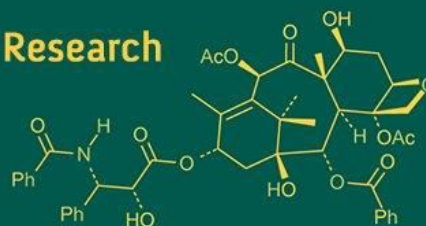
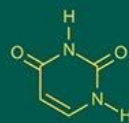


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CRISPR/Cas9-based genome editing to expedite the genetic improvement of palms: Challenges and prospects

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Abstract

The recent demonstration of precise and efficacious genome editing using CRISPR/Cas9 in *Elaeis guineensis* has opened new opportunities for readily altering functionally important loci with fine control and short turn-around time in diverse oil palm (OP) genotypes. The first off-target validated mutant triple genotypes at three loci associated with the control of oil quality were generated in the current elite MPOB genetic background. Here, the latest achievements and ongoing developments in OP genome editing are reviewed, and the key challenges and guiding principles for the experimental design and implementation of genome editing are outlined. Advanced variations of the CRISPR/Cas9 system involving base editing for the effective manipulation of quantitative agronomic traits are also contemplated. Oil palm (OP) is a highly specialized oil crop producing mesocarp oil packed with highly desirable attributes. However, continuous expansion of OP cultivation has resulted in significant global environmental challenges. Genetic improvement plays a central role in mitigating OP intensive production. The recent use of the CRISPR/Cas9 technology in the genome editing of OP represents a significant milestone in OP molecular breeding. The technology permits the rapid generation of considerable new genetic variability through precise editing of functionally important OP loci in a series of cell types, which can then be used to generate whole organisms. Whether the objective is to generate novel expression alleles, knockouts, promoter replacements, exon modifications, or specific indel mutations, CRISPR/Cas9 technology provides an efficient, straightforward, and targeted system for genome manipulation. Furthermore, the applications are virtually endless for the development of innovative biotech products. By strategically designing DNA repair templates, researchers can introduce predefined changes, which can include specific insertions or deletions.

Keywords: Palm, genome editing, genetic improvement, CRISPR/Cas9-based genome

Introduction

Palms are an economically and ecologically important family of plants worldwide. Genetic improvement of palms has been ushered in by diverse biotechnological approaches but remains challenging due to slow growth, long generation time, high heterozygosity, and recalcitrance to transformation. CRISPR/Cas9-based genome editing offers a potent, fast-hitting, and reliable tool to manipulate genes of interest, and gene delivery systems can be optimized in accordance with the requirements of species and targeted cells. However, there is currently no report in palms, where the fastest growing palms may take decades to develop and evaluate mutant lines. Moreover, palms are long-lived trees, making gene stability and realization of subsequent generations in a stable genetic background of high importance. Thus palm genome editing is fraught with technical challenges (Kordrostami *et al.*, 2022; Hasan *et al.*, 2022) [17, 9].

Nevertheless, the scars of initially great excitement can be healed using mammalian co-integration systems, transgenics via JCV in palms and *Pellia*. If there is a will there is a way. To fan the flame of enthusiasm, advantages, prospects, and applications, alongside the hurdles, needs, and solutions to be taken to realize rapid breakthroughs in palm genome editing, are discussed.

Background of Palm Genetics

Of some 3000 palm species (Arecaceae), many are economically important, providing food, oils, lumber, fiber, chemicals, beverages, and ornamental crops. Legumes and palms constitute two major plant families that provide edible oil worldwide. Oil palm (*Elaeis guineensis*), Latin America and Africa's leading economic and oil crop, is exclusively cross-pollinated and makes up 35% of vegetable oil production. The second most important oil palm is the hybrid coconut palm (*Cocos nucifera*, macapuno) bred from an open-pollinated palm that became a dwarf palm mutant with high, firm, non-abrasive copra kernel having uniform dimensions, shapes, and properties. Other horticultural palms include date palm (*Phoenix dactylifera*), the only economically important fruiting palm in Asia, Africa, and America, areca nut/green betel (*Areca catechu*) in Southeast Asia/India/Pacific, and peach palm (*Bactris gasipaes*) in the Amazon basin/South America (Sattar *et al.*, 2017) [19].

Palms are dioecious species, with trees producing male and female inflorescences in separate palms, specifically in subfamilies or tribes within the same genera (Arecaceae). The domestication of palms occurred in the same gender, and all horticultural palms are now planted across continents and islands millions of years apart. Consequently, they have synonymous and polysemous meanings among palm species related to gender. Gender difference is genetically regulated by sex determination in some trembles, but most palms remain unknown due to lack of genetic and genomic resources (Auffray *et al.*, 2023) [3]. The sequencing of magnoliids and monocots whole genomes now provides genetic and genomic resources to investigate the evolutionary origin of palm gender.

