CRISPR-Cas Systems in Modulating Fruit Ripening: Insights from Banana Research

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I. INTRODUCTION

Bananas (*Musa* spp.) are one of the most consumed fruits worldwide, contributing significantly to the diets of millions of people, particularly in tropical and subtropical regions. They rank among the top five staple crops globally in terms of both production and consumption. According to the Food and Agriculture Organization (FAO), global banana production reached approximately 153 million metric tons in 2022, with major producers including India, China, the Philippines, and Ecuador. Bananas are nutritious and may even carry the title of the first “super food” endorsed by the American Medical Association in the early 20th century as a health food for children and a treatment for celiac disease. One serving, or one medium ripe banana, provides about 110 calories, 0-gram fat, 1 gram protein, 28 grams carbohydrate, 15 grams sugar (naturally occurring), 3 grams fibre, and 450 mg potassium.

Besides their dietary importance, bananas contribute substantially to the economies of many countries, especially those in Latin America, Africa, and Asia. For example, in Ecuador, bananas represent one of the main export commodities, accounting for about 30% of the country's agricultural export revenue (FAO, 2022). Similarly, India, the largest producer of bananas, relies on the crop both for domestic consumption and as a source of livelihood for smallholder farmers. The social and economic relevance of bananas underlines the necessity of improving banana production and post-harvest management to minimize losses.

Bananas are a climacteric fruit, meaning their ripening is primarily regulated by ethylene, a plant hormone that accelerates ripening after harvest. This characteristic, however, makes bananas highly susceptible to rapid ripening and spoilage, leading to significant post-harvest losses. It is estimated that 30-40% of harvested bananas are lost before reaching consumers, primarily due to improper handling, storage, and transportation (Kumar and Turner, 2020). Post-harvest losses present a major issue for producers, consumers, and traders alike. The perishability of bananas results in a considerable reduction in their market value, particularly when premature ripening occurs during transportation or storage. This issue is even more severe in developing countries, where cold storage infrastructure is often inadequate or lacking. Consequently, premature ripening affects the entire banana supply chain, from smallholder farmers to international exporters (FAO, 2022).

Beyond economic losses, premature ripening also exacerbates food waste, contributing to global food security challenges. As the global population continues to rise, addressing this issue becomes increasingly urgent. Innovative technologies that can delay ripening and extend the shelf life of bananas are essential for reducing waste and ensuring more stable food supplies. By minimizing spoilage, such technologies can play a crucial role in enhancing both food security and the sustainability of banana production (Tian *et al*., 2021). Delaying banana ripening has been a key focus for researchers and industry stakeholders to extend storage life and reduce spoilage. Ripening is regulated by ethylene, and by

slowing its production or action, the process can be delayed. Traditional methods include modifying storage conditions, such as temperature and humidity, or using chemical inhibitors of ethylene. While effective to some extent, these methods have limitations—cold storage is energy-intensive and often inaccessible in low-income regions, and the use of chemicals raises concerns regarding safety and environmental sustainability (Kumar and Turner, 2020).

Genetic approaches offer a more sustainable solution. Conventional breeding for slow-ripening varieties has been slow and inefficient due to the banana's long reproductive cycle and polyploidy, which complicates genetic manipulation (Heslop-Harrison and Schwarzacher, 2007). Advances in molecular biology, particularly genome editing, have opened new avenues for addressing these challenges. CRISPR technology, in particular, allows precise targeting of ripening-related genes, such as those involved in ethylene biosynthesis, offering a more efficient method to delay ripening without the limitations of traditional breeding or chemical treatments (Zhu *et al*., 2020). This approach holds great promise for creating sustainable, slow-ripening banana varieties suitable for modern agricultural needs. The advent of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) technology has revolutionized genetic engineering by allowing precise, targeted DNA modifications. Coupled with the Cas9 enzyme, CRISPR enables highly specific gene editing, offering greater accuracy compared to traditional genetic modification methods. Originally discovered in bacteria as a defense mechanism, CRISPR-Cas9 has gained attention for its efficiency and versatility in editing the genomes of a wide range of organisms, including plants (Zhu *et al*., 2020).

