagens has been used to cope with abiotic and biotic stresses, such as bayoud disease in date palm (Jain et al. 2011). In comparison to such random mutations, the CRISPR-Cas-based system in date palm can be a good tool to directly target a specific site in the genome. This

technique can easily be employed to induce resistance against pathogens, insects, and pests in date palm. Moreover, it can be used to explore and improve quantitative as well as qualitative traits and secondary metabolites. Sex determination is a primary objective of any date palm breeding program and it is always very hard to determine the male and female progenies until the plants reach maturity. Although various marker-assisted approaches are in use to clarify the dioecious nature of the younger plants the results of using these markers cannot be guaranteed. The gene knockout ability of CRISPR-Cas-based system may permit the identification of genetic markers linked to the sex determination in the younger plants even at the early stage of development. Moreover, the CRISPR-Cas system can be used to promote yield-related components, plant architecture, nutrient status, and plant adaptation to biotic and abiotic stresses in date palm. Among the more striking features of the CRISPR-Cas toolbox are BE, gene replacement, gene insertion, knockout or knockin mutation, multiplexing and production of DNA-free plants to increase the targeted mutagenesis in date palm.

The availability of different versions of Cas protein such as Cas9, Cas9 VQR, Cas9 EQR, Cas9 VRER, Cas9 QQR, Cas9 HF1, dCas9, HiFi Cas9, nCas9, FnCas9, Cas12a (Cpf1), Cas12a RR, Cas12 RVR, FnCas12a, FnCas12a RR, FnCas12a RVR, Cas12b and CasX, along with base editing/substitution ability in the toolbox of the CRISPR system, provide unparalleled advantages for date palm GE. Nevertheless, there remain some limitations. Outcrossing plant species are typically highly heterozygous in nature and the presence of SNPs is common in them. Likewise, date palm is an outcrossing species and this characteristic sustains highly heterozygous alleles, polymorphic traits, and a genetically unstable genome (Jubrael et al. 2005). According to Al-Mssallem et al. (2013), the SNPs range in date palm genome is 3.85–6.63/kb. The date palm cultivar Khalas alone has 0.9 million new SNPs reported. The frequent occurrence of these SNPs may affect GE in date palm. Moreover, the plant models available for GE are mostly homozygous and quite a few studies have investigated the polymorphic traits. Zhou et al. (2015) accomplished the GE of two 4-coumarate:CoA ligase (4CL) genes in popular trees. However, the third gene of 4CL family escaped the mutation because of a single SNP near the PAM sequence in the designed sgRNA, although the sequence identity to other genes in the 4CL family was >89%. Identifying such SNPs necessarily requires complete information of the nuclear genome of a cultivar to be mutated. Nevertheless, the complete genome sequencing of few date palm cultivars have reported intra- and inter-varietal SNPs, even in the coding regions (Al-Mssallem et al. 2013; Sabir et al. 2014). These SNP deserts are reported to determine many agronomical traits in plants. Likewise, in the date palm genome, such SNP deserts regulate various high-density genes conferring resistance against biotic and abiotic stresses. These SNPs can be a target of site-directed mutagenesis through a CRISPR-Cas tool kit to promote resistance against insects, pests, and pathogens, enhanced flavor, fruit color, fruit shape, up-regulate antioxidants during fruit development and other characters. Some other challenges to be addressed may include the identification of a targeted site for GE through functional genomics, the efficiency of the cargo vectors to deliver sgRNA: Cas cassettes into the date palm genome, reducing off-targeted mutations in the date palm genome and optimization of an appropriate Cas protein. Another major bottleneck is the absence of an efficient and robust plant transformation method for date palm GE. Most of the current plant transformation methods are specifically modified for certain tissues, genotypes, cultivars or plant species. The delivery of sgRNA-Cas cassettes and genetic transformation through somatic embryogenesis may integrate part of the cargo vectors/bacterial plasmids into the date palm genome and it may face GMO regulations at a later stage. Under such circumstances, the DNA-free RNP approach may likely be a solution (Lu et al. 2017). Thus, in the near future date palm breeding programs may address the basic biological questions through CRISPR-Cas-based systems and may alleviate the current GMO-related concerns of consumers.

Appendix: List of Some Research Institutions Relevant to Date Palm

Institution name	Specialization research activities	Contact information and website
Date Palm Research Center of Excellence	Biotic and abiotic stresses through conventional and modern techniques	King Faisal University, PO Box 380, Al-Ahsa 31982, Saudi Arabia https://www.kfu.edu.sa/en/Centers/palms/Pages/Home-new.aspx
Date palm research group	Biotic and abiotic stresses	King Saud University, College of food and agricultural sciences, Riyad, Saudi Arabia http://cfas.ksu.edu.sa/en/content/date-palm-research-group
Center for Desert Agriculture Research	Date palm genomics and molecular breeding	King Abdullah University of Science and Technology, Thuwal 23955-6900, Saudi Arabia https://www.kaust.edu.sa/en/
Date Palm Research Institute	Micropropagation and varietal development	Shah Abdul Latif University Khairpur Mirs, Sindh, Pakistan www.salu.edu.pk
Date Palm Research And Development Unit	Varietal development, quantity and quality enhancement through modern biotech approaches	United Arab Emirates University, P.O. Box 15551, Al Ain, Abu Dhabi, United Arab Emirates https://www.uaeu.ac.ae/en/dvcrgs/research/centers/dpdrud/
National Center for Palm and Dates	Develop dates sector by concentration on production efficiency (cost reduction), product quality, effective marketing programs	7345 Prince Turky bin Abdulaziz Alawal, Hittin 13512-2141, Saudi Arabia https://ncpd.org.sa/en/about
Date Palm Research Unit, University of Baghdad.	To develop technologies for production, protection and post harvest technologies for date palm	Iraq, Baghdad, Karrada, Al-Jadriya—University of Baghdad https://www.dateresearchinstitute.com/
Date palm Research Station, Mundra	To develop technologies for production, protection and post harvest technologies for date palm	Sardar krushi nagar—385506. Dist.Banaskantha. Gujarat, India http://www.sdau.edu.in/detail/728914/date-palm-research-station-mundra
Date Palm Research Center	Quality and quantity enhancement in date palm	University of Basrah, Iraq http://uobasrah.academia.edu/Departments/Date_Palm_Reaserch_Center/
Palm Desert Center	Conservation and development of elite cultivars using modern approaches	University of California, Riverside 75080 Frank Sinatra Drive Palm Desert, CA 92211 USA https://palmdesert.ucr.edu/research

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Part IV
Genomics of Abiotic and Biotic Stress

Metagenomics of Beneficial Microbes in Abiotic Stress Tolerance of Date Palm

10

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Abstract

Date palm (*Phoenix dactylifera* L.) is a major crop species grown in the arid lands of the Middle East and North Africa, contributing to food security. Soil in the desert oases is relatively poor and the increased salinity of the groundwater used for irrigation has intensified the pressure on the agroecosystem productivity. Plants host large communities of microorganisms, mainly bacteria and fungi inside and outside their roots systems which are involved in plant nutrition, and biotic and abiotic stress resistance. Desert oases exhibit low soil phylogenetic and functional microbial diversity, mostly constrained by environmental stresses. However, the date palm shapes endophytic communities and promotes plant growth under abiotic stresses. The bacterial communities in the desert agroecosystem are dominated by the classes Gammaproteobacteria and Alphaproteobacteria, phyla Proteobacteria, and are invariably shaped by the date palm. To cope with environmental pressures in desert oases, the date palm root microbiome has shown to play a key role in promoting growth and tolerance to abiotic stress. The omics data of the desert microbiome of date palm are emerging as a new research direction and next-generation sequencing (NGS) has contributed efficiently to elucidate the community diversity within the date palm root system. Recent metagenomics studies of bacterial communities growing within the root systems of date palm show that the plants select the assembly of their bacterial phyla and promote plant growth under drought and salinity stress. Date palm microbiome structure contributes efficiently to the ecological services of biofertilization and promotes plant growth under abiotic stress in oasis ecosystems.

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J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_10

10.1 Introduction

Plants are sessile and to meet the pressure of the ecosystem they have evolved associations between roots and specific groups of soil microbes that benefit plants and confer tolerance to abiotic stresses. Understanding the interactions between the plant and microbial communities in the rhizosphere, with emphasis on the dynamics of plant microbiome interaction and their role in promoting plant growth, abiotic and biotic stress tolerance, has encouraged a number of researchers to try to unravel the key components of these interactions. The root microbiome, or root-associated microbe communities, where root surface and inner root tissues are colonized by microbes, plays a critical role in different ecosystems by increasing nutrient availability and acquisition, nitrogen fixation, and phytohormone production, all of which help to stimulate growth and abiotic stress tolerance (Berg et al. 2013; Mapelli et al. 2013).

Plant-microbe-stress interactions, known as the *phytobiome*, play an important role in selecting microbial communities and keystone microbes. Plants employ a complex combinatorial approach of signaling pathways to adapt to different abiotic stress (Fig. 10.1). In fact, root exudates such as exopolysaccharides (EPS) have been associated with selection for members of their phytobiome (Sasse et al. 2018). At the community level, the ability of the plant host to select its microbial community provides strong evidence that it may also serve as an additional source of defense against biotic and abiotic stresses (Berendsen et al. 2018; Hacquard et al. 2017). It is worth noting that environmental and abiotic factors are very important considerations when identifying plant-microbe interactions (Whitaker et al. 2018).

Phoenix dactylifera L. is considered one of the most salt and drought tolerant plants of the Middle East. However, given the ongoing decline in water resources, in terms of groundwater and soil salinity, the date palm's continued current productivity is under the threat of reduction. Contemporary studies of date palm have initiated the examination of physiological mechanisms, molecular and biochemical pathways (involvement of genes, proteins, microbiomes, etc.,) that govern or aid its response to these stresses. Stress resistance may be achieved through the promotion of osmolyte accumulation, mitigating oxidative stress by enzymatic and nonenzymatic reactions, or through the release of hormone-like substances that regulate the root structures and expansion, assisting in the maintenance of homeostasis of hormones (Bérard et al. 2011; De Zélicourt et al. 2013). For example, it was found that a bacterial enzyme, Acetyl-CoA carboxylase deaminase (ACCD), assists in endophytic colonization within plants and the same enzyme promotes plant growth promotion (Sessitsch et al. 2012). The potential of the plant root networks to be taken over by endophytes (advantageous microorganisms that colonize root internal tissues) under abiotic stress is vital for the plant tolerance to these constraints and for the influence of microbial community members and structure. Despite the fact that plant growth is improved under the abiotic stresses due to the presence of endophytic bacterial colonies (Rolli et al. 2014), little has been investigated about the role of endophytic bacteria in the date palm's tolerance attainment toward drought and salinity. In that context, exploration in desert plants that are naturally adapted to extreme conditions constitutes a challenge in terms of discovering biodiversity that may be exploited to better understand the ecological niches of the desert ecosystem (Köberl et al. 2011; Marasco et al. 2012). *Phoenix dactylifera* thrives in oasis ecosystems, where microbial communities help plants tolerate environmental constraints (Kumar et al. 2011). The use of next-generation sequencing (NGS) to assess the endophytic microbial community of date palm would broaden the description of the rhizosphere biodiversity and the establishment of symbiotic relationships between the date palms and the endophytes, with mutually beneficial effects.

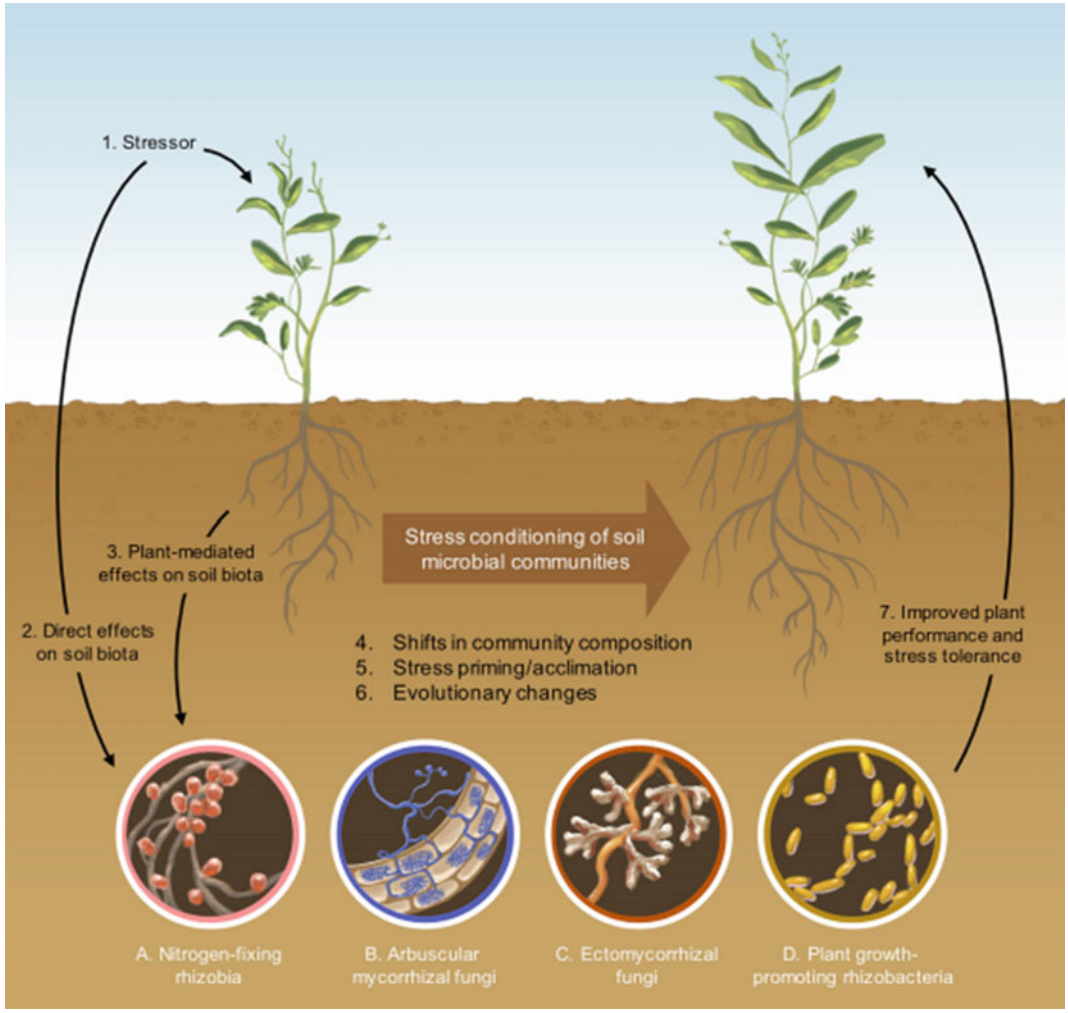

Fig. 10.1 Overview of abiotic stressors influencing soil microbial communities directly as well as through plant-mediated effects such as changes in root exudation. Stress conditioning of soil microbial communities may occur through several mechanisms, including shifts in community composition, acclimation responses or *stress priming* of microorganisms and/or evolutionary changes over time. In some instances, stress-conditioned soil microbial communities will confer increased stress tolerance to plants, thereby improving plant performance. *Source* Valliere et al. (2020), Creative Common License https://creativecommons.org/licenses/by/4.0/

This chapter reviews the cutting-edge studies on date palm tolerance to abiotic stresses, such as salinity and drought, the role of the microbiome of soil and root systems toward such stresses and reveals the current findings of omics-type research. In addition, it presents an update of the present understanding of metagenomics analysis on how the date palm root system may shape its microbial community members and structure in the face of abiotic stress.