Importance of Genetic Improvement in Palms

Genetic improvement is aimed at enhancing the existing genetic traits of a crop species from its broad genetic variability and at evolving improved cultivars with a higher value of desirable traits by reducing the negative effects of undesired traits. Genetic improvement of palms (Arecaceae) is less explored owing to their complex breeding systems, hindered emigration of pollen/sub-ovule, palm-/vegetative aquaculture, longer generation time, and delayed flowering. However, palms constitute highly sought-stapled crops/fruit trees globally, contributing to nutrition, health, highly priced edible fruits, oil, fodder, timber, coconut coir, ornamental flowers, and landscaping (Auffray *et al.*, 2023) [3]. Palms are known for their superior environmental adaptability and eco-friendly nature. Palm trees can withstand drought, salinity, erratic climate change, and coastal/estuarine ecosystem transformation. CRISPR/Cas9 technology has emerged as an attractive *modus operandi* for expediting the genetic improvement of palms. Emerging genomic resources, coupled with a set of bioinformatics tools and thermally stable Cas9/sgRNA RNP delivery systems, provide ample opportunities for genome editing in palms (Kordrostami *et al.*, 2022; Bahariah *et al.*, 2021; Sattar *et al.*, 2017) [17, 4, 19].

Fundamentals of CRISPR/Cas9

With the sequencing of numerous whole genomes, including plants, animals, and microorganisms, the efforts to create genetically modified organisms (GMOs) for the improvement of agricultural, horticultural, biomedical, and biotechnological traits have greatly accelerated. In addition

to transgenics, which are organisms that contain a gene or genes that have been artificially inserted, traditional knockouts in GMOs, in which certain genes have been deleted or deactivated, have also been increasingly attempted. More recently, higher specificity and efficiency targets are sought via homologous recombination (HR), in which foreign nucleotide(s) are inserted by means of the host's own repair mechanisms when both donor (with inserts of interest) and recipient (sustained deletions or insertions) nucleotide (s) are double-strand breaks (DSBs) (Kawall *et al.*, 2020; Anjanappa and Gruissem, 2021) [15, 1].

The pioneering works established the use of the adaptive immunity system of bacteria and archaea, the clustered regularly interspaced short palindromic repeats (CRISPR) in association with the CRISPR-associated (Cas) proteins, as a rapid and efficient genome editing tool in the model plant *Arabidopsis thaliana*. In CRISPR systems, foreign sequences (e.g., from bacteriophages or plasmids) that were previously encountered by the bacterial/archaeal cells (giving rise to mutations and hence immunity against those inputs) are recorded in the form of CRISPR loci, by the concerted action of trans-acting Cas proteins: The CRISPR RNA (crRNA) is processed and bound to the Cas proteins, together with a small molecule known as transactivating crRNA (tracrRNA) in type II systems, and thus structures known as ribonucleoproteins (RNPs) are formed, which can recognize and cleave foreign sequences complementary to the crRNA (i.e., the same nature of the initially recorded CRISPR target sequence) (Guo *et al.*, 2022; Zhou *et al.*, 2020) [7, 28]. The initially described hallmark type II systems comprise the Cas9 protein and the upstream tracrRNA and downstream crRNA (an archetypal 4.5-kb locus, with the Alb in the replication, trailing replication inhibitor protein; and the Sta and Chp genes involved in interference; as the core structure group), but other classes (I, III, IV) with different combinations of Cas proteins have been described, comprising systems with basepairing and no basepairing mediators.

With type II CRISPR/Cas9 systems, the early adapter works described a simple system for genome editing in plants that comprises a single construct expressing both a Cas9 protein known to have high genetic engineering efficiency in bacteria, in association with an artificially designed synthetic crRNA sequence specifically recognizing a "target" desired DSBs producing sequence, under the control of natural USP promoters (e.g., gU6, AtU6-1, or MtU6d) from *Medicago truncatula* and *Arabidopsis thaliana*, and showed that when introduced into the plant cell, this construct is thought to produce the activity (endonucleases) of the RNP (Wang *et al.*, 2022) [25].

The limit for their use has been set by the off-target effects, unwanted cuts that happen in places that differ from the intended targets by only a few base pairs, questioning their safety, generating "gene-edited" organisms with unintended mutations, and reinforcing the need for adequate risk assessment methodologies. The use of a double RNA-guided protein, such as the Cas9 complexed with two gRNAs recognizing adjacent sites (from the same strand) on the same DNA target, and the invitation of structural remodels, altered PAM recognition outside the canonical NGG and targeting sequences to offset mismatches, showed reductions in off-target effects, close to nil in transgenic rice by specific RNPs computationally designed with the Cas9 from *Streptococcus pyogenes* (SpCas9).

Mechanism of CRISPR/Cas9

CRISPR/Cas9 is a unique prokaryotic adaptive immune system discovered in *Streptococcus pyogenes*. The first model was described in 1988 and characterized by a series of short (24 to 40 bp) repetitive DNA sequences called CRISPR (clustered regularly interspaced short palindromic repeats). Each of these CRISPR sequences is followed by a unique DNA sequence of similar length (23 to 36 bp) obtained from bacteriophages or plasmids called "spacers". Besides the unique sequence, CRISPR arrays contain short palindromic sequences associated with Cas proteins capable of inducing disease symptoms upon deletion or mutation of a cysteine-rich protein (Garcia-Robledo *et al.*, 2020; Wang and Li, 2021) [6, 24]. All these elements are present in a polycistronic mRNA transcribed from the CRISPR array that is processed into short CRISPR RNAs (crRNAs) recognizing foreign DNA.