One of the key advantages of CRISPR over conventional techniques is its ability to make precise, site-specific changes without introducing foreign DNA into the organism. This makes it particularly relevant for crops like bananas, where public perception and regulatory concerns about genetically modified organisms (GMOs) can be an issue. CRISPR allows scientists to edit endogenous banana genes that control ripening, thus addressing both technical and regulatory challenges (Sicard and Lenhard, 2011). CRISPR's first applications in plants showcased its potential in modifying traits like disease resistance, drought tolerance, and fruit ripening. For example, in tomatoes, CRISPR has been successfully used to target ethylene synthesis genes, resulting in delayed ripening and extended shelf life (Brooks *et al*., 2014). These early successes in other fruit crops have paved the way for similar applications in bananas, where controlling ripening can have a significant impact on post-harvest losses and food security (Tian *et al*., 2021). CRISPR holds enormous promise for enhancing agricultural sustainability by improving crop traits without the controversies surrounding transgenic approaches.

The use of genetic engineering (GE) for crop improvement has grown significantly in recent years. Among the key traits targeted for enhancement, improving crop quality remains a top priority. This review highlights recent advancements in CRISPR/Cas9-driven improvements in crop quality fruit ripening and explores potential future applications of genetic engineering in this field.

II. FRUIT RIPENING: MOLECULAR MECHANISMS IN BANANAS

Ripening is a complex physiological and biochemical process that results in changes in fruit texture, flavor, color, and aroma. The process is tightly regulated by a network of hormonal signaling pathways, with ethylene being the key hormone responsible for initiating and controlling ripening in climacteric fruits like bananas.Ethylene and Climacteric Ripening

Bananas undergo climacteric ripening, characterized by a significant rise in ethylene production, followed by an increase in respiration rates, referred to as the climacteric rise. Ethylene acts as a crucial signaling molecule that triggers the activation of ripening genes, ultimately leading to various physiological changes such as fruit softening, starch-to-sugar conversion, and chlorophyll degradation. (Vaughan *et al*., 2022).

Ethylene Biosynthesis Pathway in BananasEthylene biosynthesis in bananas follows the classical Yang cycle, with S-adenosylmethionine (SAM) as the precursor for ethylene production. The two key enzymes involved in ethylene biosynthesis are 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) – converts SAM to ACC (Yang, 1987). ACC oxidase (ACO) – catalyzes the conversion of ACC to ethylene.In bananas, several genes encoding ACS and ACO isoforms have been identified, with specific expression patterns during

ripening. These genes play crucial roles in regulating ethylene production during the pre-climacteric and climacteric phases.Key Genes in Ethylene Biosynthesis are MaACS1 and MaACO1: These genes are critical for ethylene production during banana ripening. Increased expression of MaACO1 coincides with the climacteric rise, indicating its role in amplifying ethylene biosynthesis (Vaughan *et al*., 2022).

Ethylene Signaling Pathway and Its Role in Ripening.The perception and signaling of ethylene are mediated by ethylene receptors, which are membrane-bound proteins that detect ethylene and initiate the downstream signal transduction cascade. This pathway involves a series of regulatory proteins that control the expression of ripening-related genes. The major components of ethylene signaling include:

Ethylene Receptors (ETR): These receptors, such as MaETR1, negatively regulate ethylene signaling in the absence of ethylene. Upon binding to ethylene, their inhibitory effect is relieved, allowing the ripening process to commence. CTR1: A negative regulator that interacts with ETR receptors to inhibit ethylene signaling in non-ripening fruit. EIN2 and EIN3: Positive regulators that mediate the ethylene response once the signaling pathway is activated (Elitzur *et al*., 2016). Ethylene signaling plays a crucial role in regulating genes responsible for cell wall degradation, chlorophyll breakdown, and starch-to-sugar conversion. Disruptions in the ethylene signaling pathway can significantly affect the timing and uniformity of ripening.

Role of Other Hormones in Ripening.Although ethylene is the primary hormone responsible for initiating ripening, other hormones such as auxin, abscisic acid (ABA), and gibberellins (GA) also play roles in modulating the ripening process (Barua *et al*., 2015). For example, auxins are thought to inhibit ripening by suppressing ethylene biosynthesis during the early stages of fruit development. However, during ripening, the balance between auxin and ethylene shifts in favor of ethylene production, allowing ripening to proceed. Research has shown that altering the levels of these hormones can impact the timing and progression of ripening, making them potential targets for genetic modification to delay the ripening process in bananas.