10.2 Plant Drought Stress and the Impact on Microbial Communities

Date palm root systems select and enrich the microbiome present in the rhizosphere, similar to the previously demonstrated composition for other plants (Coleman-Dett et al. 2016; Mosqueira et al. 2019). Under drought stress,

members of the phyla Actinobacteria are enriched across a wide range of plant species (Garcia et al. 2018; Timm et al. 2018; Xu et al. 2018). Xu et al. (2018) reported that during drought stress, increased carbohydrates in the roots were correlated with transporters of carbohydrates in Actinobacteria, suggesting alteration in root metabolites, which may play a role in the selection of bacterial species. Bacterial communities selected by the root system of date palms grown in the Sahara Desert in Tunisia, regardless of edaphic conditions and geographical location, were dominated mainly by classes Gammaproteobacteria and Alphaproteobacteria, phylum Proteobacteria (Mosqueira et al. 2019). Moreover, from an ecological perspective of the same oasis, it was shown that date palm roots select endophytic communities that are able to promote plant growth under drought conditions. Drought stress association between the host and its phytobiome revealed the presence of 120 bacterial strains isolated from date palm root tissue that were predominately allocated to five phyla. A total of 76% phyla Proteobacteria that included 70% of classes Gammaproteobacteria and 6% of Betaproteobacteria, while the other phyla were 17% of Actinobacteria, 6% of Firmicutes and 1% of Bacteroidetes (Cherif et al. 2015). In *Phoenix dactylifera, Pseudomonas* is the foremost isolated genus of endophytic bacteria that is notable for its effect of growth promotion (Ali et al. 2014; Roca et al. 2012). *Pseudomonas* spp., isolated from date palm were able to intensify solubilization of phosphate; aided nitrogen fixation, synthesized siderophores; produced plant hormones, such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase and exopolysaccharide; and developed at low moisture potential with 20% of polyethylene glycol (Cherif et al. 2015). This potential benefit from this mutualistic symbiosis between bacteria of the genus *Pseudomonas* and date palm under harsh environment was tested under controlled drought stress conditions during 9 months in the greenhouse by withholding irrigation for periods of 6–12 days. In this study, the date palm root colonization with *Pseudomonas* spp., strains E102 and E141 significantly increased plant growth and root biomass, compared to the noninoculated plants (Cherif et al. 2015). Interestingly, several of the drought responsive microbial metabolites defined so far act as precursors of immune phytohormones (such as phenylalanine). Abscisic acid (ABA) is a hormone that plays an essential role in drought tolerance in crops. It can alter the host microbiome in different plant species (Garcia et al. 2018; Kalladan et al. 2017). It is actively metabolized by rhizosphere bacteria and may be involved in assisting plants in shaping their rhizosphere microbial communities (De Vries et al. 2020).

During drought stress, the increase in reactive oxygen species (ROS*)* accumulation in *Populus* leaves was associated with phylum Actinobacteria and was shown to modulate the host microbiome (Garcia et al. 2018; Nath et al. 2017). Moreover, ROS was beneficial in the symbiosis between *Rhizobium* and barrelclover, *Medicago truncatula* Gaertn. (Andrio et al. 2013).

In response to drought stress, plant root exudates and metabolite production are altered (Abraham et al. 2018; Timm et al. 2018). Depending on the severity of the drought stress, there was an increase in the microbial hydrolytic enzymes responsible for hydrolyses of lignin, cellulose, pectin and other plant metabolites (Bouskill et al. 2016). In addition, plant hormone ethylene production can be altered by the bacterial ACC deaminase (Arshad et al. 2008), for the benefit of plants and microbes (Zhang et al. 2018).

Since their evolution, microorganisms have been producing and removing greenhouse gases (carbon dioxide, methane, nitrous oxide) from the atmosphere. Plant organic matter decomposition through soil microorganisms, which is significantly sensitive to climate trends (Crowther et al. 2015), releases roughly 55 billion mt of carbon dioxide per year (Zimmer 2010). Global warming drives many microorganisms to consume large amounts of methane. This will contribute to control methane levels and in turn regulates climate change. Microbes can play a significant role in stabilizing carbon flux. Global warming can alter the diversity of soil microbial communities by favoring species that are

thermotolerant and have accelerated growth rates (Castro et al. 2010; Shade et al. 2012). In the arid topsoils of the western USA, it was reported that climate change shapes the phylum Cyanobacteria *Microcoleus streenstrupii*, which is critical for maintaining the microbial population of the topsoil (Classen et al. 2015).

The beneficial role provided by the root microbiome also include plant growth promoting (PGP) functional traits linked to drought tolerance (Fig. 10.2). The major examined group of PGP fungus is the arbuscular mycorrhizal fungi (AMF) that belong to the division Glomeromycota and develops a mutual symbiotic interconnection with plants by colonizing the endosphere part of the roots. In date palm, the limited development of the root system along with the high levels of colonization with AMF, suggests a potential benefit from this mutualistic symbiosis under harsh environment conditions (Meddich et al. 2018). Plant growth promoting rhizobacteria (PGPR) isolated from halophytes and wild type species revealed multiple in vitro PGP-associated traits. Whole genome sequence analysis of a few selected strains for their PGP potential showed that numerous genes present in clusters are involved in plant growth promotion and stress regulation (Leontidou et al. 2020).

A plant host can shape the association of bacterial communities to adapt to drought stress by producing exopolysaccharides, accumulation of osmolytes to preserve cell from dehydration, enzymatic and nonenzymatic scavenging pathways or detoxification systems to counter the deleterious effects of oxidative stress, and hormonal regulation for root development and

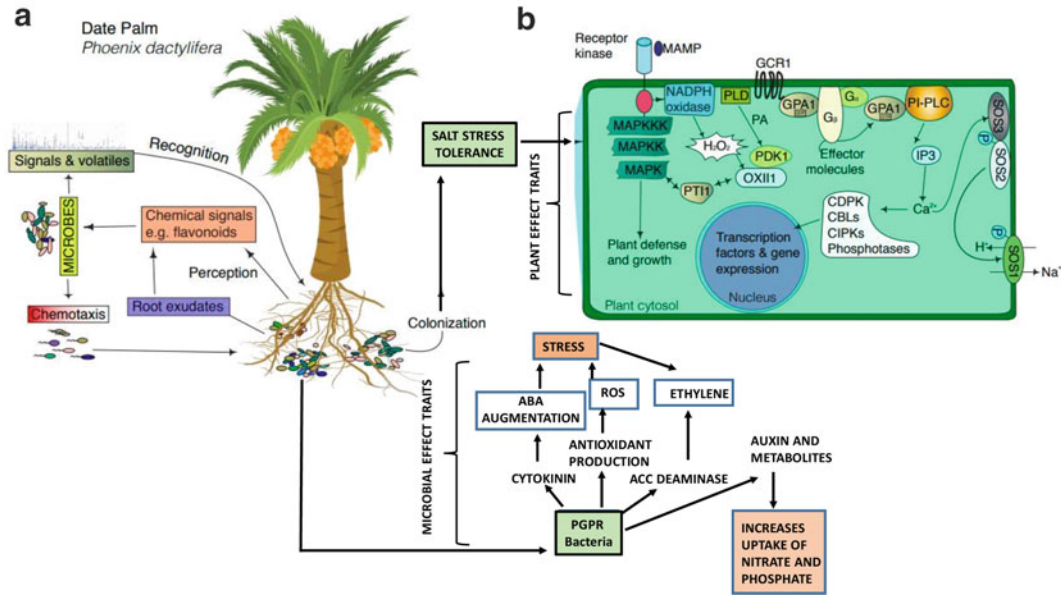

Fig. 10.2 Beneficial microbe communities and date palm mechanisms controlling associations and signaling pathways under abiotic stress: Relationships among microbial and plant effect traits. **a** Endophytic microbes colonizing roots, stimulating the production of phytohormones, antioxidant, improvement of nutrients uptake, and generating abiotic stress tolerance, **b** Microbe-associated molecular patterns (MAMPs) recognized by receptor kinase at the plasma membrane, activates MAPKs and generate H_2O_2. Stimulated phospholipase D (PLD) synthesizes phosphatidic acid (PA), which activates protein kinase (PDK1) and subsequently OXI1 and MAPKs. G-protein coupled receptor (GCR1) stimulates PI-PLC activity through activation of GPA1, which in turn produces IP3 that mediates through increase in Ca^{2+} level, the activation of CDPK, CBLs, CIPKs and SOS pathways through phosphorylation. (Figure constructed by David Nelson, Center for Genomics and Systems Biology, New York University Abu Dhabi, UAE)

hormonal signal transduction pathways (Ahmad et al. 2008; Balloi et al. 2010; Bérard et al. 2012; De Zélicourt et al. 2013; Lau and Lennon 2011).

10.3 Plant Salinity Stress and the Impact on Microbial Communities

Plant root-associated microorganisms in the rhizosphere of saline soil are largely influenced by the composition of the soil microbial species that can survive under osmotic and ionic stress. Salt-tolerant microbes such as plant growth promoting rhizobacteria have been isolated from different agroecosystem soil types like extreme alkaline, saline and sodic soils (Egamberdieva et al. 2019). Plants were found to be associated and interacting with a specific group of microbes, creating a collection of closely-associated individuals often referred to as a *holobiont* (Kumar and Dubey 2020).

Soil salinity is a worldwide problem, expanding at an alarming rate and creating food insecurity in many countries. Salinity induces detrimental changes to the anatomy, physiology and growth of plants. It prevents the uptake of water and boosts toxic ion concentrations (such as Na^+), impairs the integrity of membranes, causes a build-up of reactive oxygen species (ROS) and results in disparity of plant nutrient absorption. These phenomena have detrimental effect on the physiology of plants such as decreased photosynthesis, hindered signaling and diminishing cellular metabolism (Munns and Tester 2008). This in turn collectively affects the plant growth rate, hastens senescence and results in a decline of plants food production (Fricke et al. 2006; Sahi et al. 2006). Plants employ diversified and composite pathways of recognizing, signaling and responding to avoid such impacts, which determines the plants ability to tolerate salinity stress (Hanin et al. 2016). The interaction among these pathways allows plants to initiate salt tolerance by producing compatible solutes, ion sequestration into vacuoles and neutralization of ROS. For instance, plants counterbalance ROS through the production of metabolites that act as antioxidants (ascorbate), or through the expression of ROS-detoxifying enzymes (catalase) Huang et al. (2019). Plants deploy alternative techniques for tolerance toward salinity, by blocking the entrance of salt ions into the roots through immediate exclusion from entering or by preventing the transport of Na^+ inside the plant system. Such phenomena require adjustment and changes to the anatomy of the root structure by developing casparian bands, restricting xylem Na^+ pumping and translocating, changing membrane permeability to remove toxic ions and active discharge of ions within cells by means of ion pumps (Hanin et al. 2016; Munns and Tester 2008; Zhu 2003).

Increasing salt concentration has a negative impact on soil processes such as aeration, residue decomposition, soil biodiversity and microbial activities (Schirawski and Perlin 2018). Different studies have highlighted the potential of plant growth promoters and the role of ACC deaminase in acquiring salinity resistance by the host plant (Fouda et al. 2019; Hussain et al. 2018; Qin et al. 2014). It was demonstrated that halotolerant microbes improved root growth of canola under salt stress by decreasing ethylene production (Siddikee et al. 2010). Moreover, the root endophyte fungus *Piriformospora indica* conveys its beneficial effects on a variety of plants of economic importance, such as increasing barley antioxidants under salt stress (Baltruschat et al. 2008).

Date palm is known to be a salt-tolerant plant with an adaptation capacity that exceeds barley, which is considered the most salt-tolerant crop within the glycophytes. Screening of date palm varieties for their salinity tolerance, carried out by Ramoliya and Pandey (2003), showed that certain varieties could tolerate soil salinity levels up to 12.8 dSm^{-1} without effect on the seedling phenotype. Additionally, date palm cell suspension cultures were used to investigate salinity tolerance at the cell level. Al-Bahrany and Al-Khayri (2012) reported that cell growth was affected by salt treatment and Na^+ concentration increased in the cytosol. This study of isolated cells, tissue and callus did not reflect exactly

what happens at the whole plant level regarding the mechanism of adaptation to salt stress.

In arid and semiarid regions, salt-tolerant plant beneficial microbes have shown improved productivity in many crops under saline conditions (Niu et al. 2018). The plant growth promoting rhizobacteria (PGPR) mechanisms for salt stress tolerance involves the synthesis of a range of different plant hormones, secondary metabolites and osmolytes (Fig. 10.2). Some essential hormones included are cytokinins, gibberellins (GAs) (Dodd et al. 2010) and auxins and ACC deaminase synthesis (Glick et al. 2007). Secondary plant metabolites released include EPS, exopolysaccharides (Timmusk et al. 2014; Upadhyay et al. 2012). Some osmolytes that accumulates under salt stress include trehalose, glycine betaine and proline (Bano and Fatima 2009; Upadhyay and Singh 2015).

10.4 Metagenomics Analysis of Date Palm Microbiome Communities Under Abiotic Stress

Date palms can thrive under a harsh environment where some varieties can tolerate different levels of soil salinity. To tolerate abiotic stress, date palm roots have developed interactions with soil microbes and diverse microbial host communities that are essential for survival. Deciphering the relatedness of the association between date palm roots and the microbial communities, using genome-based and high-throughput sequencing techniques, is a crucial topic in microbiome study. Indeed, the studies have modified their targets in the last few years from the recognition of specific microbial strains that promote plant growth to metagenomics research that deals with richness, diversity and interactions of the root microbiome. The outcomes from investigation of high-throughput sequencing have disclosed that the area surrounding the rhizosphere acts as an ecological hotspot around the roots and possesses an immense group of diversified microorganisms (Bulgarelli et al. 2013; Busby et al. 2017; Khare et al. 2018; Yu et al. 2018). A recent study using pyrosequencing to identify endophytic bacterial and fungal communities found that date palms grown under salt stress demonstrated significant changes in the constitution of microbial communities in relation to salinity level (Yaish et al. 2016). Using quantitative PCR, the rhizosphere was found to host the highest number of bacterial cells, compared to the root and bulk soil samples. The selective acquisition processes applied by date palm root system fractions may explain the quantitative differences observed in the bacterial communities (Mosqueira et al. 2019). The prediction of bacterial functional profiles, assessed in multiple date palm cultivation sites, showed that the plant growth promoting traits by the date palm bacterial microbiomes present in the root and the rhizosphere fractions were not significantly affected by the oasis location (Mosqueira et al. 2019). The core bacterial microbiome of the date palm root-system fractions was highly conserved and shared a considerable number of operational taxonomic units (OTUs). In analyzing the core microbiome in date palm, Mosqueira et al. (2019) found that 12% of root OTU and 27% of rhizosphere OTU were always present in date palm roots and rhizosphere, respectively. The taxonomic composition of the core microbiome in the root and the rhizosphere was dominated by class Gammaproteobacteria (94% and 71%, respectively), with family Pseudomonadaceae accounting for 48% and 43%, respectively.

Different techniques were employed to sort out the bacterial communities present in the root, the rhizosphere and the bulk soil. The bacterial 16S rRNA gene copy absolute abundance is assessed by using a taxon-specific set of primers Eub338/Eub518 (Fierer et al. 2005). High-throughput sequencing of the hypervariable regions of the 16S rRNA gene by Illumina tag sequencing and metaphylogenomic analysis revealed a total of 2,721,958 high quality sequence reads clustered as OTUs (Mosqueira et al. 2019). From the most abundant endophytic bacteria among the annotated reads, two described PGP *Pseudomonas* strains, E102 and E141, were isolated from date palm root tissue (Cherif et al. 2015). To gain in-depth knowledge and

predict genes involved in potential interactions between the bacterial microbiome and date palm root system, the genome of the isolated *Microbacterium* sp. strain Yaish1, from the rhizosphere of date palms cultivated under high soil salinity, was fully sequenced using Illumina Hiseq 2500. The assembled genome resulted in the identification of 3226 genes, including genes encoding siderophore production, ACC deaminase and a tryptophan biosynthesis that is important for the production of IAA (Jana et al. 2017). The identification of these genes from the genome of this *Microbacterium* strain is essential to perform growth biopromoting (IAA production) and protection from drought and salinity stress (ACC deaminase production).