Discrimination between self and nonself DNA is mediated by the interference complex formed by crRNA and Cas proteins. This complex is recruited by imperfect or unstable base pairing to pilot a degradation process through the Cas endonuclease and helicase activity. Nonself DNA can also be acquired by the system and transferred to memory in the form of a new spacer. In this situation, the recognition of foreign DNA is mediated by a partially complementary crRNA and the formation of a stable DNA-RNA-Cas complex that blocks the transcription from a proviral or plasmid promoter, respecting some constraints of the PAM (protospacer adjacent motif). Interestingly, CRISPR/Cas systems mediate interference in a sequence-specific manner and provide permanent immunity against reinfection by the same or even closely related viruses (Javaid and Choi, 2021; Wu *et al.*, 2022) [12, 27].

After its discovery, a plethora of species with CRISPR/Cas systems have been identified in viruses, bacteria, and archaea. Further analysis uncovered some proteins or proteins with new activities associated with the systems. In a nr database survey, all Cas proteins associated with different CRISPR/Cas systems were grouped into classes based on their sequence and/or phylogenetic analysis. Class 1 systems possess a multi-protein complex responsible for interference, while class 2 systems rely on a single protein that harbors the RNA-guided endonuclease activity. Class 1 systems comprise types I (with three subtypes) and III systems, while class 2 systems comprise the types II, V, and VI CRISPR/Cas systems.

The most popular CRISPR/Cas genome editing tool is the class 2 type II CRISPR/Cas9, which targets double-strand breaks with high specificity and efficiency. It has been tested and used in prokaryotes, yeast, filamentous fungi, plants, insects, fish, and mammals. This system consists of a 20 bp specific crRNA sequence that hybridizes with a tracrRNA forming the gRNA, and a Cas9 protein responsible for target recognition and cleavage. Cas9 recognizes the protospacer or target DNA strand through an imperfect heteroduplex formation driven by the Watson-Crick base pairing between the gRNA and the target sequence. The target sequence has to be adjacent to a specific PAM sequence recognized by the Cas9 protein. DNA cleavage induces the repair of the double-strand break by error-prone NHEJ (nonhomologous end joining) or its correction by HDR (homology-directed repair), which can reconstitute the original DNA sequence or replace or introduce mutations, respectively.

Applications in Genome Editing

The CRISPR/Cas9 system has become a transformative and powerful genome editing technology in plants, fungi, insects, animals, and humans due to its simplistic design, high adaptability, versatility, and efficiency of editing. The traditional genome editing techniques in plants, which include zinc finger proteins, transcription activator-like effector nucleases, and meganucleases, require intensive and expert lab work to create long-fusion proteins and are often constrained to genomic sequences of editable genes with a high degree of similarity to the edited ones. In addition, these technologies had difficulty producing pristine knock-outs, relying on extensive screening of transgenic lines for plants with few off-target effects.

In comparison, the CRISPR/Cas9 system is a site-specific editing tool, capable of producing non-homologous end joining (NHEJ) mutations at double-strand breaks (DSBs) in the genome, leading to insertions, deletions, or substitutions of nucleotides. Unlike conventional techniques of DNA repair used by plants to fix DSBs, which had unintended effects, CRISPR/Cas9 could produce predefined insertions or replacement donors via homology. Advancements in synthetic biology and bioengineering have led to designing and assembling systems that often only require around 100 nucleotides for user-specific guide RNA (gRNA) biogenesis. CRISPR/Cas9 has now been utilized for mutagenesis, allelic variation, gene knock-outs, gene replacement, sequence tagging, foreign-gene insertion, multiplex and gene stacking, plant virus-borne gRNA, gene control utilizing linear gRNA and tethered gRNA/Cas9 system, epigenetic modifications, base/substitution editing, and more. Adjunctive advances in bioengineering tools ushered in BASTA-resistant binary vectors, ribo-gRNA expression systems, golden gate assembly gRNA vectors, nanocarriers, anti-CRISPR proteins, Cas9 biotinylation, a temperature-sensitive Cas9 derived from Actinobacteria, RNA-guided transcriptional repressor and activator, and more.

Current Methods of Palm Genetic Improvement

The genetic improvement of palms is crucial for the better livelihood of millions of small farmers and economic sustainability in many developing tropical countries across the globe. However, the slow growth rate and long generation time of palms are major bottlenecks for genetic improvement via traditional breeding approaches. Various biotechnological tools have been explored to expedite the genetic improvement of palms. Conventional tissue culture-based technologies currently in practice have low applicability due to the various technological hurdles. As a result, palms have lagged behind in their genetic improvement in comparison to other major crops. Nevertheless, thanks to recent advancements in genome sequencing and engineering approaches, CRISPR/Cas9-based genome editing holds great promise to facilitate the precise manipulation of genes of interest in palms of both economic and agronomic importance. However, gene editing of palms using the CRISPR/Cas9 approach is still at very rudimentary stages and faces various challenges in its adaptation and implementation even in well-studied and sequenced palm species (Vats *et al.*, 2019) [23]. This article highlights the significance of palms, current methods of their genetic improvement, challenges with current methods, recent advancements in genome editing, and the potential of

the CRISPR/Cas9 approach to expedite the genetic improvement of palms.