III. CRISPR-CAS TECHNOLOGY: A BRIEF OVERVIEW

The CRISPR story began in 1987 while studying the *iap* enzyme involved in isozyme conversion of alkaline phosphatase in *Escherichia coli*, Nakata and colleagues reported a curious set of 29 nt repeat downstream of the *iap* gene (Ishino *et al*., 1987). Unlike most repetitive elements, which typically take the form of tandem repeats like TALE repeat monomers, these 29 nt repeats were interspaced by five intervening 32 nt non-repetitive sequences. Mojica and colleagues eventually classified interspaced repeat sequences as a unique family of clustered repeat elements present in >40% of sequenced bacteria and 90% of archaea (Mojica *et al*., 2000). Jansen and Mojica (2002) coined the acronym CRISPR to unify the description of microbial genomic loci consisting of an interspaced repeat array. Several clusters of signatures CRISPR-associated (cas) genes were identified to be well conserved and typically adjacent to the repeat elements (Jansen *et al*., 2002). During 2005, systematic analysis of the spacer sequences separating the individual direct repeats suggested their extra-chromosomal and phage-associated origins (Mojica *et al*., 2005). This insight was tremendously exciting, especially given previous studies showing that CRISPR loci are transcribed (Tang *et al*., 2002) and that viruses are unable to infect archaeal cells carrying spacers corresponding to their own genomes (Mojica *et al*., 2005). These combined findings led to the speculation that CRISPR arrays serve as an immune memory and defence mechanism, and individual spacers facilitate defence against bacteriophage infection by exploiting Watson-Crick base-pairing between nucleic acids (Mojica *et al*., 2005; Pourcel *et al*., 2005).

Horvath and colleagues uncovered the first experimental evidence for the natural role of a type II CRISPR system as an adaptive immunity system, in the bacterial strain *Streptococcus thermophilus* demonstrating a nucleic-acid-based immune system in which CRISPR spacers dictate target specificity while Cas enzymes control spacer acquisition and phage defence (Barrangou *et al*., 2007).Biochemical characterizations reported by the groups of Charpentier, Doudna, and Siksnys showed that purified Cas9 from *Streptococcus thermophilus* or *Streptococcus pyogenes* can be guided by crRNAs to

cleave target DNA *in vitro*. Furthermore, a single guide RNA (sgRNA) can be constructed by fusing a crRNA containing the targeting guide sequence to a tracrRNA that facilitates DNA cleavage by Cas9 *in vitro* (Jinek *et al*., 2012).

A pair of studies simultaneously showed successful engineering of type II CRISPR systems from S. *thermophilus* and S. *pyogenes* to accomplish genome editing in mammalian cells. Heterologous expression of mature crRNA-tracrNA hybrids as well as sgRNAs was reported (Cong *et al*., 2013; Mal *et al*., 2013).

3.1Biology of CRISPR/Cas9

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated proteins) modules are adaptive antivirus immunity systems that are present in most archaea and many bacteria and function on the self-nonself discrimination principle (Makarova *et al.*, 2013). These systems incorporate fragments of alien DNA (known as spacers) into CRISPR cassettes, then transcribe the CRISPR arrays including the spacers, and process them to make a guide crRNA (CRISPR RNA) which is employed to specifically target and cleave the genome of the cognate virus or plasmid. Numerous, highly diverse Cas (CRISPR-associated) proteins are involved in different steps of the processing of CRISPR loci transcripts, cleavage of the target DNA or RNA, and new spacer integration (Makarova *et al*., 2011).

The action of the CRISPR-Cas system is usually divided into three stages: Adaptation or spacer integration.Processing of the primary transcript of the CRISPR locus (pre-crRNA) and maturation of the crRNA which includes the spacer and variable regions corresponding to 5' and 3' fragments of CRISPR repeats.DNA (or RNA) interference (Barrangou *et al*., 2013)

Two proteins, Casl and Cas2 that are present in the great majority of the known CRISPR-Cas systems are sufficient for the insertion of spacers into the CRISPR cassettes (Yosef et al., 2012). These two proteins form a complex that is required for this adaptation process; the endonuclease activity of Cas1 is required for spacer integration whereas Cas2 appears to perform a non-enzymatic function (Nunez et al., 2014). The Cas1-Cas2 complex represents the highly conserved "information processing" module of CRISPR-Cas that appears to be quasi-autonomous from the rest of the system.