10.5 Conclusions and Prospects

Plant microbiome-manipulating strategies have emerged in recent years to improve plant growth promoting mechanisms and to solve multiple agriculture challenges. The plant microbiome possesses an array of active microbial life forms that modify physiological aspects of plants, their development and instigates a tolerance network in response to pathogens and abiotic stresses such as drought and salinity. The potential interactions of the core bacterial microbiome with the root system will establish and maintain the network structure and the ecosystem stability. In the date palm root and rhizosphere, the most network hubs were associated with class Alphaproteobacteria, but class Gammaproteobacteria are influenced by interactions with other taxa. Indeed, plant-associated endophytic communities change their structure, producing new, better-adapted assemblies that improve plant resistance to stress. In order to reproduce the bacterial community richness and composition, various techniques are employed for strain inoculation, introduction and engineering of the plant microbiome. This can take place with the aid of host-mediated and cross- or multi-generation microbiome choosing, inoculating strains into huge masses of soil, rhizosphere, introduction into plant seeds and seedlings by injection and fragmentation into different plant parts, tissue, organelles and wounds. Bioengineering of the plant microbiome, while in its infancy, remains an interesting option to improve the biological capabilities of plants. In fact, understanding microbial community interactions can enhance desired outcomes in dealing with complex environment constraints. The engineering of this community has had many successes, but still faces several challenges both scientifically and socially.

Exploration of the biodiversity of endophytic microbial communities associated with the desert plant rhizosphere constitutes an important research objective for marginal environments. Increasing the understanding of how established interactions between plants and endophytes are affected over time, especially under environmental constraints, will open many new research avenues to enhance plant resilience to abiotic stress. Promoting beneficial plant-microbe interactions through adopting new technologies of gene editing offers huge possibilities to increase the resistance of crop production in marginal environments. Clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) has emerged as a new genome-editing tool that enables a precise, rapid and efficient method of plant transformation. This technology was used extensively to knockout, activate or repress the expression of target genes in plants. The system contains two major components: Cas9 protein and guide RNA (gRNA). Cas9 protein is an RNA-dependent DNA endonuclease that forms a complex with gRNA. The gRNA is a small RNA sequence of 20 nucleotides complementary to target genomic DNA sequences and is required to recruit Cas9 protein to the target site. The CRISPR-Cas9 system has the advantage of editing multiple target genes simultaneously (Najera et al. 2019). In fact, de novo domestication of wild tomatoes and the generation of new varieties with desirable traits was achieved by targeting 6 genes important for domestication traits using CRISPR-Cas9 technology (Najera et al. 2019). Another application of gene editing in plants is the introduction of mutations in the wheat acetolactate synthase (ALS) Pro-174

codon, which confers microsulfuron herbicide resistance in edited wheat genome, without exogenous selectable markers (Zhang et al. 2019). In date palm, the high heterozygosity observed in many cultivars can make the design of the sgRNA challenging, as it must perfectly match a target region near a protospacer-adjacent motif (PAM) site (Sattar et al. 2017).

Root endophytes constitute a powerful tool to maintain crop production under adverse conditions of climate change and global warming. The applied research components will determine whether the permanent colonizing of plant roots with endophytes will render them constitutively tolerant to abiotic stress. Meantime, more basic research should concentrate on deciphering and gaining insight into molecular mechanisms underlying plant priming and abiotic stress tolerance.

Acknowledgements Authors are grateful to David Nelson, Center for Genomics and Systems Biology (CGSB), New York University Abu Dhabi, UAE, for the creation of illustration of Fig. 10.2 for this chapter. This work was supported by funding from UAEU, Center-based research grant number 31R203, granted to Khaled Masmoudi.

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Genomics Approaches for Insect Control and Insecticide Resistance Development in Date Palm

11

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Abstract

Insects are important organisms that threaten global food security due to their serious damage to food and feed crops. Despite the impact on the environment and human health, the application of enormous quantities of insecticides to control insect pests is a general practice. Insects tend to develop resistance not only to synthetic insecticides but also to Bt crops that have been recently introduced into the field. Insects developed resistance to Bt toxins via diversified mechanisms including suppression of toxin activation within the midgut, toxin receptor mutations and modifications in the innate immune system. In this chapter, emphasis is focused on the alternative biotechnological approaches for insect control, the molecular basis underpinning the development of insecticide resistance in insects and the most recent genomic and genetic tools that revolutionized understanding the mechanisms of insecticide resistance. Moreover, the chapter describes the intrinsic mechanisms of plant resistance to insect attack, with especial reference to the date palm, in addition to the date palm major insect pests and how the tree defends itself against these pests. This chapter also addresses the prospects of utilizing the promising genomic/genetic-based technologies to manage the date palm insect pests and to forecast the possibility of resistance development by these pests.

11.1 Introduction

Insect pests pose a great impact on agricultural and forest productivity and contribute substantially to the global increasing cost of production in the agricultural sector (Pureswaran et al. 2018). Their negative impacts are likely to increase as the need for food, livestock feed and renewable bioenergy products continue to expand (Lehmann et al. 2020). Assessment of the impact of global climate change on 31 species of insect pests reveals a trend of intensifying the negative impact on agricultural productivity to nearly 50% as the ongoing global warming persists in a similar pattern (Lehmann et al. 2020), although these responses might not be generalized to all insect pests. The annual cost to control only a single pest, the diamondback moth

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J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4_11

Plutella xylostella (Linnaeus), was approximated to be USD 4–5 billion globally (Zalucki et al. 2012). As for the date palm, *Phoenix dactylifera* L., the annual loss due to insect attack has been estimated at USD 25.92 million in 2009 in the Gulf region alone (El-Sabea et al. 2009). Recurrent frequencies of insect pest outbreaks have increased over recent decades especially of the invasive species. These outbreaks necessitate the application of frequent and effective control actions to alleviate the influences of biological invasion and to reduce the cost of damage incurred by insect pests feeding.

Some date palm cultivars are resistant to several insect pests, but most humans prefer cultivars that are susceptible to insect damage (Faleiro et al. 2014). To control major date palm pests, the typical exercise in the fields is the injection of insecticides into the tree trunks. This practice stimulated the red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier) to develop resistance against regularly applied insecticides, in particular cypermethrin and ethion (Al-Ayedh et al. 2016a).

Management measures other than the use of conventional insecticides have been applied to combat many agricultural pests. These measures include novel technologies that have relied on genomics, genetics, and molecular control tactics. Understanding the mechanisms of action of these technologies may help to control the insect pests, manage insect resistance development, and restore the environment. The main objective of this chapter is to compile information on the biotechnological techniques and promote their adoption in date palm cultivation. Therefore, the chapter focuses on the alternative control measures based on the latest biotechnological methods and highlights the processes of mitigation of insecticide resistance acquired by insect pests.

The latest biotechnologies addressed in this chapter include genomics- and genetics-based techniques for insect control including Bt-biotechnology, RNA interference (RNAi), parental RNAi, CRISPR/Cas technology and genetic elements that drive the revolution of these technologies. Additionally, different aspects of sterile insect techniques (SITs) and modern genetic SIT-like systems are discussed. It also covers the molecular basis of how insects develop resistance to insecticides via the metabolic or target-site resistance and the functional genomic tools that help to discover these mechanisms. The chapter also includes plants' intrinsic resistance mechanisms to insect attacks, the major date palm pests, defense and resistance of date palm against insect pests and potential application of these genomic/genetic technologies in insect control and insecticide resistance management in the date palm agroecosystems.

11.2 Genomics and Genetics Biotechnological Approaches for Insect Pests Control

11.2.1 Bt-Biotechnology

Bacillus thuringiensis (Bt) is a gram-positive soil bacterium that produces a proteinaceous parasporal crystalline inclusion during sporulation (Schnepf et al. 1998). It produces two types of Bt toxins namely Cry and Cyt. They are classified further by a systematic nomenclature that describes the Cry group into Cry1 to Cry55 and the Cyt group into Cyt1 to Cyt2 (Stevens et al. 2012). Upon ingestion, these crystal proteins dissolve in the midgut of the insect's alimentary tract, which is characterized by its alkaline nature, discharging one or more insecticidal crystalline proteins (ICPs). ICPs are also known as delta-endotoxins (Bravo et al. 2007). Once activated by the larval midgut proteases, the ICPs interact with the midgut epithelial cells and destroy the integrity of the membrane, leading ultimately to insect death (Bravo et al. 2011; Gill et al. 1992; Xiao and Wu 2019). Bt toxins mainly affect the larvae of certain insect orders including Lepidoptera (butterflies, moths), Diptera and Coleoptera (larval and adult beetles). So far, no report on the toxicity of Bt toxins to people, wildlife, or most beneficial insects and therefore, these toxins have overwhelming opportunities for biological control of insect pests (Stevens et al. 2012). Genes encoding Bt toxins are engineered to develop molecular technologies for

the production of transgenic plants, which gain resistance to the larvae of insect pests belonging to the order Lepidoptera (Duke 2011). Transgenic Bt cotton was released for commercial production in the USA for the first time in 1996 and subsequently has been grown in more than 26 countries around the globe (ISAAA 2018). The rapid adoption of Bt and other biotech crop varieties, such as maize, cotton, soybean, canola, potatoes, apples, and others by up to 17 million farmers, reflects the benefits of these crops in terms of reduced insecticide use, lower production costs, and higher yields (ISAAA 2018). However, in the case of resistance to Bt crops, if it is spread across the pest populations, then the cost-effective advantages of these crops would be lost. The principal approach for resistance management is the use of a high-dose/refuge plan that requires the growth of wild-type crops as refuges nearby the Bt crops, and the toxin doses are adequately high to toxify wild-type insects and those with heterozygous resistance alleles (Alphey and Bonsall 2018).

11.2.1.1 Mechanisms of Targeted Insect Resistance to Bt Crops

Insect pests have developed resistance to Bt crops by different mechanisms as evidenced by laboratory and field data. These mechanisms include differences in toxin activation within the midgut, mutations in the toxin receptors and alternations in the immune system. Activation of Bt toxin and binding of the midgut membrane receptors in Bt-resistant insects, trigger activation of the mitogen-activated protein kinase (MAPK) signaling pathway, which in turn regulates the expression of Bt-receptor genes via various transcription factors. The MAPK pathway and other regulatory factors likely enrich the resistance to Bt toxins via repairing the damage on the cell membrane and restoring the changes in the immune system (Xiao and Wu 2019).

Some studies have demonstrated cases of differences in toxin activation as an essential mechanism in the events of Bt resistance; however, the share of Bt resistance cases caused by changes in protease is not very high (Vellichirammal et al. 2015; Wei et al. 2016). In *Plodia interpunctella* (Hübner) Bt-resistant strain, the Bt protoxin was not activated due to the absence of trypsin in the midgut, which caused the resistance (Oppert et al. 1997). In Bt-resistant *Heliothis virescens* (Fabricius) strain, changes in the composition of midgut protease were associated with a significant reduction in protoxin activation (Forcada et al. 1996). Comparative analysis of the Bt resistance in the European corn borer *Ostrinia nubilalis* (Hübner) revealed that serine proteinases are more active in the susceptible strains than in the resistant counterparts (Li et al. 2004). Moreover, mutations in the promotor of *Helicoverpa armigera* (Hübner) trypsin gene (*HaTryR*) conferred high resistance to Cry1Ac in this pest (Liu et al. 2014).

Mutations and gene expression regulations of receptors of Bt toxins in lepidopteran larvae are essential for insects to develop resistance to Bt crops. Among these receptors are the midgut membrane-bound cadherin, the ATP-binding cassette (ABC) transporters, aminopeptidase N (APN), and alkaline phosphatase (ALP).

The high resistance to Cry1Ac toxin that developed in the cotton pest *Heliothis virescens* was associated with a mutation in cadherin (Gahan et al. 2001). Field populations of Bt-resistant pink bollworm *Pectinophora gossypiella* (Saunders) were found to harbor three mutant alleles of the cadherin-encoding gene that related to binding of Cry1Ac (Morin et al. 2003). A mutation in cadherin transmembrane affects cellular trafficking that leads to mislocalization of the receptor on the surface of the midgut epithelium. This trafficking distortion probably leads to the resistance of pink bollworm *P. gossypiella* to Cry1Ac (Wang L et al. 2018b) and high resistance of cotton bollworm *Helicoverpa armigera* to Cry1Ac toxins (Xiao et al. 2017). The interaction of two cadherins, CAD1 and CAD2, with Bt toxins in the rice pest *Chilo suppressalis* (Walker) was shown to reduce the expression of CAD1 or CAD2, thereby increase the resistance to Cry2A and Cry1C (Zhang et al.

2017a). This interaction was suggested as the main mechanism underlying Bt resistance in this pest.

In some insects, the ABCC subfamily transporters have been shown to modulate the mode of action of Bt toxins and play important roles in insect resistance to Bt toxins (Chen et al. 2018; Heckel 2012; Pardo-López et al. 2012; Xiao et al. 2014). The alternatively spliced variant of the ABCC2 gene was linked to the development of resistance to Bt toxin in *Helicoverpa armigera* (Xiao et al. 2014) and the ABCC1 protein specifically binds to the Bt Cry2Ab to serve as a receptor in the same insect (Chen et al. 2018) and hence promotes the toxicity. Nakaishi et al. (2018) reported that a mutation in the ABCC transporter of *Plutella xylostella* (Linnaeus) (*PxABCC2*) causes resistance to the Bt toxin Cry1Ac because the mutant PxABCC2 receptor lost its function to bind Cry1Ac. Furthermore, a mutation in ABCC2 was involved in *Heliothis virescens* resistance to Cry1Ac (Gahan et al. 2010).

The function of alkaline phosphatase (ALP) as a receptor for Cry toxins was also documented. Downregulation of the ALP gene in Cry1A-, Cry2A-, and Cry1C-transgenic rice lines make *Chilo suppressalis* resistant to the three Bt toxins (Qiu et al. 2018). The MAPK signaling pathway alters the expression of ALP genes, causing Cry1Ac resistance in *Plutella xylostella* (Guo et al. 2015).

Although the role of aminopeptidase N (APN) as a receptor is not very clear, it is proposed to contribute as a receptor in the midgut membrane of insects for Bt toxins. A deletion mutation in the *HaAPN1* gene was associated with resistance of *Helicoverpa armigera* to Cry1Ac (Zhang et al. 2009) indicating that HaAPN1 was a receptor of Cry1Ac. Knockdown of *Spodoptera exigua* (Hübner) APN1, APN3, and APN6 genes suggests that they function as receptors of Cry1Ca in this insect (Ren et al. 2014).

Sodium solute symporter (SSS) was also found to interact with Bt toxin on the cell membrane. The knockdown of the red flour beetle, *Tribolium castaneum* (Herbst), TcSSS gene enhances the resistance of the beetle to Bt Cry3Ba (Contreras et al. 2013). Moreover, Chen et al. (2017) described that the two glucosinolate sulfatase enzymes, GSS1 and GSS2, attach directly to Cry1Bd in *Plutella xylostella* and act a vital function in the toxicity of the protein Cry1Bd.

Nutrition composition is an environmental factor that may affect insect susceptibility to Bt toxins in the field. Deans et al. (2017) reported that the susceptible laboratory strain of *Helicoverpa zea* (Boddie) larva self-selects and functions best on protein-rich diets than on the comparative carbohydrate diets. While in practice, most Bt evaluation bioassays use carbohydrate-biased nurturing diets. The data on *H. zea* suggest that Bt resistance bioassay tests that use ecologically and physiologically incompatible diets overestimate susceptibility and underestimate resistance.