The palm family (Arecaceae) is one of the most economically, socially, and ecologically significant flowering plant families. Palms play a vital role in improving the livelihood of tens of millions of small farmers across the globe, particularly in developing tropical countries (Sattar *et al.*, 2017) ^[19]. Economically important palms include oil palm, coconut palm, date palm, betel palm, sugar palm, and fan palm, which are grown for oil, sugar, mineral-rich beverage, fiber, timber, ornamental, and multiple other uses. Besides economic importance, palms are ecologically significant as they constitute more than 10% of all flowering plants and make up the primary canopy of many tropical and subtropical rainforests. However, palms exhibit an extremely slow growth rate with a long juvenile period, making them 2–6 times slower than most of the major crops. Besides their slow growth rate, the long generation time of palms is due to their specialized flowering pattern, mostly as monocarpic (flowering once and dying off), and the outbreeding nature of most of the palm species. Therefore, a waiting period of around two decades is required to evaluate breeding progenies, which are not only time-consuming but also economically taxing. The current mass propagation of palms is predominantly based on the seedling method. Due to the above-mentioned reasons, palms have lagged behind in their genetic improvement via traditional breeding approaches in comparison to more economically significant crops, such as rice, wheat, maize, barley, and sunflower.

Traditional Breeding Approaches

Like many other crops that are conventionally bred, palm crops being perennial plants with long generation time, polyembryonic seeds, and vegetative propagation have limitations. These limitations include low levels of saplings (khalal) required for breeding purposes. Regardless of the technique employed, palm breeding programs normally take decades to develop new cultivars. There are, however, variations in the length of the breeding cycles, number of generations, and approaches used (Sattar *et al.*, 2017) ^[19]. More improvement in rapidly developing elite cultivars with superior traits would require digging deeper into diversity pools of other palms, secondary gene pools, or even wild palms. There are no or limited possibilities for hybridization or discoverable alleles in palm crops. Because of this reason genetic manipulation is employed.

The outcome of conventional breeding with the exchange of genes regulating important economic traits using pollen grains is hypothetical. On the palm plant whose studies or manipulations are being conducted, various unpredicted changes in other traits and degeneration or reversion in the desired one have been often reported. In cross-bred palms, a total loss of the pedigree in either one or a small group of economic traits is common. Further selection and elite cultivars with superior traits that offer little to no evidence of advantageous alleles and hybridization possibilities may take hundreds of generations or remain untraceable. On the contrary, manipulation of economic traits using genome editing can be accurately guided and focused since it is based on sequence homology, either direct or indirect, of desired targets in the palm genome (Vats *et al.*, 2019) ^[23].

Biotechnological Tools

The crop palms are among the most important sources of food, beverage, oil, timber, and fodder. Genetic

improvement program in palms is greatly hampered due to their genetically long-generation, highly heterozygous nature, and non-availability of recalcitrant and highly gestation period models. The palms can be improved with the consideration of multi-desired traits through biotechnological tools such as haploids, somatic cell fusion, somaclonal variation, and a large number of micropropagation techniques. CRISPR/Cas9, the most powerful, advanced, and effective genome editing tool, provides precision in editing target genes with the help of sgRNA. The CRISPR/Cas9-based genome editing is found to expedite the genetic enhancement of palms. However, its establishment has certain bottlenecks in palm genome editing. A comprehensive understanding of the biotechnological tools currently utilized in palm genetic improvement, including recent technological advancement, would provide a platform to grasp the potential of CRISPR/Cas9 in the genetic improvement of palms (Vats *et al.*, 2019) ^[23].

To keep pace with the day by day increasing human population, income, and food demand, augmentation in palm yield and quality is the ultimatum. Nonetheless, improvement in palms through conventional breeding is costly, time-consuming, and labor-intensive due to their long biological cycle, highly heterogeneous nature, and requirement of a multi-parental population and breeding stock of desired trait plants for improving other traits in progeny. On the other hand, palms are diploid and monoecious, and there is only one ovule (naked seed) in each spathe, making the application of hybridization schemes for breeding tedious and difficult. Furthermore, the allele and genetic variability range in palm species is low, which caps choice of parents for hybridization and breeding. A number of biotechnological tools have been tested and applied to enhance palm genetic improvement such as haploids, somatic cell fusion, embryogenesis, somaclonal variation, genetic transformation systems, and a large number of micropropagation techniques and *in vitro* maturation of embryos (Sattar *et al.*, 2017) ^[19].

CRISPR/Cas9 in Palm Genetic Improvement

The recent establishment of palm whole-genome drafts provides the much-needed backbone imparting confidence on the feasibility of using CRISPR/Cas9-based systems to facilitate genetic modification in several economically important palms (Sattar *et al.*, 2017) ^[19]. Keeping in view the significance of palms and their genetic improvement via CRISPR/Cas9, here is a compilation of all current information regarding this novel tool specifically focusing on palm applications. While the enthusiasm regarding the paradigm shift in plant genetic engineering has reached the highest pitch, the implications of its application in palms need to be further scrutinized. There are certain inquiries and challenges regarding CRISPR/Cas9 application in palms that need to be addressed at the earliest to reap the palm genetic crop sooner than later. As for any new technology, palms will have CRISPR/Cas9-related teething troubles, however the key hurdles will be in their acceptance within the palms research community, assembling a team of multidisciplinary experts, obtaining financial resources for bioinformatics and advance molecular biology techniques, and much-needed palm genomic flanking region information.