The second stage, processing of pre-crRNA into the guide crRNAs, is performed either by a dedicated RNA endonuclease complex or via an alternative mechanism that involves bacterial RNase III and an additional RNA species (Deltcheva et al., 2011). The mature crRNA is bound by one (type II) or several (types I and III) Cas proteins that form the effector complex, which targets the cognate DNA or RNA (Jinek *et al*., 2012). Effector complex of type I systems is known as Cascade (CRISPR-associated complex for antiviral defense) (Brouns *et al*., 2008).

Classification of CRISPR associated protein

CRISPR systems have been grouped into six distinct types (I-VI) (Table 1) according to current classification of CRISPR/Cas loci and each employs a unique set of Cas proteins along with crRNA for CRISPR interference.

Table1: Classification of CRISPR associated protein's

Sl. No.	Types	Associated Proteins
1	Type I	Cas1, Cas2, Cas3, Cas5/Cse2, Cas6, Cas8, Cas4
2	TypeII	Cas1, Cas2, Cas4/Csn2, Cas9/Csn1
3	TypeIII	Cas1, Cas2, Cas6, Cas10, Csm2/Cmr5, Csm4/Cmr3, Csm5/ Cmr1
4	TypeIV	Csf1, Csf2, Csf3, DinG
5	TypeV	Cas1, Cas2, Cas4, Cpf2
6	TypeVI	Cas1, Cas2, C2c2,

(Source: Wright *et al.,* 2016)

3.2Mechanism of CRISPR/Cas9

Once introduced exogenously into the target cells, the Cas9-gRNA complex recognizes the (Protospacer adjacent motif) PAM sequence (NGG for S. *pyogenes* Cas9) and binds to the target DNA fragment by base pairing between the crRNA and DNA. Two Cas9 subunits, the HNH and RuvC domains, nick the crRNA-binding DNA strand and the opposite DNA strand, respectively, resulting in a site-specific double-stranded break (DSBs) three-nucleotides upstream the PAM sequence. Jiang *et al*. (2015) proposed that the Cas9-gRNA complex undergoes substantial

conformational changes for target DNA binding. A pre-ordered PAM recognition region in the Cas9-gRNA complex binds with the GG of NGG and the 10-nt cRNA seed sequence adjacent to the PAM sequence further pairs with the target DNA. Later Cas9 reaches a cleavage competent state and induces DNA cuts, thereby activating the DNA repair system of the host. PAM sequences occur every 16 nucleotides in a random DNA sequence, meaning that multiple gRNAs might be designed and applied within one or several genes simultaneously enabling multiple targeting (Xue *et al*., 2014).

The cellular DNA repair system fixes DSBs mainly through NHEJ or HDR. NHEJ is considered to be error-prone because it results in insertion or deletion (indel) mutations. These indel mutations often lead to frameshift and dysfunctional protein products. The size and type of indels is not predictable through NHEJ. HDR, which requires a donor template containing homologous sequences, can be used to insert desired DNA fragments into target sites. In this way, a gene can be targeted for disruption, termed knockout (KO), or used as a docking site for target sequence insertion, termed knock in (KI). Typically, the DSB is repaired by the copying of DNA sequences from a repair template that is homologous to the DSB-flanking region. If such a template is provided exogenously, genetic changes such as, point mutations and epitope tags, can be introduced into the chromosome at or near the DSB site. This permited scientists to construct a "dead" or nuclease-inactive Cas9 (dCas9). dCase has no or incomplete nuclease activity but retains the capability to act as an RNA-guided DNA binding protein 79 (Doudna and Charpentier, 2014). If one domain, either NH or RuvC, is inactive, dCase induces a nick in only one DNA strand. To improve on-target DSB specificity, a double-nicking approach can be used to increase the overall number of bases that are specifically recognized in the target DNA. By using pairs of gRNAs and a dCas mutant, properly spaced cooperative nicks can mimic DSBs and mediate efficient indel formation (Hsu *et al.,* 2014; Ran *et al*., 2013; Sander and Joung, 2014). Because off-target nick sites are precisely repaired, this multiplexed nick strategy can improve specificity by up to 1,500x relative to the wild type Cas9 (Ran *et al*., 2013).

IV. CRISPR APPLICATIONS IN FRUIT CROPS

Collection of traits improved by CRISPR/Cas9 and CRISPR/Cas12 in different fruit. (Martín *et al* 2023).