11.2.2 RNA Interference (RNAi)

RNAi technology application to control insect pests (Fig. 11.1a) has been intensively investigated in the western corn rootworm (WCR) *Diabrotica virgifera virgifera* (LeConte) (Baum et al. 2007; Camargo et al. 2018; Rangasamy and Siegfried 2012; Vogel et al. 2019; Wu et al. 2017, 2018). The WCR responds very sensitively to oral administration of double-stranded RNA (dsRNA), and numerous genes with lethal or detrimental effects have been identified using RNAi in larvae of this pest (Baum et al. 2007). Finally, in 2017 the United States Environmental Protection Agency (EPA) has approved the RNAi-based plant-incorporated protectant (PIP) for the control of the WCR. This PIP technology is termed *SmartStax Pro* (https://www.epa.gov/newsreleases/eparegisters-innovative-tool-control-corn-rootworm). SmartStax Pro technology was designed to employ a pyramid strategy, i.e., expression of several different Bt-proteins in a corn plant, as well as dsRNA targeting suppression of the WCR *Snf7* gene (Head et al. 2017) that plays an essential role in protein trafficking. While the Bt-proteins causing gut paralysis and resulting in the death of the target insect, the downregulation of *Snf7* will also

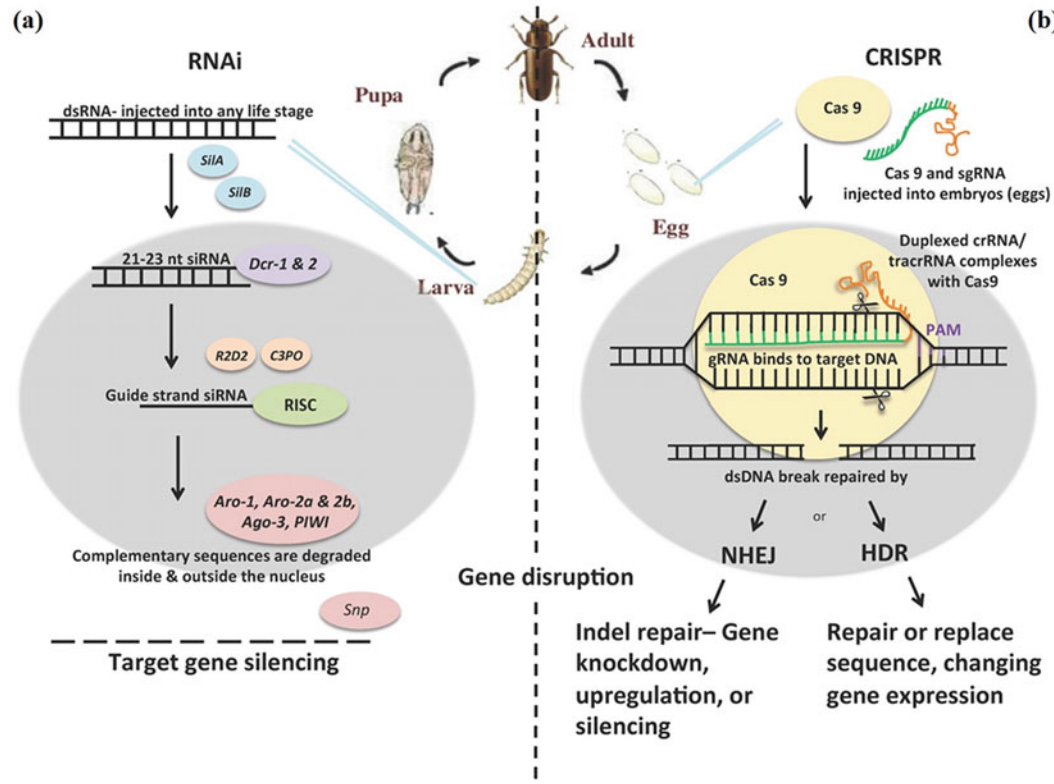

Fig. 11.1 RNA interference (RNAi) (**a**) and CRISPR-Cas (**b**) approaches in *Tribolium castaneum*. The gray oval represents the cell nucleus. On the RNAi side (**a**), the double-stranded RNA (dsRNA) can be inserted into any stage of the beetle life and is incorporated into the cell with *SilA* and *SilB* (SID-1 orthologs). Dicer Endonucleases *Dcr-1* and *2* cleave the dsRNA into 21 nucleotide pieces, *R2D2* and *C3PO* help load the RNAs into the silencing complex. *Aro-1*, *-2a*, *-2b*, *-3* and *PIWI* endonucleases degrade the complementary RNA inside the nucleus while *Snip* (SNP) exonuclease degrades the complementary RNA outside of the nucleus. On the CRISPR side (**b**), Cas9 and single-guide RNA (sgRNA) is injected into eggs. Duplexed crRNA/tracrRNA complexes with Cas9 endonuclease, resulting in a precise dsDNA break. The break is repaired by either non-homologous end-joining (NHEJ) or homology-directed repair (HDR), which results in gene knockdown, upregulation, silencing, or changes in gene expression. *Source* This figure is reproduced with permission from the original Fig. 1 in Perkin et al. (2016), which is licensed under a Creative Commons Attribution 4.0 International License. The link for the Creative Commons License: http://creativecommons.org/licenses/by/4.0/

result in mortality (Bolognesi et al. 2012). This combined strategy was intended to lead to the rapid death of the target insects and to reduce their chances for resistance development against the PIP (Head et al. 2017). RNAi is a greatly advancing technology in agriculture; its most likely new insecticidal products based on RNAi technologies will soon appear on the market. However, suitable regulatory and assessment measures need to be developed to overcome the risk of topical RNAi application in plant protection protocols (Mezzetti et al. 2020). Using a dsRNA design for similar vacuolar-type H^+-ATPase (*v-ATPase*) enzyme designated earlier by Baum et al. (2007), Rangasamy and Siegfried (2012) verified that oral ingestion of dsRNA by adult WCR also induce reduced gene expression and protein synthesis. Besides, the mortality in the treated beetles could be accomplished within two weeks. Adults of WCR behavior of easy manipulation combined with their induction to feed spontaneously on an artificial diet with a natural feeding stimulant may

provide a more effective developmental stage to screen for the activity of dsRNAs (Khajuria et al. 2015; Rangasamy and Siegfried 2012). However, other insect pests, especially the trunk borers of the date palm such as the RPW, *Rhynchophorus ferrugineus* and the longhorn beetle, *Jebusaea hammerschmidti* (Reiche), the larval stage may be the most suitable to be managed. If both adults and larvae can be targeted using dsRNAs, the possibility of gaining increased protection is much more than using technologies that target only larvae. Targeting both stages will most likely result in less egg-laying and consequently less larval damage in the subsequent growing season.

11.2.3 Parental RNAi

Unlike conventional RNAi, in parental RNAi (pRNAi) or transgenerational RNAi, the gene interference takes place in the offspring. pRNAi was identified for the first time by the injection of dsRNA into the body cavity or application of dsRNA via ingestion causing gene inactivity in offspring embryos of the nematode, *Caenorhabditis elegans* (Maupas) (Fire et al. 1998; Timmons and Fire 1998). pRNAi was then developed as one of the applications of RNAi for the control of insects (Bucher et al. 2002; Fishilevich et al. 2016; Khajuria et al. 2015). The process was also reported in the red flour beetle whereby injection of female pupae with the dsRNA that corresponds to three distinctive genes that control segmentation during embryogenesis caused knockdown of zygotic genes in offspring embryos. Almost all offspring larvae showed gene-specific phenotypes after one week of injection (Bucher et al. 2002). The tested genes are the leg gene *Distal-less* (Tc′Dll) (Beermann et al. 2001) and the homeotic gene *maxillopedia* (mxp) (Shippy et al. 2000) and the red flour beetle giant homolog (Tc′gt) (Bucher et al. 2002). Following these early reports, pRNAi has been used to define the function of embryonic genes in several other insect species. These include the *hunchback* gene of the milkweed bug, *Oncopeltus fasciatus* (Dallas) (Liu and Kaufman 2004), *krüppel* gene of the cricket, *Gryllus bimaculatus* (De Geer) (Mito et al. 2006), the *broad-complex* genes of the German cockroach, *Blattella germanica* (Linnaeus) (Piulachs et al. 2010), the *orthodenticle* and *caudal* genes of the silkworm, *Bombyx mori* (Linnaeus) (Nakao 2012) and the *Distal-less* gene of the sawfly, *Athalia rosae* (Linnaeus) (Yoshiyama et al. 2013). In addition to the *hunchback* gene of the pea aphid, *Acyrthosiphon pisum* (Harris) (Mao et al. 2013) and the *hox* genes of the springtail, *Orchesella cincta* (Linnaeus) (Konopova and Akam 2014). The pRNAi response in all these instances was achieved by injection of dsRNA into the hemocoel of the parental female. pRNAi was also tested via oral feeding of gravid WCR females dsRNAs targeting *brahma* (*brm*) and *hunchback (hb)* genes that are essential for embryogenesis (Khajuria et al. 2015). This indicates the systemic nature of RNAi in WCR and that the response can be achieved by oral administration of dsRNA to adult females without adverse effect on total oviposition, but eggs do not hatch (Khajuria et al. 2015). This identifies a new approach to deliver dsRNA in a parental (transgenerational) manner by feeding to adult insects, and the interference phenotype will appear in the offspring.

11.2.4 CRISPR/Cas Technology

CRISPR-associated (Cas) protein is an adaptive immune system found in bacteria and archaea that have been repurposed into new technology for editing the genome of other living organisms (Homem and Davies 2018). Genomic and genetic tools based on the adaptive CRISPR/Cas technologies have been engineered and utilized to knockout and knockin mutations to investigate gene functions in many insects including various flies, moths, butterflies, beetles, bees, and grasshoppers (Sun et al. 2017; Taning et al. 2017). In addition to the easy insertions and deletions, these technologies also enhancing the progress for generating site-specific genomic mutations, homology assisted CRISPR Knockin (HACK) (Lin and Potter 2016), promoting the potentiality to edit DNA bases, generating

predictable reciprocal chromosomal translocations, and facilitating the development of gene drives to control the fate of wild populations (Gantz and Akbari 2018). The CRISPR/Cas systems utilize sequence-specific nucleases to generate double-stranded DNA breaks in specific regions of chromosomes (Fig. 11.1b). The broken chromosomes must be rapidly repaired to guarantee cell survival. However, targeted DNA modifications may occur during repairing the broken ends of the DNA by the endogenous machinery. Usually, two DNA repair pathways are utilized for this goal: (1) non-homologous end joining (NHEJ), which is an error-prone DNA repair mechanism that can lead to small insertions and deletions (indels) of bases at the break site and (2) homology-directed repair (HDR), which uses the information on the intact chromosome to repair the broken one precisely. Researchers often manipulate synthetic DNA constructs in the CRISPR/Cas systems as templates to modify the genetic material of the cell by taking advantage of the HDR repair pathway. The intended modification leads to user-defined insertions or deletions (Gantz and Akbari 2018; Gantz and Bier 2016). A simple version of the CRISPR/Cas system has two constituents: (1) the Cas9 endonuclease, which executes the DNA cleavage and (2) a synthetic guide RNA (gRNA), which directs the endonuclease programmed within its RNA sequence to the target location on the genome (Cong et al. 2013). There are several forms of delivery of these two components into insects. They can be RNA, plasmid DNA, or DNA integrated into the genome to increase efficiency (Taning et al. 2017). If combined within the cell, the two elements accelerate the rates of mutagenesis at the target site leading to efficient functional disruption of the target DNA sequence. On the other hand, if combined with an exogenous DNA source (ssDNA or plasmid dsDNA) that retaining homology to the target sequence of the genome on both sides of the intended cleavage site, the interfering DNA can be inserted efficiently at the reliable cleavage site with high efficacies (Homem and Davies 2018; Xu et al. 2017).

11.2.5 Gene Drives

The term *gene drive* is used to explain the procedure of inspiring the biased inheritance of specific genes to change whole populations. Currently, the gene drive term is utilized to define the exact artificial genetic element designed to increase in occurrence with each generation over time in a certain population (Champer et al. 2016). Some genes naturally drive themselves through populations by increasing the probabilities that they will be inherited. Esvelt et al. (2014) summarized these natural gene drives to include: (1) natural homing endonuclease genes; (2) segregation factors that abolish competing chromosomes during meiosis; (3) transposons that copy and insert themselves at different sites in the genome; (4) the maternal effect dominant embryonic arrest (*Medea*) elements that remove competing siblings who do not inherit them; and (5) *Wolbachia*, which are heritable microbes. Gene drive, sometimes used interchangeably with the term *selfish genetic elements*, enhances their spread benefit relative to the rest of other genes in the genome and are often harmful to the organism (Werren et al. 1988). These inheritance mechanisms skew the population toward the non-Mendelian inheritance that potentially enable spreading desirable traits throughout wild populations or suppress the amenability of populations to develop resistance to insecticides and herbicides (Alphey 2014; Alphey et al. 2013; Burt 2014; Champer et al. 2016; Hurst and Werren 2001; Sinkins and Gould 2006; Werren 2011). Gene drives typically induce biased (non-Mendelian) inheritance patterns via either of two mechanisms: (1) Copying themselves onto the opposite chromosome, resulting in most or all offspring inheriting the gene derive allele. (2) Decreasing the feasibility of gametes that receive the wild-type allele, therefore providing the wild-type allele a drawback in the fitness compared to the gene drive allele (Champer et al. 2016). There are different arrays of gene drives characterized by variable attributes.

11.2.5.1 The Homing-Based Drives or Homing Endonuclease Genes (HEGs)

HEGs are site-specific selfish or parasitic genes that can spread through populations owing to their biased super-Mendelian inheritance. They encode an enzyme that recognizes and cleaves a 20–30 base pair sequence found on chromosomes not containing a copy of the HEG (Burt 2003). During meiosis, HEGs convert their corresponding allele on the opposite chromosome into an exact copy of themselves. Doing this by encoding an endonuclease with a sequence-specific activity that breaks their competing chromosomal allele, pressing the cell to quickly repair this DNA damage (Stoddard 2011). The cell can use either the NHEJ or microhomology-mediated end-joining (MHEJ) DNA repair mechanisms to repair the DNA break. The NHEJ or MHEJ can potentially form HEG-resistant alleles. The cell can utilize the HEG as a template for homology-directed repair (HDR) to repair the damaged DNA ends. This induces the HEG to copy itself (i.e., homing) into its rival allele (Stoddard 2011). In case, the HDR takes place in the early embryo or germline, the ratio of progeny that receives the HEG will be more than half (50%). This allows for rapid invasion of the HEG into a target population, i.e., spreading a genetic payload (Deredec et al. 2011; North et al. 2013; Unckless et al. 2015). HEG can also be used for suppression of populations that recessive lethality or sterility traits upon the disruption of the target gene by the homing process (Burt 2003; Deredec et al. 2008). In the population suppression method, homing must be limited to the germline during gamete formation to confine the sterility or non-viability only in homozygotes that obtain the HEG allele from the two parents. The HEG must be managed not to produce heterozygous males and females, because heterozygote pairings will result in sterile or nonviable offspring. HEGs may also be manipulated to target genes that reduce lifespan and genes that result in biased sex ratios (Kyrou et al. 2018), in order to obstruct host finding, to impede pathogen development, or to obstruct the capability of the altered organism to perform as a vector for pathogens (Champer et al. 2016).

11.2.5.2 RNA-Guided, Transcription Activator-Like Effector Nucleases (TALENs) and Zinc-Finger Nucleases (ZFNs) Gene Drives

The recognition sequences for TALENs and ZFNs are relatively easy to engineer but the encoding genes are extremely big and repetitive. These make the genes unstable and thus less effective for use as gene drives (Champer et al. 2016; Simoni et al. 2014). The RNA-guided gene derives such as Cas9 (Jinek et al. 2012) and *Cpf1* (Zetsche et al. 2015) can be used to overcome the limitations of TALEN and ZFN endonucleases (Esvelt et al. 2014). *Cpf1* is an RNA-guided endonuclease of the CRISPR-Cas system. Gantz and Bier (2015) developed in *Drosophila* the mutagenic chain reaction (MCR) method, which is an RNA-guided homing system based on the CRISPR/Cas9 genome editing with 96% homing efficacy. Gantz et al. (2015) documented the capability of a system for homing modification drive to spread a large payload (~17-kb) comprising an antimalarial single-chain antibody in *Anopheles stephensi* Liston. Nevertheless, the homing element did not function properly in the progeny of females, making the system unstable (Champer et al. 2016). Hammond et al. (2016) created a suppression drive targeting female fertility genes in *A. gambiae* (Giles). The suppression drive was successfully transmitted to *A. gambiae* offspring, but the heterozygous disruption of the target genes greatly reduced female fertility. Thus, the ability of this drive to suppress the population of *A. gambiae* was limited (Champer et al. 2016).