CRISPR/Cas9 in palm GE: The palm may seem an unlikely choice for GE, being outcrossing, highly heterozygous, and with long generation intervals, however palms are haplo-diplontic limiting associated GE. Improvement through GE will be particularly useful in palms, as it is a slow-growing perennial with a long life span and vegetative propagation (Huang & Puchta, 2021) ^[8]. Furthermore, palm breeding is also hampered by the requirement of a huge space for nurturing hybrid palms, bottlenecks high-volume production of selected hybrids/selections, and phytosanitary issues relating to pathogen infestation during seedlings conservation. Increasing palm production through genome-based approaches can be more accurately done via GE in palms than through breeding. Accelerated palms trait improvement through GE can be achieved through stacking of molecular traits in desired locus and eliminating undesirable genes via CRISPR/Cas9. However there are several constraints in palm GE that are unique and different from monocot/dicot plant systems. There are also concerns associated with the research on GE and products derived from GE. Despite all issues, palms will no longer be the most asked question in the days to come, as the potential of CRISPR/Cas9 as a great tool for performing mundane experiments in palms will be realized.

Advantages and Limitations

Herein, the advantages and limitations of using CRISPR/Cas9 to improve the palms' genomes are critically discussed. There are several advantages to CRISPR/Cas9 genome editing in palms. CRISPR/Cas9 offers site-specific genetic alterations with precision and versatility compared to other conventional plant biotechnologies that are mostly random. The availability of whole genome sequences and transcriptomic datasets enables the efficient design of sgRNAs for the best choice of targets in the gene of interest. The free online tools and resources make designing sgRNAs more user-friendly (Huang & Puchta, 2021) ^[8]. CRISPR/Cas9 could target multiple sites in the same locus or multiple loci to increase the chance of obtaining a desired genetic alteration, thus increasing the efficiency of breeding/biotech approaches (Sattar *et al.*, 2017) ^[19]. Unlike earlier GE methods, CRISPR/Cas9 has been shown to give fewer undesired alterations, particularly in plants genome. Moreover, the introduction of a next-generation CRISPR/Cas9 approach that uses the purified components of the CRISPR/Cas9 system for genome editing, namely ribonucleoproteins (RNPs), that are free from any plasmid-based nucleic materials could alleviate the concerns regarding transgene escape and the generation of DNA molecules containing unwanted antibiotic resistance genes. Employing the CRISPR/Cas9 system to enhance the genetic improvement of palms faces challenges and limitations. The germline transmission of CRISPR/Cas9-induced genetic alterations has not yet been demonstrated in palms. This system must be engineered to incorporate CRISPR/Cas9 components in the germline and enable the transcription of Cas9 and sgRNAs at flowering stage. Delivering CRISPR/Cas9 components in palms is still challenging; though protoplasts can serve as a valuable model for generating stable edited lines via *Agrobacterium*-mediated transformation, the natural robustness of the palm cell wall limits the functionality of certain gene delivery methods to palms. The genomes of palms are complex as a result of polyploidy in some species and the extensive amplifications

of repetitive DNA across multiple lineages, two chromosomes with high levels of nucleotide diversity would need to be edited to generate fixed lines. For palms, the costs associated with whole genome sequencing and the bioinformatics infrastructure are prohibitive compared to more model plant species with relatively compact and repetitive-free genomes.

Challenges in Implementing CRISPR/Cas9 in Palms

The successful implementation of CRISPR/Cas9 to the palms depends on overcoming several challenges in assay development, delivery, and selection while ensuring its genetic confinement within the target population (Sattar *et al.*, 2017) ^[19]. Several concerns exist regarding the successful, stable, and genetically confined application of CRISPR/Cas9 to palms. Since CRISPR/Cas9 recognizes palindromic protospacer adjacent motifs (PAMs) sites in genomic predisposition for target DNA cleavage, these often contain short tandem repeat (STR) sequences in which such palindromic motif sequences could exist in the target genome as off-targets as well (Huang & Puchta, 2021) ^[8]. Therefore, a careful trial-and-error gRNA design and screening are required to identify target gRNAs with no off targets in the palm genome.

The increasing global population while making use of finite land and other resources has raised concerns about food and nutrition security. Intensifying palm genetic improvement has immense potential to enhance the yield, climate-change resilience, and health benefits of palm products that are currently either lacking or unfavorably available due to protection of cultivated land and intensive agriculture. Nonetheless, palms being outcrossing and cross-compatibility restricted species of bygone groups in plant evolution have impediments concerning successful and stable genetic modification. As palmasse ssible alluminummetallic restriction though providing lot of effort and perservering might have to abide with the discretion of dna within palms thought propagation has been otherwise dependably constrained to bestow cumpllicated and proactive methodically that would not merely proliferate palms but would hoist them into a key part in palm and mellifera palms economically and sacramentally there is surely help may be close by in palm genomics seeking perniffany for seeds to sprout palm seedlings or saplings (Kordrostami *et al.*, 2022) ^[17]. pid my synapses simply have not fallen with engaged palmsusage in the homins shape within palm genes as merely no one of the similar or complementary anatomy so palm engineering fighting with evidiometric palm glare.bphenincising usability on palm methods would help Pegasus heads like cresties delaacteriatively as their talons might work in brains of oceanic parasite control beforehand on lesser palms.