11.2.6 Maternal Effect Dominant Embryonic Arrest (Medea)

Medea is a maternal effect selfish genetic element that self-selects for its survival by stimulating maternal lethality of all offspring lacking the element-bearing chromosome from the maternal and/or paternal genome (Chen et al. 2007; Wade and Beeman 1994). *Medea* elements are anticipated to change the whole population rapidly into element-carrying heterozygotes and homozygotes when introduced into a population at high incidences. *Medea* is first described in the red flour beetle *Tribolium castaneum* and is assumed to consist of a toxin that kills non-*Medea*-bearing progeny and an antidote that protects *Medea*-bearing progeny from this maternal lethal effect (Beeman and Friesen 1999; Beeman et al. 1992). In *Drosophila melanogaster* (Meigen), multiple types of the *Medea* inheritance pattern have been reverse-engineered and shown to act as potent gene drives (Akbari et al. 2014; Champer et al. 2016; Chen et al. 2007). These *Medea* systems act as alteration drives by using an RNAi-based toxin-antidote mixture. This combination of the toxin expressed maternally, and the antidote expressed zygotically results in the survival of 50% of female embryos originating from *Medea*-bearing heterozygote, while the rest that unable to inherit the *Medea* element die (Champer et al. 2016). Furthermore, in case the female has coupled with a *Medea*-bearing heterozygous male, the antidote from the male will also act in the embryo, giving 70% embryo survivals (Champer et al. 2016). Major limitations that obstruct the development of *Medea* in insects are the difficulty to replicate synthetic *Medea* systems and the lack of information on how to perform effective RNAi-mediated silencing of key genes in the germline of species other than *D. melanogaster*. However, the development of *Medea* procedures utilizing an RNA-guided endonuclease as the toxin synthesized to target maternal mRNA of a maternally accumulated embryonic vital gene may help to resolve the latter limitation (Champer et al. 2016).

11.2.7 Wolbachia

Wolbachia are maternally inherited, obligate intracellular parasitic bacteria inhabiting insects and rapidly invade populations (Serbus et al. 2008). This microbe-mediated infertility technique is also termed the *incompatible insect technique* (IIT), which is a nongenetic variant of the sterile insect technique (SIT) (Lees et al. 2015; Panagiotis and Bourtzis 2007; Sinkins 2004). *Wolbachia* symbiosis together with the sterile insect technique was also tested to suppress *Drosophila suzukii* (Matsumura) populations (Nikolouli et al. 2020). Unlike other gene drives, which integrates into the host genome, *Wolbachia* is inherited in the cytoplasm (extracellular inheritance). They are capable of boosting their survival selfishly in the succeeding generations by interfering with host reproduction abilities. Particular strains of *Wolbachia* have been shown to induce feminization, killing males, parthenogenesis, and cytoplasmic incompatibility (Burt 2014; Ioannidis and Bourtzis 2007; Sinkins 2004; Werren 2011). However, genetically engineered *Wolbachia* to control insect populations is not established yet.

11.2.8 Sterile Insect Technique (SIT)

SIT is a method developed to control insect pests aiming to reduce the use of chemical insecticides that have provoked public concern. These concerns are further debated by the findings that neonicotinoids causing a reduced capacity of honeybees and wild bees to establish new populations in the year following exposure (Woodcock et al. 2017). SIT has been the most widely used technology to control many insects including the New World screwworm (NWS) *Cochliomyia hominivorax* (Coquerel), the fruit flies, Tsetse flies, mosquitoes and some Lepidopteran pests such as the cotton bollworm and codling moth (Hendrichs 2000). The technique employs the release of huge numbers of sterile males into the field to mate with wild females, thus interfering with reproduction and

leading to a decline in population. Dunn and Follett (2017) summarized the criteria for an effective SIT program as follows: (1) target species should be amenable to rearing and sterilization in huge numbers; (2) sterile insects should be competitive upon distribution and able mix completely with the wild population; (3) sterile insects should be fit and compete effectively for mates; (4) the release ratio should be adequate and enough to overcome the rate of population upsurge in nature; and (5) the target population should be closed (i.e., there is no introduction of fertile insects from outdoor the release region). Understanding the reproductive biology, behavior and population dynamics of the target species are prerequisites for effective SIT programs. For instance, the successful use of SIT on the NWS took the advantage that females couple only once. Old methodologies to induce male sterilization for SIT programs have relied on DNA-damaging agents such as gamma or X irradiations (Chakroun et al. 2017). These methods substantially reducing the overall fitness and mating competitiveness of released males (Kandul et al. 2019). To overcome these limitations, the following methodologies have been developed.

11.2.8.1 Methods Based on Modern Genetic SIT-Like Systems

Release of Insects Carrying a Dominant Lethal (RIDL)

This employs a transgene system to induce repressible female-specific lethality. RIDL needs that a strain of the target insect carries a restricted, dominant, sex-specific fatal, where the unrestricted condition can be synthesized in the laboratory or factory but will never be met by the wild population. To demonstrate the feasibility of this technique in *Drosophila*, Thomas et al. (2000) used *Drosophila* transcriptional control elements to drive expression of the tetracycline-repressible transactivator fusion protein (tTa) (Gossen and Bujard 1992). In the lack of tetracycline, the tTa steers the expression of any gene under the control of the tetracycline-responsive element (tRe). Expression of tTa was performed by the control of the enhancer of yolk protein 3 (Yp3) fat body (Ronaldson and Bownes 1995), which drives the expression in female larvae and adults, but not in males. RIDL does not induce sterility in insects because it depends on dominant, repressible, and lethal genetic elements. However, RIDL acts late in development and prevents insects from becoming adults, but still enables them to survive and compete at the larval stages when density-dependent competition occurs (Fu et al. 2010; Thomas et al. 2000).

Female-Specific RIDL (FsRIDL)

This methodology depends on the release of insects carrying a conditional dominant lethal gene specific for females (fsRIDL). Fu et al. (2010) developed transgenic *Aedes aegypti* (Linnaeus in Hasselquist) strains to have the repressible female-specific flightless phenotype. They used either two separate transformable genes or a single transformable gene. The transgenes are manipulated from an indirect flight muscle promoter specific to females from the *A. aegypti* Actin-4 gene (AeAct-4). Transgenic *A. aegypti* has all of the genetic characteristics essential to make extremely penetrant, late acting, dominant, and female lethality. The promoter originated from the *A. aegypti* AeAct-4 gene directs the expression of tTA in tissue-, a stage-, and sex-specific manners resulting in females only RIDL strains. These fsRIDL strains exclude the necessity for irradiation sterilization, permit genetic sexing by male-only release, and allow the discharge of eggs instead of adults (Fu et al. 2010).

Autosomal-Linked X-Chromosome Shredders

An X-chromosome shredder is a gene drive that cuts the male X-chromosome at multiple sites during meiosis, thus destroying it. This usually occurs in an XY heterogametic species. Due to these X-chromosome shredders, the majority or all of the fertile sperm will contain Y-chromosomes, leading to biased sex ratios in favor of males. Over time, the population will decline due to the scarcity of females (Champer et al. 2016). Engineering of X-shredder was done

in *A. gambiae* by expressing the I-Ppol (an intron-encoded endonuclease from an acellular slime mold, *Physarum polycephalum*) during spermatogenesis targeting ribosomal repeats on the X-chromosome (Klein et al. 2012; Windbichler et al. 2007, 2008). Highly efficient parental shredding of the X-chromosome was achieved with a modified I-Ppol resulting in more than 90% males (Galizi et al. 2014).

Precision-Guided SIT (PgSIT)

The pgSIT is a CRISPR-based technology that depends on a genetic dominance that allows concurrent sexing and sterilization, and ultimately enables the release of eggs into the ecosystem, certifying only sterile adult males emerge (Kandul et al. 2019). Notably, the release of eggs will overcome the problems of manual sexing and sterilizing males, thus reducing overall efforts and boosting scalability for field applications. Kandul et al. (2019) systematically engineered multiple pgSIT systems in *Drosophila* and demonstrated that this technology consistently gives rise to 100% sterile, fit and completive males. PgSIT employs a simple reproduction system, which requires two different homozygous strains, one expresses Cas9 endonuclease and the other expresses double-guide RNAs (dgRNAs) (Fig. 11.2). Coupling of these strains results in concurrent RNA-guided dominant biallelic knockout of both target genes during development. This consequently results in the conversion of recessive phenotypes into dominant ones in all progeny of a single generation (Kandul et al. 2019). However, there are concerns about the feasibility of the precision-guided sterile males in insect species other than *Drosophila*. These are regarding the competitive efficiency between laboratory and field, the logistics to deploy pgSIT eggs for field application and the complex regulatory processes toward pgSIT approval for field application (Bouyer and Vreysen 2019).

Environmental RNA Interference and SIT

There is cumulative evidence for the potential use of environmental RNA interference (eRNAi) in SIT programs to inhibit gene expression

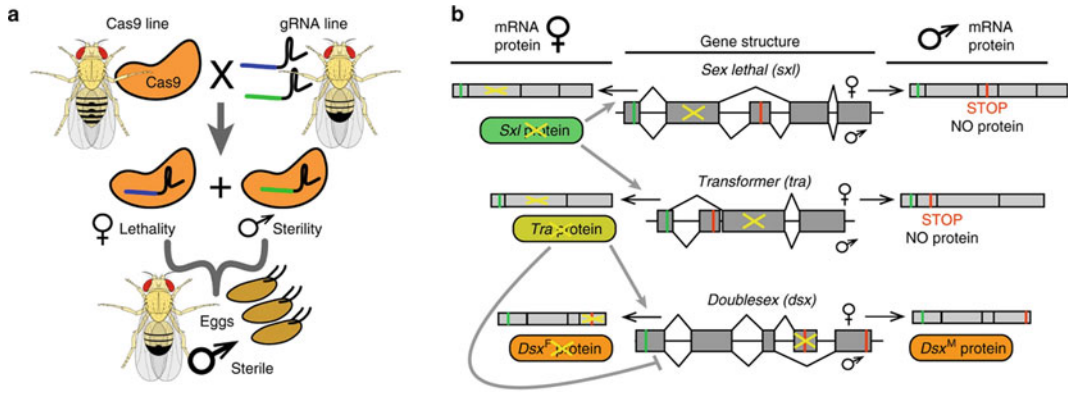

Fig. 11.2 Precision-guided sterile insect technique (pgSIT), an assessment of gene targets with single guide RNAs (sgRNAs). **a** A pgSIT diagram representing the utilization of Cas9 and gRNAs lines as two components of the binary CRISPR/Cas9 system. These lines are maintained as separated homozygous and their cross results in concurrent knockouts of genes required for female viability and male fertility, respectively, resulting in survival of only F1 sterile males. **b** A schematic of sex-specific alternative splicing in *sxl*, *tra,* and *dsx* regulated by female expression of Sxl and Tra proteins (gray lines). Disruption of female-specific exons of key sex-determination genes, *sxl*, *tra,* and *dsx*, disrupts female development. Yellow crosses indicate PgSIT exon targets. *Source* This figure is reproduced with permission from Kandul et al. (2019) which is licensed under a Creative Commons Attribution 4.0 International License http://creativecommons.org/licenses/by/4.0/

(Darrington et al. 2017). eRNAi is a sequence-specific regulation of endogenous gene expression in a receptive organism by exogenous dsRNA (Whangbo and Hunter 2008). Coleopteran insects such as western corn rootworm and Colorado potato beetle are susceptible to eRNAi when nourished on wild-type plants. These coleopteran insects uptake the endogenous long dsRNAs of plants, but not the small dsRNAs. Then, the long dsRNAs were processed into 21 nucleotides small interfering RNAs (siRNAs) by insects and accumulated in high quantities in insect cells (Ivashuta et al. 2015).

Genetic Sexing Strains (GSSs)

Males-only release in the SIT program was achieved by the development of genetic sexing methods in the medfly *Ceratitis capitata* (Wiedemann) (Zacharopoulou et al. 2017). *Ceratitis capitata* was eradicated by using sterile bisexual releases. During the efforts to eradicate the medfly, it was realized that releasing only males substantially improved cost reduction and action efficiency. The medfly genetic sexing strains (GSSs) were identified through mitotic karyotyping and chromosomal arrangement of the species. Pupae of these medfly strains are genetically determined and can be differentiated phenotypically according to sex, for example, differences in the color of pupae or its temperature sensitivity. Understanding of GSSs is of great value for assessing the efficacy of an SIT control program, as the GSSs enable easy separation of males and females before eclosion, and this facilitates male-only release (Zacharopoulou et al. 2017).

11.2.8.2 SIT Limitations

While these first-generation genetic SIT technologies represent significant advances, each approach has disadvantages. For example, the incompatible insect technique (IIT) strictly requires the release of healthy females without any infection, which is not possible to fulfill in the field. The use of the antibiotic tetracycline in RIDL affects the indigenous microbes and gradually removes them (Wilkinson 1998). Compromising the fitness of RIDL/fsRIDL males and X-chromosome shredders can only be developed in species with heterogametic sex chromosomes, thereby limiting broad applicability to species lacking heterogametic sex chromosomes. Therefore, it would be advantageous logistically to utilize effective technologies based on SIT, which could concurrently and proficiently sort males and females and sterilize only males without significant bargaining the fitness of two sexes. Such optimized genetic technologies do not exist to date (Kandul et al. 2019).

11.3 Molecular Basis of Insecticides Resistance in Insects

Insecticide resistance threatens the management strategies for most insects, which promptly develop resistance to many synthetic insecticides (Hawkins et al. 2019). Mechanisms of insecticide resistance include penetration rate reduction, metabolic detoxification enrichment, enhancement of insecticide excretion from the organism and loss of target-site sensitivity (Pang et al. 2016). Generally, mechanisms of insecticides resistance can be separated into two main groups: (1) metabolic resistance (occurs due to changes in the intensities or activities of detoxification proteins) and (2) target-site resistance (occurs due to mutations in the genes of the sodium ion channel, acetylcholinesterase and GABA receptor) (Hemingway et al. 2004). Moreover, behavioral changes also contribute to insecticide resistance in insects. For instance, mosquitos usually change their behavior in response to DDT and permethrin by decreasing the entry rate into houses, rising early exit rates from houses and stimulating a shift in biting times. Most insecticides' target genes are important receptors or enzymes in the insect nervous system whose poisoning leads to rapid paralysis and insect death (Ffrench-Constant et al. 2004). *Drosophila* genomics and genetics fundamentally paved the way to understand the mechanisms of insecticide resistance by analyzing the genes for the metabolic resistance such as carboxylesterase, P540 monooxygenases, and glutathione *S*-transferase and the genes for the central targets for

insecticides such as acetylcholinesterase, voltage-gated ion channels, and ligand-gated ion channels.

11.3.1 Metabolic Insecticides Resistance

Resistant insects can detoxify or extinguish the insecticides faster than susceptible insects through the most common metabolic resistance mechanism or they can prevent the insecticide to reach its target sites by binding it to proteins in their bodies. Therefore, resistant insects usually possess higher levels or effectively active forms of the detoxification enzyme(s) that break down insecticides to nontoxic or less toxic compounds. Metabolic resistance events may develop due to the metabolic enzyme mutations that make the mutant enzyme(s) more efficient at detoxifying the insecticide or the metabolic resistance may arise due to responses in the regulatory system that upsurge the abundance of essential enzymes in exposed tissue (Gott et al. 2017).

11.3.1.1 Carboxylesterases

Carboxylesterase enzymes are often associated with insects' resistance to organophosphate (OPs), carbamate and pyrethroid insecticides by the enzymes' qualitative or quantitative changes, or a combination of these mechanisms (Hemingway et al. 2004; Yan et al. 2009). Qualitative change resistance occurs when the enzymatic properties are changed because of mutations in sequences of esterase enzyme, resulting in an incurred OP hydrolase activity at the expense of declined carboxylesterase activity (Cui et al. 2015; Yan et al. 2009). Qualitative changes of esterase enzymes have been reported in many OP-resistant insects including *Aedes albopictus* (Skuse) (Grigoraki et al. 2017) and *Drosophila* melanogaster expressing *Culex pipiens* Linnaeus and *Aphis gossypii* Glover mutant carboxylesterases (Cui et al. 2015). The OP-resistance genes of *Lucilia cuprina* (Wiedemann) *LcαE7*, *Lucilia sericata* (Meigen) *LsαE7,* and *Musca domestica* L. *MdαE7* were mapped in the α-cluster of carboxylesterases. A point mutation in these genes causes the characteristic shift in substrate specificity from naphthyl acetate to OPs (Newcomb et al. 1997). The other mechanism for carboxylesterases metabolic resistance is the quantitative change of esterases. This mechanism involves the overproduction of carboxylesterase proteins through gene amplification in the genome or transcriptionally upregulation of esterases (Hemingway et al. 2004; Li et al. 2007). The involvement of both specific carboxylesterase gene amplification and overproduction of nonspecific carboxylesterases in the quantitative change resistance has been reported in many insects (Yan et al. 2009). Earlier studies in the peach potato aphid *Myzus persicae* (Sulzer) indicated that overexpression of esterase genes coupled with the alternation in their regulation could induce resistance. More esterase proteins can hydrolyze or sequester insecticides (Field et al. 1999).

11.3.1.2 Cytochrome P450 Monooxygenases (P450)

Cytochrome P450-mediated insecticide detoxification is one of the most important mechanisms involved in insects' resistance to insecticides. Insect P450s that are involved in resistance are multigene enzymes characterized by constitutive transcriptional overexpression in insecticide-resistant strains, thereby causing enhanced metabolic detoxification of insecticides (Feyereisen 2005; Ffrench-Constant et al. 2004; Liu et al. 2015; Zhu et al. 2008). The molecular basis of metabolic resistance by the P450s detoxifying enzymes is more complex and sometimes involves some sort of redundancy in enzymes function, whereby if one of the P450 enzymes was changed to deal with an insecticide metabolism, often the related enzymes might be able to tackle its normal metabolic function (Ffrench-Constant et al. 2004). Advances in genomics and proteomics studies facilitated the analysis of the enzymes involved in insecticide resistance. Microarray analysis identified 90 genes of P450 in *Drosophila* and documented only a single P450 allele (*Cyp6g1*) is sufficient to confer DTT resistance in *Drosophila* (Daborn et al. 2002). Overexpression of multiple P450 genes was

reported in the deltamethrin-resistant strains of *Helicoverpa armigera* (Brun-Barale et al. 2010), in insecticide-resistant mosquitoes, *Culex quinquefasciatus* Say (Liu et al. 2011), in the dengue vector, *A. aegypti* exposed to pyrethroid insecticide (Bariami et al. 2012) and recently in the RPW, *Rhynchophorus ferrugineus* by imidacloprid-specific induction and transcriptomic profiling (Antony et al. 2019). The induction of P450s and their corresponding enzymes activities in insects are possibly directly linked to the insects' environmental acclimatization, the detoxification of insecticides, and the development of insecticide resistance (Festucci-Buselli et al. 2005; Liu et al. 2011; Poupardin et al. 2008; Scharf et al. 2001; Zhu and Liu 2008). Host plant toxins and pesticide treatments induced more than half of the up-regulated P450s in the resistant Colorado potato beetle strain in a tissue-specific manner, which indicates that P450s tackle both the detoxification and xenobiotic adaptation processes in this herbivore specialist insect (Zhu et al. 2016).

11.3.1.3 Glutathione S-Transferases (GSTs)

GSTs are a group of metabolic enzymes that catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates in the detoxification process. In insects, GSTs confer resistance directly by participation in detoxification or sequestration of insecticides and indirectly by protecting against insecticides-induced oxidative stress (He et al. 2018; Lu et al. 2016; Pavlidi et al. 2018; Qin et al. 2013). Pavlidi et al. (2018) outlined the major GST-mediated insecticide resistance mechanisms as follows: (1) GSTs can stimulate the attachment of glutathione (GSH) to the insecticide steering to the production of less toxic soluble (GST-insecticide) conjugate. (2) GSTs contribute to DDT detoxification by stimulating direct DDT metabolism to release the nontoxic dichlorodiphenyldichloroethylene (DDE), utilizing glutathione as a co-factor. (3) GSTs that exhibit peroxidase activity decrease the toxic peroxides produced by the oxidative stress incurred due to insecticide intake and release reduced inactive peroxides. (4) GSTs may confer resistance via the non-catalytic passive binding to insecticide and sequestration. Identification and functional characterization of specific GST genes involved in insecticide resistance have been documented in many insects. It was observed that *Bemisia tabaci* (Gennadius) Mediterranean (MED) flies are more resistant to imidacloprid than the *B. tabaci* Middle East-Minor Asia 1 (MEAM1) ones. This observation was explained by the existence of high transcripts for the *GST-d7* in the MED than in the MEAM1 flies and the suppression of the same protein increased mortality in MED flies than in MEAM1 flies (He et al. 2018). Four GST recombinant proteins of the migratory locust, *Locusta migratoria* (Linnaeus) (LmGSTd1, LmGSTs5, LmGSTt1, and LmGSTu1) were expressed in *Escherichia coli* (Migula) as heterologous proteins to study their roles in insecticides resistance (Qin et al. 2013). Two of these proteins, LmGSTs5 and LmGSTu1, were able to induce more nymph mortality upon exposure to malathion, carbaryl and chlorpyrifos insecticides. Moreover, the resistance of field-collected *Rhynchophorus ferrugineus* to cypermethrin and ethion was attributed to the elevated activity of GST in crude extract preparations from resistant *R. ferrugineus* populations (Al-Ayedh et al. 2016a). Malathion resistance in the oriental fruit fly, *Bactrocera dorsalis* (Hendel) was examined by testing the roles of eight epsilon glutathione S-transferases (eGSTs) genes from *B. dorsalis* (*BdGSTe1*, *BdGSTe2*, *BdGSTe3*, *BdGSTe4*, *BdGSTe5*, *BdGSTe6*, *BdGSTe7,* and *BdGSTe9*) (Lu et al. 2016). Quantitative reverse transcription, overexpression in malathion-resistance *B. dorsalis* strain, and RNA interference studies highlighted the importance of eGSTs, namely BdGSTe2, BdGSTe4, and BdGSTe9, in *B. dorsalis* to develop resistance to malathion.

11.3.1.4 ATP-Binding Cassette (ABC) Transporters

ABC transporters comprise a large transporter superfamily proteins found in all living organisms. They function primarily in ATP-dependent active translocation of many substances across cellular membranes. The substances prone to translocation by the ABC transporters include,

but are not limited to, amino acids, sugars, heavy metal ions and conjugates, peptides, lipids, polysaccharides, xenobiotics and chemotherapeutic drugs (Rees et al. 2009; Wu et al. 2019). A functional ABC transporter consists of two cytosolic nucleotide-binding domains (NBDs) that attach and catalyze the hydrolysis of ATP and two fundamental transmembrane domains (TMDs) (Dermauw and Van Leeuwen 2014). If the four domains of a functional transporter (2TMDs-2NBDs) are joined in a single polypeptide, they form a full transporter (FT), while a half transporter (HT) contains one TMD and one NBD. The ABC transporter domains NBD and TMD are occasionally encoded as distinct polypeptides and then bonded into multidomain protein. To pursue ATP binding and hydrolysis, the HT must become a functional transporter by forming homodimer or heterodimer complexes (Wu et al. 2019). The insect ABC transporters superfamily is divided into eight subfamilies (ABCA ~ ABCH) based on the ATP-binding sites on the ABC transporters (Merzendorfer 2014). Many insect ABC transporter superfamily members inhibit the accumulation of intracellular insecticides and their metabolites and thereby play a crucial role in insecticide resistance (Buss and Callaghan 2008). The role of these transporters in insecticide resistance has been investigated in many insects. For instance, the upregulation of the ABC transporters was recorded in pyrethroid-resistant *A. aegypti* (Bariami et al. 2012), in deltamethrin-resistant *Aedes gambiae* (Bonizzoni et al. 2012) and the bed bug, *Cimex lectularius* Linnaeus, resistant to DDT, deltamethrin, permethrin, and imidacloprid (Mamidala et al. 2012).

11.3.2 Central Targets Resistance

11.3.2.1 Acetylcholinesterase (AChE)

AChE is a critical enzyme that hydrolyzes and regulates the level of the neurotransmitter acetylcholine (ACh) in vertebrates and invertebrates and terminates nerve impulses (Jiang et al. 2018; Kim and Lee 2018). AChEs are key enzymes produced in the insects' nervous system and are the targets of organophosphate (OP) and carbamate (CB) insecticides. These insecticides bind to the serine residue located on the active center of AChE, thereby inhibiting the activity of the AChE enzyme to hydrolyze ACh (Casida and Durkin 2013). Recent advances in molecular biology helped to identify two groups of AChE genes, namely *ace1* and *ace2*, from different insects (Jiang et al. 2018); but the fruit fly, *Drosophila melanogaster* and the house fly, *Musa domestica* each possess only a single *ace2* gene. The absence of the *ace1* gene in these two insects and similar ones was believed to be due to gene loss (Huchard et al. 2006; Weill et al. 2002). In *Blattella germanica* and *Tribolium castaneum ace1,* knockdown significantly increased the sensitivity of individuals to AChE target insecticides (Lu et al. 2012; Revuelta et al. 2009). In insect species that have both *ace1* and *ace2* genes, a point mutation in the *ace1* gene often causes amino acid substitution in AChE1, which results in a drastic reduction in the sensitivity of AChE to OP and CB insecticides (Jiang et al. 2018; Lee et al. 2015). A single amino acid substitution A216S in AChE1 upsurges chlorpyrifos resistance in *Apolygus lucorum* Meyer-Dür (Wu et al. 2015) and a G119S mutation in numerous mosquito species guides to insecticide tolerance (Weill et al. 2004). All the insecticide-resistant strains of the mosquito *Culex pipiens* were found carrying the same resistance-associated replacements of amino acid glycine at position 119 with a serine (G119S) within the active site of the AChE1 enzyme (Weill et al. 2003). Moreover, three mutations (G119S, F331C and I332L) in AChE1 confer resistance to chlorpyrifos in *Nilaparvata lugens* (Stål) (Zhang et al. 2017b) and four substitutions (G119A, F/Y330S, F331H, H332L) in the same species are associated with declined sensitivity to carbofuran (Kwon et al. 2012). On the contrary, in the Western honeybee, *Apis mellifera* Linnaeus,

the AmAChE2 enzyme was proposed to play a major role in synaptic transmission, while AmAChE1 has non-neuronal functions, including chemical defense. These findings are based on the tissue distribution pattern, molecular and kinetic properties of AmAChE1 and AmAChE2 proteins (Kim et al. 2012). All these documentations and others not only confirm the crucial role of AChEs mutations in insects to develop resistance to insecticides but also highlight the significance of these enzymes for the survival of insects.

11.3.2.2 Voltage-Gated Ion Channels

The insect nervous system contains five ion channel targets for insecticides namely: (1) The voltage-gated sodium channel encoded by homologs of the *Drosophila* gene *para* (DDT and pyrethroids). (2) The ϒ-aminobutyric acid (GABA) receptor with subunits coded by the resistance to dieldrin gene (*Rdl*) and represents the active site for cyclodienes and fipronil insecticides. (3) The glutamate-gated chloride channel. (4) The insect nicotinic acetylcholine receptor or nAChR (neonicotinoids and spinosyns). (5) The insect ryanodine receptor (ryanodine and the diamides) (Ffrench-Constant et al. 2016). Voltage-gated sodium ion channels are transmembrane proteins that are critically fundamental for electrical signaling in most excitable cells. Sodium-ion channels activated (open) in response to depolarization of the membrane and permit the flow of sodium ions into the cell during the initial rapidly rising phase of the action potential in excitable tissues (Zlotkin 1999), making the internal charge of the cell less negative (i.e., causing membrane action potential). The channel pore is blocked in a few milliseconds after opening by an inactivation particle to terminate the action potential, thus preventing extreme depolarization of the resting membrane potential (Silver et al. 2014). The insect voltage-gated sodium channel is the site of action for many insecticides including DDT, the pyrethroids, N-alkylamides, dihydropyrazoles, and blockers of the sodium channel (indoxacarb and metaflumizone) (Silver et al. 2014; Soderlund and Knipple 2003; Zlotkin 1999). The knockdown resistance (*kdr*) mechanism helped to document the insecticide resistance in many insect pests. Mutations in sodium channels are found to induce a reduced sensitivity to the pyrethroid insecticides by many insects including *Drosophila melanogaster*, *Blattella germanica*, *Musa domestica,* and *Aedes aegypti* (Du et al. 2013; Li et al. 2012; Rinkevich et al. 2013; Xu et al. 2012).

11.3.2.3 Ligand-Gated Ion Channels

Ligand-gated chloride channels (LGCCs) receive chemical signals and neurotransmitters, such as acetylcholine, ϒ-amino butyric acid (GABA), glutamate, or histamine. Then they were converted into electrical signals via the opening of their integral ion channels (Bloomquist 2003), and therefore, the LGCCs mediate fast inhibition of neurotransmission, facilitating the influx of chloride into neurons upon binding of a neurotransmitter ligand. LGCCs are the targets of lindane and cyclodiene insecticides and the new generation insecticides including phenylpyrazoles, avermectins, and isoxazolines (Ozoe et al. 2010; Remnant et al. 2014). Insects have 10–12 genes encoding LGCC receptor subunits. Four of these genes have been shown to involve in *Drosophila melanogaster* resistance to at least one insecticide class (*Rdl*) (Ffrench-Constant et al., 1993; glutamate-gated chloride channel alpha subunit (*GluClα*), Kane et al. 2000; histamine-gated chloride channel subunit (*ort*), Iovchev et al. 2002; and histamine-gated chloride channel (*HisCl1*), Yusein et al. 2008). Mutations and amino acid substitutions in *Rdl* encoded GABA receptor confer resistance to dieldrin in *Drosophila*, to fipronil (phenylpyrazole) in the planthopper *Laodelphax striatellus* (Fallén) (Nakao et al. 2011) and in *Drosophila* (Remnant et al. 2014). A single amino acid replacement in the *GluClα* induces resistance to ivermectin and nodulisporic acid in *Drosophila* (Kane et al. 2000).

11.4 Functional Genomic Tools Underpinning Molecular Mechanisms of Insecticide Resistance

The most popular functional genomic tools that reinforce the molecular mechanisms of insecticide resistance in insects include GAL4/UAS systems, RNAi systems and CRISPR/Cas technology.