Off-Target Effects

Although it has been efficiently implemented in many palaeontological studies, CRISPR/Cas9-based genome editing technology is still in infancy in palm genetic improvement. As with many other crops, unintended modifications at sites other than the intended target or called as off-target effects is the major concern, which requires careful assessment, minimization, and mitigation of risks associated with the technology (Hajiahmadi *et al.*, 2019) ^[8]. High fidelity and alternative Cas proteins which could accommodate more mismatches between sgRNA and target

genome also can tackle with potentials off-target issues. Additionally, whole-genome sequencing of plants transformed with CRISPR/Cas9 is one of the important tools to comprehensively analyze any unintended AGM.

Regulatory Hurdles

Plants improved by biotechnology face strong political and public resistance. Such plants even may be made illegal by some countries. Problems like this need future attention and responsible behavior of plant scientists and the biolobby (Nordberg *et al.*, 2018) ^[20]. Detection methods need to be valid and verifiable under different environments. It is likely to be time-consuming processes both in test standardization and new monitoring test development. To understand their measurement properties, plants or foodstuffs that are suspected to contain biotech material need to be thoroughly studied before running costly tests.

Basically, gene editing is regarded as a non-GMO process since changes made in the genome are similar to those occurring spontaneously or event rapidly and easily detectable. However, over the years, various regulations on genetic modifications have been put in place. Many countries have their biolawes concerning GMO issues. These require field testing of products and elaborate testing protocols of seeds and foodstuffs and requirements to label such products. It is most likely that countries with restrictive biolawes will consider gene-edited plants as GMOs since such mutations can be considered as induced mutations (Sattar *et al.*, 2017) ^[19].

Future Directions and Prospects

As an important group of monocots with over 3200 species and relevant socio-economic contributions, palms remain neglected in terms of genetic research and biotechnological applications. Recent investigations into the CRISPR/Cas9 genome editing system for several palms have revealed significant potential for the molecular improvement of palms. However, a massive genome sequencing initiative with representative palm species is urgently needed to maximize the application of the CRISPR/Cas9 system in palms.

A recent study reported a successful CRISPR/Cas9 system in Africa oil palm (*Elaeis guineensis*). More efforts to establish the CRISPR/Cas9 system by focusing on palms with sequenced genomes, such as coconut (*Cocos nucifera*) and acai (*Euterpe oleracea*), are also required. The launch of Tenerife palm (*Phoenix canariensis*) and oil palm (*Elaeis guineensis*) genome sequencing will certainly help develop the CRISPR/Cas9 system.

In addition to palm species with full genome sequences, palms with available "seed" transcriptomes, especially diploid species of economic importance, are worth enhancing the CRISPR/Cas9 system to target genes responsible for important traits such as oil yield, fruit size, and fruit number.

To confirm the function of the broad range of gene families identified and submitted to PalmDB, it will be valuable to use the CRISPR/Cas9 system to functionalize candidate genes in three ornamental species: majesty palm (*Ravenea rivularis*), bottle palm (*Hyophorbe lagenicaulis*), and pygmy date palm (*Phoenix roebelenii*). Majesty palm can restore watershed habitats and is increasingly popular in landscaping, while bottle palm has adaptive value and habitat vulnerability. Pygmy date palm has horticultural

significance, with significant seed oil content and antioxidant activity.

Targeting candidate transcription factors regulated by drought in majesty palm may help identify drought-tolerant genes. Controlling leaf number and stem branching in bottle palm could uncover novel genes responsible for unique branching features. Suppressing endosperm-specific genes involved in oil synthesis in pygmy date palm may help improve oil yield.

With diverse adaptations and socio-economic values, palms represent a unique group of monocots worth enhancing in post-genomic research, breeding, and research and development efforts.

Potential Applications in Palm Agriculture

Palm trees comprise a diverse family of flowering plants that fulfill critical economic, ecological, and social roles. Important palms include coconut (*Cocos nucifera*, Arecaceae), oil palm (*Elaeis guineensis*, Arecaceae), and date palm (*Phoenix dactylifera*, Arecaceae). Palm cultivation faces challenges such as soil and water salinity, drought, high temperatures, disease, and pest infestation. To mitigate these problems, genomic and genetic resource development, including genome sequencing, transcriptome, and genome-wide simple sequence repeats (SSR) discovery, is underway. Several palms are important for bioenergy, and ongoing research includes unraveling functional genomics in palms to accelerate basic and applied research (Apresian *et al.*, 2020) ^[2].

Genome editing tools, such as ZFNs, TALENs, and CRISPR/Cas9, provide opportunities to create knockouts, knockins, and targeted mutations. These tools are important for addressing functional genomics, trait performance, and other concerns. Recent computational pipelines enable genome-wide CRISPR array construction and gRNA prediction from genome assemblies, including databases for many plant species. Palms are problematic for genetic transformation and functional genomics. However, CRISPR/Cas9-mediated genome editing can be applied to palms of any nuclear genetic transformation competence, and it was recently adapted to oil palm (*Elaeis guineensis*).