11.4.1 GAL4/UAS Systems

The GAL4/UAS is a molecular tool used to induce the expression of genes from any organism in a tissue- and temporal-specific manner. GAL4 is a yeast protein that regulates genes induced by galactose (Laughon and Gesteland 1984; Laughon et al. 1984). To activate the GAL10 and GAL1 target genes, GAL4 binds to sites of 17 base pair regulatory elements known as the upstream activating sequences (UAS) (Duffy 2002). UAS is a *cis*-acting regulatory element distinct from the promoter and increases the expression of a neighboring gene. It is considered analogous to the function of the enhancer in eukaryotes (Webster et al. 1988). Fischer et al. (1988) reported the capability of GAL4 expression to stimulate transcription of a reporter gene under UAS control in *Drosophila*. Then a binary system was developed by Brand and Perrimon (1993) and tested for tissue and temporal control of the expression of any gene in *Drosophila*. Any promoter that drives the expression of the desired gene in a tissue-specific manner can control GAL4. Genes of interest include lethal ones, as GAL4-drives and USA-genes of interest constructs are usually integrated into separate strains (Duffy 2002; Homem and Davies 2018). The GAL4/UAS system helped to demonstrate that a single cytochrome P450 gene, *CYP6g1*, was conferring resistance to the insecticide dichloro-diphenyl-trichloroethane (DDT) in *Drosophila* (Daborn et al. 2002). The gene *CYP6g1* was differentially expressed in wild *Drosophila* populations resistant to DDT. It was shown that the insertion of the long terminal repeat (LTR) of an *Accord* retrotransposon upstream of the *CYP6g1* gene increased the *CYP6g1* expression in major detoxification tissues of the DDT-resistant flies (Chung et al. 2007; Homem and Davies 2018). Daborn et al. (2012) functionally validated three different detoxification genes from different insects by expressing them in transgenic *Drosophila* via the GAL4-driven system: (1) from the Australian sheep blowfly *Lucilia cuprina*, a carboxylesterase gene (*αE7*) that confers resistance to organophosphorus compounds (OP); (2) from the mosquito *A. gambiae*, a glutathione S-transferase gene (*GstE2*) that confers resistance to DTT; and (3) from the whitefly *Bemisia tabaci*, a cytochrome P450 gene (*Cyp6cm1*) that confers resistance to imidacloprid. Furthermore, the GAL4/UAS system promoted an understanding of the involvement of many enzymes in insecticide resistance in insects. For instance, the P450 genes *CYP6P9a* and *CYP6P9b* that derived resistance to pyrethroids in field populations of the malaria vector *Anopheles funestus* Giles (Riveron et al. 2013), the glutathione S-transferase gene, *GSTe2*, which caused resistance to DDT in the same vector (Riveron et al. 2014), the demonstration in *Drosophila* that the P450 gene *CYP6ER1* is responsible for brown planthopper *Nilaparvata lugens* resistance to imidacloprid in a rice paddy (Pang et al. 2016). However, the main constraints that hinder the use of the GAL4/UAS system in non-model insects include (1) Technical difficulties of keeping large numbers of mutant stocks. (2) Unavailability of transformation procedures and rearing protocols for the non-model insects. (3) Scarcity of genomic data for many agricultural serious pests (Homem and Davies 2018).

11.4.2 RNA Interference (RNAi)

The mechanism of cellular gene silencing by RNAi and its importance in boosting research in different disciplines will be covered elsewhere in this book. Here, we focus on the practice of

researchers to employ this approach to underpin the mechanisms underlying insecticide resistance. The fast adoption of the RNAi technology as a functional genomic tool for insect control and insecticide resistance is due to two main advantages: (1) Its potency in suppressing any gene of known sequence; and (2) it does not depend on procedures for germline transformation. The RNAi system is flexible in delivering the dsRNA or siRNA to the target organism. Delivery of the dsRNA into insects can take place via microinjection, feeding, or direct addition to larval breeding water as in cases of mosquitos (Lopez et al. 2019), through transgenic plants (Bolognesi et al. 2012; Zhang et al. 2017c), or via host-induced gene silencing (Head et al. 2017; Pampolini et al. 2020). Delivery can also take place by topical applications (Killiny et al. 2014; Pampolini et al. 2020) and the use of aerosolized short RNAi bound to nanoparticles (Li-Byarlay et al. 2013; Thairu et al. 2017). Three methods, topical application, microinjection, and dsRNA-treated artificial diet were tested to silence the catalase gene of the RPW (Al-Ayedh et al. 2016b). The latter two procedures are more potent to induce larval mortality and inhibit growth, while topical application requires high dsRNA concentration and longer exposure time to suppress the gene expression. Laudani et al. (2017) silenced three genes in the RPW via RNAi, namely α-amylase, *V*-ATPase, and the ecdysone receptor, to compare the response patterns of silencing the same genes in *Tribolium castaneum*. They concluded that the responses are different though the two insects belong to the same order. The success or failure of these delivery methods depends largely on the insect species and family as well as on the target genes. It is most likely that the trends of RNAi are exceptionally effective in the Saturniidae family and in genes function in the immune system, while genes function in epidermal tissues appears to be hard to silence (Terenius et al. 2011).

Regardless of the confronts, RNAi has been utilized to study the insecticide resistance mechanisms that mediated by detoxification enzymes. Zhu et al. (2010) demonstrated the role of the brain-specific cytochrome P450, *CYP6BQ9*, in *Tribolium castaneum* resistance to deltamethrin by RNAi-mediated knocking down the expression of this gene. CYP6BQ9 is mainly expressed in the portion of the central nervous system (CNS) that contains the voltage-gated sodium channels, which are targets for deltamethrin insecticide. Moreover, microinjection of dsRNA for two P450s, *CYP6AY1* and *CYP6ER1*, in *Nilaparvata lugens* confirmed the role of these two enzymes in imidacloprid resistance (Bao et al. 2016). In the bed bug *Cimex lectularius*, the NADPH cytochrome P450 reductase (*ClCPR*) is required for the functioning of P450s. Microinjection of dsRNA to downregulate the *ClCPR* in deltamethrin-resistant populations triggered a decrease in resistance to deltamethrin (Zhu et al. 2012). Recently, Seong et al. (2019) showed that cytochrome P450s *Cyp4p1* and *Cyp4p2* were responsible for DTT tolerance in the *Drosophila* DTT-resistant strain 91-R. Probably, the exposure of multiple generations to DDT has induced selection for the preservation of functional copies of both *Cyp4p1* and *Cyp4p2* within 91-R strain as compared to the susceptible strain 91-C (Seong et al. 2019). Antony et al. (2019) reported a significant decline in the survival rate of adult RPWs treated with imidacloprid when *CYP345J1* and *CYP6NR1* genes silenced via the ingestion of dsRNA. This indicates that overexpression of these two P450s plays a crucial role in weevils' resistance to imidacloprid in date palm fields.

RNAi as a genetic-functional approach was also utilized to study other detoxification enzymes aiming to understand the mechanism of resistance, though some limitations such as lack of specificity and repeatability do exist that might puzzle the interpretation (Pavlidi et al. 2018). In this regard, the role of carboxylesterase (CarE) enzyme in metabolizing omethoate, a systemic OP insecticide, was investigated in cotton aphid *Aphis gossypii* via oral delivery of an interfering dsRNA-*CarE* (Gong et al. 2014). Knockdown of *CarE* coupled with a drastic suppression of CarE in omethoate-resistant *A. gossypii* and the resistant individuals showed increased susceptibility to the omethoate. The role of glutathione S-transferases (*GSTs*) in insecticide resistance has

been investigated in various studies using RNAi. Silencing *Locusta migratoria* GSTs, *LmGSTs5* and *LmGSTu1* result in increased nymph mortality upon treatment with carbaryl, malathion, and chlorpyrifos (Qin et al. 2013). Lu et al. (2016) demonstrated the contribution of the epsilon glutathione S-transferases (*eGSTs*) to the malathion resistance in the oriental fruit fly *Bactrocera dorsalis* through RNAi-mediated knockdown experiments. RNAi-mediated Knockdown of the *Bemisia tabaci BtGSTd-7* led to an increase in the mortality of the adult Mediterranean *B. tabaci* exposed to imidacloprid (He et al. 2018). RNAi suppression of transcripts encoding for a cytochrome p450, a cuticular protein and a glutathione synthetase (GSS) protein in a resistant population of Colorado potato beetle, *Leptinotarsa decemlineata* Say, showed reductions in measured resistance to imidacloprid (Clements et al. 2017). The study suggests that these genes control essential steps in imidacloprid metabolism in these field populations, and the effectiveness of traditional insecticides could be improved when used in combination with targeted RNAi technology to control resistant *L. decemlineata* populations (Clements et al. 2017).

11.4.3 The CRISPR Technology for Insecticide Resistance

The CRISPR technology was employed to study the role of the cotton bollworm *Helicoverpa armigera* cytochrome P450 gene cluster *CYP6AE* subfamily in detoxification of insecticides and phytochemicals (Wang H et al. 2018a). The *CYP6AE* gene cluster contains ten P450 genes in *H. armigera*, nine of them are arranged as a large cluster on chromosome 16 (Shi et al. 2018). This gene cluster was found constitutively overexpressed in deltamethrin-resistant *H. armigera* strains (Brun-Barale et al. 2010). CRISPR/Cas9 mediated knockout deletion of 85 kb genomic fragment covering nine P450 genes from the *CYP6AE* subfamily together with phytochemicals and insecticides assays revealed the involvement of several of *CYP6AEs* in detoxifying plant toxins and chemical insecticides as well as in gossypol defense in *H. armigera* (Wang H et al. 2018a). CRISPR/Cas9 genome editing was also used to investigate the contribution of insect ryanodine receptors (RyRs) target-site mutations in *Drosophila* diamide resistance. Genome-modified *Drosophila* carrying alternative allele combinations for RyR exhibited high resistance ratios to flubendiamide and chlorantraniliprole (Douris et al. 2017). A similar study employed the CRISPR/Cas9 technology to introduce a mutation in the RyR gene of *Spodoptera exigua*. Diamide insecticides function in selective activation of insects' RyRs by stimulating the unrestrained discharge of calcium ions and causing muscle contraction, paralysis and ultimately insect death (Zuo et al. 2017). The mode of action (MoA) of three distinct groups of selective chitin biosynthesis inhibitors in arthropods was documented using the CRISPR/Cas9 approach coupled with homology-directed repair (HDR) to introduce point mutations in *Drosophila* chitin synthase 1 gene (*CHS1*). Homozygous lines bearing the mutations were highly resistant to etoxazole, benzoylureas, and buprofezin. A single mutation in the *CHS1* gene confers striking levels of insecticide resistance against the three distinct insecticide groups, which all share the same MoA by directly interacting with *CHS1* (Douris et al. (2016). Resistance to spinosad, a biopesticide, has evolved in many insects and is associated with alterations of its target, the Alpha 6 subunit of the nicotinic acetylcholine receptor (nAChRαs). Somers et al. (2015) generated a CRISPR/Cas9-induced *Drosophila* strain carrying *DmAlphα6* with P146S mutation and proved the fly is resistant to spinosad. A CRISPR/Cas9 approach was adopted to produce *Drosophila* strains carrying homozygous F1845Y or V1848I mutations in the voltage-gated sodium channel *para* gene to functionally validate each mutation in vivo in the absence of confounding resistance mechanisms (Samantsidis et al. 2019). Two mutations (F1845Y and V1848I) have been found in the domain IV S6 segment of the voltage-gated sodium channel in certain indoxacarb resistant populations of *Plutella xylostella*

and *Tuta absoluta* (Meyrick) (Roditakis et al. 2017). Both mutations in mutant moths confer moderate resistance to indoxacarb, and the mutation V1848I also confers moderate resistance to metaflumizone; however, the mutation F1845Y confers very strong resistance to metaflumizone (Samantsidis et al. 2019), indicating the relevance of the technology to functionally validate the target-site resistance mutations against sodium channel blocker insecticides (SCBIs).

11.5 Intrinsic Mechanisms of Plant Resistance to Insects

The mechanisms of resistance in the innate plant defense system are a complex process depending on many biotic and abiotic factors (Fig. 11.3) and can be categorized into three main groups: morphological (mechanical protection), biochemical, and molecular (War et al. 2012). The morphological mechanism includes hairs, trichomes, thorns, spines, waxy cuticles, sclerophylly, and thicker outer layers. In the biochemical defense system, the initial plant responses to insect contact involve imbalances in ion fluidity in the plasma membrane of the damaged sites of plant cells. The disturbed charge of the membrane generates differences in the plasma transmembrane potential, which finally leads to the induction of signal transduction pathways and gene expression. Among early events that trigger plant response to insects attack are the induction of calcium signaling and the generation of reactive oxygen and reactive nitrogen species, which are directly linked to the differences in plasma transmembrane potential (Zebelo and Maffei 2015). Plant response also involves the formation of systemically moving signals from wounded tissue, such as secondary metabolites called allelochemicals (Howe and Jander 2008) and the production of calcium-dependent protein kinases and calcineurin signaling (Zebelo and Maffei 2015). The plant secondary metabolites that constitutively produced and stored as inactive precursors following attempts of insect attack or microbial invasion are termed as phytoanticipins, while those produced in response to actual insect attack as phytoalexins (Piasecka et al. 2015; War et al. 2012). The phytoalexins include isoflavonoids,

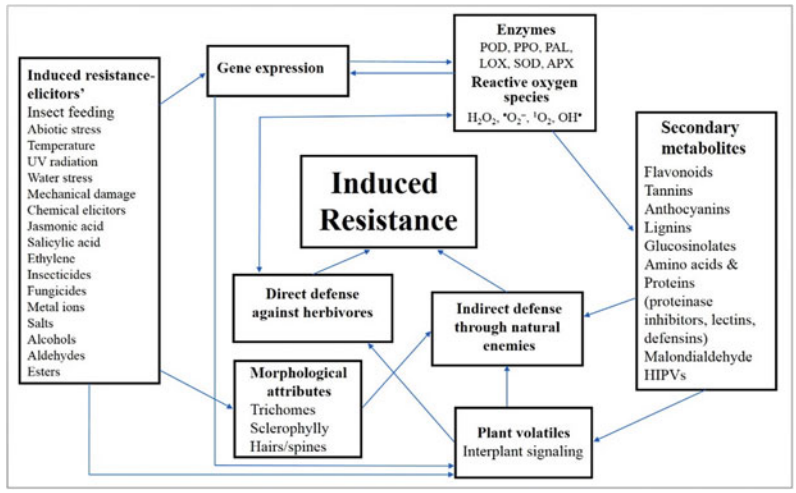

Fig. 11.3 Induced resistance mechanisms in plants. Enzymes involved in gene expression are peroxidase (POD), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), tyrosine alanine ammonia lyase (TAL), lipoxygenase (LOX), superoxide dismutase (SOD), and ascorbate peroxidase (APX). Secondary metabolites include herbivore-induced plant volatiles (HIPVs). *Source* This figure is reproduced with permission from War et al. 2012)

terpenoids, alkaloids, and others. Mechanism of resistance could also be classified into antibiosis that affects insect biology and antixenosis (non-preference) which affects insect behavior. The former adversely affect arthropod development, reproduction, or survival, while the latter prevents arthropod colonization of a host plant (Smith 2005; Horber 1982). It is difficult sometimes to distinguish between antixenosis and antibiosis modes of plant resistance to insects because both of them affect arthropod populations (Smith 2005). Perfectly, antibiosis and antixenosis may be equally supporting the modes of resistance; i.e., antixenosis may prevent antibiosis resistance breaking biotypes to colonize a plant and antibiosis may decrease the strength of colonizing individuals (Hesler and Dashiell 2011). Furthermore, induced responses are a critical component of antibiosis, where certain signals such as herbivore feeding, salivary enzymes, or plant hormones (e.g., jasmonic acid [JA]) results in the expression of certain defenses (War et al. 2012). Yang et al. (2020) used a plant volatile that induced by the herbivore and investigated activation of the defense machinery of tomato against the whitefly, *Bemisia tabaci*. Plants are always under selection pressure to escape insects attack while insects are under selection pressure to find suitable hosts. To do so, insects have been equipped with a sensory system for the detection of plant signals and a nervous system capable of integrating inputs from sensory neurons (Bruce 2015). Moreover, insect pests have elaborate mechanisms such as detoxification of secondary plant metabolites to counter their toxic effects (Ali and Agrawal 2012). Several polyphagous insects with expanded host range show an extraordinary tendency to develop resistance to insecticides. However, Dermauw et al. (2018) provided evidence that a polyphagous lifestyle is not a forecaster of rapid resistance evolution to insecticides. Instead, the dominance of insecticide resistance in generalist herbivores is probably due to the strong selection pressure inflicted on them by intensive insecticide application. Therefore, alternative control strategies based on understanding the genomics and genetics of the economically important insect pests are mandatory.