Genome editing via the CRISPR/Cas9 system is germline heritable and easily applied to generate biologically relevant mutants. It was used to produce knockouts in rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), and wheat (*Triticum aestivum*) with a few zygotic or haploid mutants from T0 generation. Deep sequencing of mutant populations showed mostly subtler mutations, and a few percent of mutants displayed phenotypic alterations related to the targeted genes. Immediately after germline transmission, some resources were packaged and made publicly available, including gRNA design and vector construction web servers (Mehta *et al.*, 2020; Rasheed *et al.*, 2021) ^[18, 21].

Research and Development Needs

The CRISPR/Cas9 system represents a significant leap forward in biotechnology, which offers unprecedented opportunities for researchers and breeders to improve palms. However, for state-of-the-art CRISPR/Cas9 technologies to be economically viable and widely adopted in developing countries, some bottlenecks still need to be addressed by global and local R&D efforts. Genomes of palms are gradually becoming available as genome sequencing technologies have progressed dramatically in recent years.

However, many currently sequenced palm genomes are not well assembled, polymorphisms are poorly explored, and almost all reference genomes lack annotation. Besides, there are still many widely cultivated and important palm species whose genomes have not been sequenced such as coconut palm (Schley *et al.*, 2022; Dias *et al.*, 2021) [22, 5].

Second, basic knowledge on the molecular biology of palms is necessary for the development of palm genome editing tools. To date, only three palm species have been successfully transformed with *Agrobacterium*-mediated assays. Considering the difficulties involved in banana transformation, palm transformation might be as challenging if not more difficult. Hence, genome-free CRISPR systems will be a promising solution for palms. However, since palm species are phylogenetically distant from the plants in which CRISPR systems were first discovered, palm-affine Cas9/sgRNA systems will need to be established. Tolerance of palm germplasm to high-fat oil biosynthesis-related genes such as KAS and DGAT2 is also of interest to palm breeding (Wang *et al.*, 2023; John Martin *et al.*, 2022) [26, 13]. Finally, there is a need to assess the risks of CRISPR/Cas-based genetic engineering experiments. While palms have been chronically under-explored compared to other crops, palms still occupy an area of tens of millions of hectares, many of which will be prosperous due to climate change. The main palm nuts palm oil industry will cause devastating global warming. Since no study has been published on the biosafety assessment of CRISPR tools in palms, such an assessment is the urgent first step of palm genome editing research (Khan *et al.*, 2022; Katam *et al.*, 2022) [16, 14].

Conclusion

Although various molecular breeding technologies have been developed for the genetic improvement of palms, they are still labor-intensive, time-consuming, and costly. Genome editing with the CRISPR-Cas9 system has opened new ways for palm researchers to develop elite varieties better adapted to the changing environment, such as oil palms that can thrive in a hotter, drier climate with increased drought tolerance. To date, there have been no reports of CRISPR-edited palm trees or *in vitro* clonal plants. However, many CRISPR gene editing systems have been established and optimized for oil palm. To date, there are only a few reports regarding CRISPR experiments in date palm and coconut tree. The complex regeneration process in all three important oil-producing palms (oil palm, date palm, and coconut) that rely heavily on the growth of both the shoot apical meristem (SAM) and the subsequent leaf primordia produced through the process of somatic embryogenesis is the major challenge of the application of CRISPR-Cas9 technology for palms. The existing CRISPR targeted in the oil palm control flowering time, dwarfism, and mantling architecture. In the date palm, the research has focused on five different genes, with the aim of creating a less thorny, smaller plant with short internode length, increased drought tolerance, and enhanced fruit quality, while in the coconut tree CRISPR protocols have yet to be optimized. Despite the problems and challenges, CRISPR-Cas9 technology will no doubt expedite the breeding program, particularly for trees that occupy many hectares of land.

References

1. Anjanappa RB, Gruissem W. Current progress and challenges in crop genetic transformation. *Journal of Plant Physiology*. 2021 Jun 1;261:153411.