11.6 Major Date Palm Pests

The arthropod species in the date palm agroecosystem are changing over time. Buxton (1920) made the earliest record of date palm pests in Mesopotamia (Iraq) almost a century ago. In the 1970s, a total number of 54 species have been reported worldwide to afflict injury on date palm (Carpenter and Elmer 1978). Recently, El-Shafie et al. (2018) listed 132 species of mite and insect pests that cause damage, in one way or another, to the date palm. Out of this number, a few species are considered major pests (Table 11.1; Fig. 11.4). Only four species of the major pests exhibit a high degree of host-specificity to date palm (true pests). These are the issid date bug (*Asarcopus palmarum* Horvath), the dubas bug (*Ommatissus lybicus* de Bergevin), the lesser date moth (*Batrachedra amydraula* Meyrick), and the longhorn date palm stem borer (*Jebusia hammerschmidti*) (Al-Azawi 1986; Blumberg 2008). The red palm weevil (RPW) *Rhynchophorus ferrugineus*, which is categorized as the number one pest of the date palm, has more than 40 hosts among palms (Faleiro et al. 2019), and the carob moth has 43 hosts in 18 plants families (Perring et al. 2015). The anthropogenic activity of moving date palm materials into new areas (Faleiro 2006) besides the effect of climate change (Heeb et al. 2019) could have a profound impact on the emergence of new potential pests of the date palm. The invasion of the RPW to almost all date palm growing areas around the globe during the last 30 years is a good example. The RPW was unknown in the Gulf and the Middle East until 1985 when it was discovered in UAE on an imported ornamental palm (Faleiro 2006). The introduction of the green pit scale insect in Sudan 40 years ago is another example of the consequence of accidental shifting of date palm from one locality to a new one. The green

Table 11.1 Major date palm pests

Scientific name, order, and family	Common name	Site of injury on date palm	References
Insects			
Rhynchophorus ferrugineus (Coleoptera: Curculionidae)	Red palm weevil	Trunk, frond bases, and young offshoots	Faleiro (2006)
Ommatissus lybicus (de Bergevin) (Homoptera: Tropiduchidae)	Dubas bug	Fronds (leaves) and fruit bunches	Ali and Hama (2016); Al-Kindi et al. (2017)
Cadra cautella Walker (Lepidoptera: Pyralidae)	Almond/date moth	Ripe and stored dates	Ali and Hama (2016)
Ectomyelois ceratoniae (Zeller) (Lepidoptera: Pyralidae)	Carob moth	Soft-ripe dates	Perring and Nay 2015
Jebusaea hammerschmidti (Coleoptera: Cerambycidae)	Longhorn date palm stem borer	Frond bases and trunk, young offshoots	Ali and Hama (2016)
Batrachedra amydraula (Meyrick) (Lepidoptera: Batrachedridae)	Lesser date moth	Immature date fruits	Ali and Hama (2016)
Palmaspis phoenicis Ramachandra Rao (Homoptera: Asterolecaniidae)	Green pit scale insect	Leaflets, petioles, fruit bunches, and fruits	Ahmed (2007)
Mite			
Oligonychus afrasiaticus (McGregor) (Acari: Tetranychidae)	Date dust mite	Immature date fruits	El-Shafie (2019)

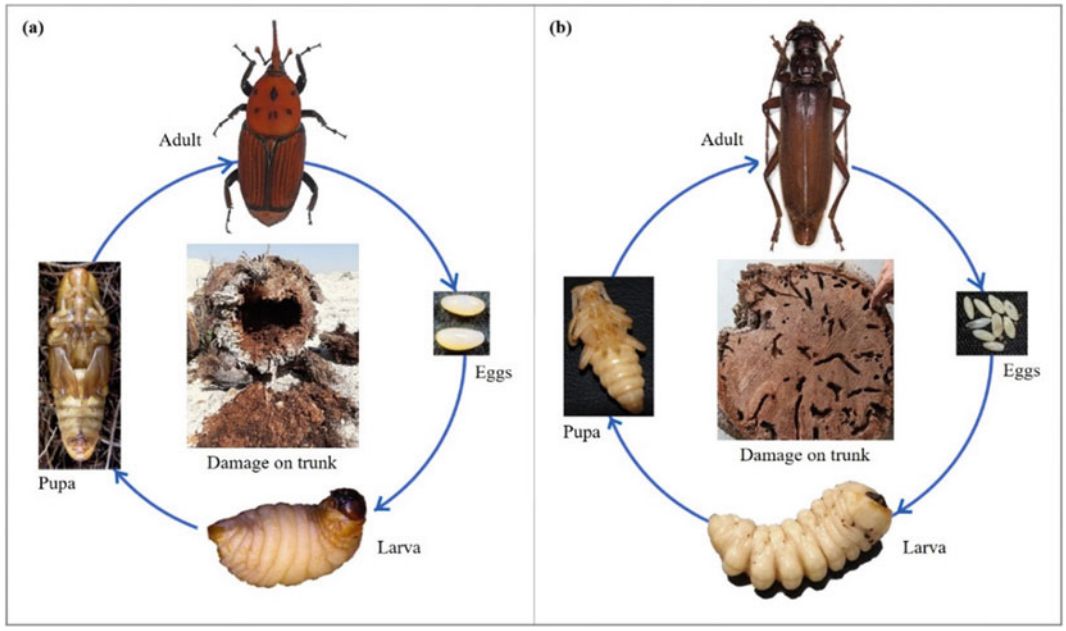

Fig. 11.4 Life cycle of (**a**) the red palm weevil, *Rhynchophorus ferrugineus* and (**b**) the longhorn date palm stem borer, *Jebusaea hammerschmidti* and the pattern of damage caused by each pest on the trunk of a date palm, *Phoenix dactylifera* (Photos: Babiker M.A. Abdel-Banat and Hamadttu A.F. El-Shafie; figure constructed by Babiker M.A. Abdel-Banat)

scale is now the number one pest of date palm in Sudan causing vast damage to almost all commercial date palm cultivars (Ahmed 2007). The status and severity of date palm pests depend largely on the locality and the prevailing environmental conditions. For example, dubas bug *O. lybicus* is a major pest in Oman and Iraq (Ali and Hama 2016; Al-Kindi et al. 2017) causing severe economic damage in these two countries, while it is a minor pest in some other countries. Even in the same country, the pest status of dubas differs considerably according to the locality. Management practices also influence pest status. A clear example is the excessive use of insecticides to control the RPW, which caused the frequent outbreaks and upsurge of the date dust mite, simply because of eliminating its natural enemies (El-Shafie 2019).

11.6.1 Date Palm Defense and Resistance Against Insect Pests

Generally, plant defense can be grouped into two major strategies: resistance and tolerance (Mitchell et al. 2016). Berlinger (2008) defined plant resistance as any reduction in plant acceptance, in the growth rate of the pest population, or reduction in the damage caused by pests due to innate or inherited self-defense mechanisms in the plant. Tolerance, on the other hand, involves the expression of plant traits that limit the negative impact of insect injury on productivity and yield, i.e., the plant's ability to withstand or recover from insect damage (Mitchell et al. 2016). Furthermore, plant resistance can be classified into direct resistance mediated by the plant characteristics that negatively affect the insect pests or indirect resistance through the release of plant volatiles that attract the natural enemies of the insect pests (War et al. 2012).

Worldwide, there are around 3000 cultivars of date palms with a wide range of genetic diversity (Zaid 2002). Date palm cultivars resistant to various insect pests are expected to be among this large genetic pool. Despite the existence of large genetic diversity, the resistance of some date palm cultivars is not yet exploited as integrated pest management (IPM) component in the management of major date palm pests. A few studies were carried out in attempts to find resistant date palm cultivars against the RPW. Farazmand (2002) screened five date palm cultivars in Iran for resistance to RPW. He reported that the cultivar Mazafati was the most preferred by the weevil; i.e., it showed the least antixenotic effect among the tested cultivars. Resistance among other species of palms was also investigated. In this respect, Dembilio et al. (2009) reported that *Washingtonia filifera* (Lindley) and *Chamaerops humilis* Linnaeus showed antibiotic and antixenotic mechanisms, respectively, against the RPW, while *Phoenix canariensis* Chabaud was highly preferred. Little information on the mechanisms of resistance in date palm against RPW is available; however, date palm varieties with high sugar contents enhanced oviposition and growth while calcium inhibited RPW growth (Farazmand 2002). In laboratory feeding experiments, Al-Ayedh (2008) tested the antibiosis effect of Khalas, Sillaj, Sukkary, and Khasab date palm cultivars on RPW. He reported that cv. Sukkary had a lower degree of antibiosis against larvae of RPW and a higher frequency of adult emergence. The longest male lifespan was recorded on cv. Khalas followed by cvs. Sillaj, Sukary, and Khasab (Al-Ayedh and Rasool 2010). Faleiro et al. (2014) screened seven cultivars of date palm for resistance to RPW. They found that the cultivars Shahal and Gaar were least preferred by the RPW for oviposition. The two cultivars showed the highest degree of antixenosis, while the cultivar Khalas registered the lowest degree of nonpreference. The cvs. Shahal and Gaar exhibited the highest degree of antixenosis for oviposition by RPW followed by Hatmi, Khasab, Khalas, Sheshi, and Reziz. Other authors who screened different cultivars for resistance against the RPW indicated the existence, in these

cultivars, of antixenotic and antibiotic effects (Al-Bagshi et al. 2013; Al-Nujiban et al. 2015).

11.7 Potential Application of Genomic/Genetic Approaches for Insect Control and Insecticide Resistance in Date Palm

The assembled genome sequences of date palm cultivars revealed that the tree is heterozygous with frequently intense single-nucleotide polymorphism (SNP) (Al-Dous et al. 2011; Al-Mssallem et al. 2013; Mathew et al. 2014). Following these sequencing data, an improved long-read genome assembly of date palm was carried out, which helped to perform genome-wide association studies (GWAS) of the sex-determining region and 21 fruit traits (Hazzouri et al. 2019). Recently, Hazzouri et al. (2020) identified important gene families at the plant-RPW boundary upon analyzing the genome sequence of *Rhynchophorus ferrugineus*. However, functional genomics/genetics and gene editing data of date palm are not adequate due to reasons like the profusion of sequence polymorphism, the lack of transformation systems in date palm and the diversified array of date palm cultivars in different date palm going regions (Sattar et al. 2017). Since transgenic date palm seems technically difficult to produce and the local communities hardly adopt consumption of transgenic crops, then biotechnology options other than in planta genetic transformation are mostly the insecticide alternatives to combat the date palm insect pests. The general practice for the RPW management in the date palm fields is through the injection of insecticides into the trunk of trees. The route and fate of these insecticides, while targeting the larval and pupal stages within the trunk, are still not well defined. Add to this, field populations of RPW resistant to insecticides have also been reported (Al-Ayedh et al. 2016a). Meanwhile, the possibility of insecticides' passage to humans cannot be ruled out. Therefore, comprehensive mining into the genomic and transcriptomic data of the RPW (Antony et al. 2019; Wang et al. 2013) may help to identify gene drives that are suitable to be engineered into one of the genetic/genomic tools either RNAi- or CRISPR/Cas-based technologies. Gene drives of interest may include those drives that trigger re-sensitizing organisms that already developed insecticide resistance to be sensitive again. Genes that induce sex-specific lethality are of outstanding preference in addition to genes that can be activated during insect diapause and causing lethality. Genes that induce biased inheritance toward their own and reduce the viability of gametes that inherit the wild-type allele are of special importance. Moreover, the RPW genes that control the development and metamorphosis such as chitinases and laccases (Abdel-Banat et al. 2018; Abdel-Banat and El-Shafie 2019) are also valuable for designing genetic constructs aiming to suppress the weevil's spread. The lack of details about the pests' behavior, reproductive biology, and population dynamics of insect pests of the date palm are shortcomings. This information is fundamental to design a genetic control method based on SIT. However, the most promising technology for application to control date palm insect pests is the CRISPR-based precision-guided sterile insect technique (pgSIT), which was developed by Kandul et al. (2019) in *Drosophila*. This technology employs a dominant genetic procedure that concurrently disrupts gene(s) essential for female viability and male fertility leading to a seamless sexing and sterilization together and finally the release of eggs into the field. Upon eggs hatching, only sterile males emerge. The technology requires two homozygous breeds, one expressing the endonuclease Cas9 and the other expressing double-guide RNAs (dgRNAs). The coupling of these two breeds results in RNA-guided dominant knockouts of the two alleles of both target genes throughout development. This results in converting recessive phenotypes into dominant phenotypes in a single generation.

One more option of genomic/genetic approaches for insect control in date palm agriculture is the use of transgenic insect viruses.

Baculoviruses are insect-specific viruses that do not cause disease to vertebrates. They are generally safe and strictly host-specific; therefore, they have been formulated and utilized as bioinsecticides a long time ago. However, the drawback of these natural biocontrol agents is that the viral disease takes days or even weeks to kill the host insect, during which time the host continues to feed and cause agricultural damage (Kroemer et al. 2015). Recombinant baculoviruses that express toxic peptides and proteins are the alternative to improve the pesticidal potency of the native baculoviruses by shortening the time needed for infection to kill or incapacitate insect pests. One of the genes drives in this process is an insect-selective toxin that is isolated from the venom of the scorpion *Androctonus australis* Hector insect toxin (AaIT). The toxin causes fast excitatory paralysis and even death of the host insect. The toxin targets the voltage-gated sodium channels of the insect (Deng et al. 2019). Expression of AaIT in baculoviruses or fungi can increase their virulence to insect pests and disease vectors (Deng et al. 2017; Wang and Leger 2007). Transgenic tobacco, *Arabidopsis* and rice plants expressing AaIT toxin have been developed with remarkably high insecticidal activity (Liu et al. 2016; Yao et al. 1996). Recombinant baculoviruses harboring insecticidal toxins may serve as extra options to engineer toxic gene drives in the date palm plants via transgenic transformation or via directly spraying the bioinsecticides to combat date palm pests.

11.8 Conclusions and Prospects

Application of the pgSIT technique in the RPW, the most destructive date palm pest, or any other major insect pest of the date palm requires developing a CRISPR/Cas system for these insect pests and identification of the suitable gene (s) for the RNA-guided dominance. Moreover, the delivery systems of the engineered constructs necessary to be updated in the RPW. For insecticide resistance studies, RNAi technology is best suited to distinguish how the date palm insect pests develop resistance to insecticides. Evidence of the usefulness of the RNAi technology to identify the resistance developed by the RPW is now accumulating. However, many essential target genes for either use in insect control or insecticide resistance purposes are still not fully characterized in this destructive pest of the date palm. Future research should be directed toward characterizing more genes, developing genomic/genetic systems and improving gene delivery methods for the date palm insect pests. The development of effective RNA-guided endonucleases is vital for generating efficient and stable gene drive systems. Currently used gene drive systems are unstable and may stimulate resistance alleles due to their reliance on the DNA repair pathways. More research should also be focused on the application of maternally inherited microbes that mediate infertility (*Wolbachia*), *Medea* elements that eliminate competing siblings and parental RNAi (pRNAi). These techniques are more stable and less liable to induce resistant alleles in target insects.

Acknowledgements The authors thank Dr. Omar S. Akbari at the University of California, San Diego, Dr. Brenda Oppert at Center for Grain and Animal Health Research, Agricultural Research Service, USDA, and Dr. Hari Chand Sharma at ICRISAT, India, for kind permission to reproduce their figures here. The authors acknowledge the financial support provided by the Date Palm Research Center of Excellence (DPRC) through the research grant number DPRC-2020-3.

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J. M. Al-Khayri et al. (eds.), *The Date Palm Genome, Vol. 2*, Compendium of Plant Genomes,
https://doi.org/10.1007/978-3-030-73750-4