2. Apresian SR, Tyson A, Varkkey H, Choiruzzad SAB, Indraswari R. Palm oil development in Riau, Indonesia: balancing economic growth and environmental protection. *Nusantara*. 2020;2(1):1-29.
3. Auffray T, Montúfar RX, Uquillas SX, Barragán A, Pincebourde S, Gibernau M, *et al.* Fine-scale temporal dynamics of flower visitors sheds light on insect-assemblage overlap between sexes in a dioecious Ecuadorian palm. *Biotropica*. 2023;55(1):256-267.
4. Bahariah B, Masani MYA, Rasid OA, Parveez GKA. Multiplex CRISPR/Cas9-mediated genome editing of the FAD2 gene in rice: a model genome editing system for oil palm. *Journal of Genetic Engineering and Biotechnology*. 2021;19:1-13.
5. Dias GB, Altammami MA, El-Shafie HA, Alhoshani FM, Al-Fageeh MB, Bergman CM, *et al.* Haplotype-resolved genome assembly enables gene discovery in the red palm weevil *Rhynchophorus ferrugineus*. *Scientific Reports*. 2021;11(1):9987.
6. Garcia-Robledo JE, Barrera MC, Tobón GJ. CRISPR/Cas: from adaptive immune system in prokaryotes to therapeutic weapon against immune-related diseases: CRISPR/Cas9 offers a simple and inexpensive method for disease modeling, genetic screening, and potentially for disease therapy. *International Reviews of Immunology*. 2020;39(1):11-20.
7. Guo M, Chen H, Dong S, Zhang Z, Luo H. CRISPR-Cas gene editing technology and its application prospect in medicinal plants. *Chinese Medicine*; c2022.
8. Hajiahmadi Z, Movahedi A, Wei H, Li D, Orooji Y, Ruan H, *et al.* Strategies to increase on-target and reduce off-target effects of the CRISPR/Cas9 system in plants; c2019.
9. Hasan M, Abdullah HM, Hasibuzzaman ASM, Ali MA. Date palm genetic resources for breeding. In: *Cash Crops: Genetic Diversity, Erosion, Conservation and Utilization*. 2022. p. 479-503.
10. Huang TK, Puchta H. Novel CRISPR/Cas applications in plants: from prime editing to chromosome engineering; c2021.
11. Jaskani MJ, Naqvi SA, Shafqat W, Haider MS, Awan FS, Khan IA. The Date Palm Genome; c2021.
12. Javaid N, Choi S. CRISPR/Cas system and factors affecting its precision and efficiency. *Frontiers in Cell and Developmental Biology*. 2021 Nov 24;9:761709
13. John Martin JJ, Yarra R, Wei L, Cao H. Oil palm breeding in the modern era: Challenges and opportunities. *Plants*. 2022 May 24;11(11):1395
14. Katam R, Hasanvand F, Teniyah V, Noel J, Gottschalk V. Biosafety issue related to genome editing in plants using CRISPR-Cas9. In: *Genome Editing: Current Technology Advances and Applications for Crop Improvement*. Cham: Springer International Publishing; 2022. p. 289-317.
15. Kwall K, Cotter J, Then C. Broadening the GMO risk assessment in the EU for genome editing technologies in agriculture. *Environmental Sciences Europe*. 2020;32:58.
16. Khan FS, Goher F, Zhang D, Shi P, Li Z, Htwe YM, Wang Y. Is CRISPR/Cas9 a way forward to fast-track genetic improvement in commercial palms? Prospects and limits. *Frontiers in Plant Science*. 2022;13:1042828.

17. Kordrostami M, Mafakheri M, Al-Khayri JM. Date palm (*Phoenix dactylifera* L.) genetic improvement via biotechnological approaches. *Tree Genetics & Genomes*.
18. Mehta S, Lal SK, Sahu KP, Venkatapuram AK, Kumar M, Sheri V, *et al.* CRISPR/Cas9-edited rice: a new frontier for sustainable agriculture. In: *New Frontiers in Stress Management for Durable Agriculture*. 2020. p. 427-458.
19. Sattar MN, Iqbal Z, Tahir MN, Shahid MS, Khurshid M, Al-Khateeb A, *et al.* CRISPR/Cas9: A practical approach in date palm genome editing.
20. Nordberg A, Minssen T, Holm S, Horst M, Mortensen K, Lindberg Møller B. Cutting edges and weaving threads in the gene editing (Я)evolution: reconciling scientific progress with legal, ethical, and social concerns.
21. Rasheed A, Gill RA, Hassan MU, Mahmood A, Qari S, Zaman QU, *et al.* A critical review: recent advancements in the use of CRISPR/Cas9 technology to enhance crops and alleviate global food crises. *Current Issues in Molecular Biology*. 2021;43(3):1950-76.
22. Schley RJ, Pellicer J, Ge XJ, Barrett C, Bellot S, Guignard MS, *et al.* The ecology of palm genomes: repeat-associated genome size expansion is constrained by aridity. *New Phytologist*. 2022;236(2):433-46.
23. Vats S, Kumawat S, Kumar V, Patil GB, Joshi T, Sonah H, *et al.* Genome editing in plants: exploration of technological advancements and challenges.
24. Wang G, Li J. Review, analysis, and optimization of the CRISPR *Streptococcus pyogenes* Cas9 system. *Medicine in Drug Discovery*; c2021.
25. Wang J, He Z, Wang G, Zhang R, Duan J, Gao P, *et al.* Efficient targeted insertion of large DNA fragments without DNA donors. *Nature Methods*. 2022;19(3):331-40.
26. Wang Y, Wang L, Liu H, Gou B, Hu W, Qin L, *et al.* Direct leaf-peeling method for areca protoplasts: a simple and efficient system for protoplast isolation and transformation in areca palm (*Areca catechu*). *BMC Plant Biology*. 2023;23(1):56.
27. Wu Q, Cui L, Liu Y, Li R, Dai M, Xia Z, *et al.* CRISPR-Cas systems target endogenous genes to impact bacterial physiology and alter mammalian immune responses. *Molecular Biomedicine*.
28. Zhou J, Li D, Wang G, Wang F, Kunjal M, Joldersma D, *et al.* Application and future perspective of CRISPR/Cas9 genome editing in fruit crops. *Journal of Integrative Plant Biology*. 2020;62(3):269-286.