CRISPR-Cas Delivery	Fruit Trees	Target Gene Edited	Improved Traits
A. tumefaciens-mediated	Apple	*PDS*	Enhanced biosynthesis of carotenoid
		PDS and *TFL1*	Albino phenotype and early flowering
		MdDIPM1 and *MdDIPM4*	Fire blight resistance
		ALS	Chlorsulfuron resistance
		CNGC2	*B. dothidea* resistance
		MsPDS	Albino phenotype
		MdMKK9	Increased anthocyanin content
			Detection of viruses and viroids with CRISPR
	Banana	*RAS-PDS1* and *2*	Albino phenotype
		PDS	Albino phenotype and dwarfing

CRISPR-Cas Delivery	Fruit Trees	Target Gene Edited	Improved Traits
		eBSV	Control of virus pathogenesis
		MaGA20ox2	Dwarf phenotype
		LCYε	Carotene biosynthesis
		MaACO1	Fruit ripening
	Cacao	*TcNPR3*	*Phytophthora tropicalis* resistance
	Citrus	*CsPDS*	Method optimization
		Cs7g03360	Phenotypic changes in Carrizo leaves
		CsLOB1	Citrus canker resistance
		CsLOB1 promoter	Citrus canker resistance
		PDS and *CsLOB1*	Albino phenotype and canker resistance
		CsWRKY22	Citrus canker resistance
		CsLOB1	Citrus canker resistance
		pC-PDS1 and *Pc-PDS2*	Chlorophyll and carotenoid content
		CsLOB1	Citrus canker resistance
	Grape	*IdnDH*	High tartaric acid biosynthesis
		VvPDS	Increased carotenoid biosynthesis
		VvWRKY52	*Botrytis cinerea* resistance
		VvPDS	Albino phenotype
		VvMLO7	Powdery mildew resistance
		VvPR4b	Downy mildew resistance
		MYBA5/6/7 and *TAS4a/b*	Anthocyanin accumulation
		VvMLO3 and *VvMLO4*	Powdery mildew resistance
		TMT1 and *TMT2*	Reduced sugar accumulation
		TMT1 and *DFR1*	Flavonoid accumulation
	Kiwifruit	*CEN4* and *CEN*	Terminal flower and fruit
		AcPDS	Albino phenotype (leaves)
		AcBFT2	Reduced dormancy and early bud break
	Papaya	*CpDreb2*	Gene disruption for water stress

CRISPR-Cas Delivery	Fruit Trees	Target Gene Edited	Improved Traits
		PpalEPIC8	*Phytophthora palmivora* resistance
		Ppal15kDa	*Phytophthora palmivora* resistance
	Pear	*TFL1*	Early flowering
		PbPAT14	Dwarf yellowing phenotype
		PDS and *ALS*	Albino phenotype and chlorsulfuron resistance
PEG-meditated	Apple	*DIPM-1, -2* and *-4*	Fire blight resistance
	Banana	*PDS*	Method optimization
	Chestnut	*PDS*	Method optimization
	Grape	*MLO7*	Enhanced biotic resistance against powdery mildew
		DMR6 and *MLO6*	Downy and powdery mildew resistance
		GFP	Method optimization
	Orange	*CsNPR3*	Induced biotic stress tolerance
		PH5	Method optimization

Jiang *et al*. (2019) advanced the application of CRISPR/Cas9 technology by targeting multiple genes involved in ethylene biosynthesis simultaneously. The researchers focused on *MaACS1* and *MaACO1*, which encode ACC synthase and ACC oxidase, respectively. These enzymes catalyse sequential steps in the ethylene production pathway. By designing gRNAs specific to both *MaACS1* and *MaACO1*, a multiplex gene editing approach was achieved. The edited banana plants exhibited a more substantial reduction in ethylene production than single-gene edits. Gas chromatography results confirmed markedly lower ethylene emission, and physiological assays showed a significant delay in ripening, with fruits remaining green and firm for an extended period.

Down regulating ethylene biosynthesis using CRISPR/Cas9 not only extends shelf life but also impacts various physiological and biochemical processes in bananas. Studies by Liu *et al*. (2021), revealed that CRISPR-mediated *MaACO1* mutagenesis led to reduced expression of genes associated with cell wall degradation and softening enzymes, thereby maintaining fruit firmness. Additionally, downregulated ethylene biosynthesis can influence sugar metabolism. Research by Gao *et al*. (2020), showed that CRISPR-edited bananas with reduced *MaACS1* expression exhibited altered expression of genes involved in starch degradation and sucrose metabolism, potentially contributing to extended shelf life.

Hu *et al*. (2021) reported that the CRISPR/Cas9 gene-editing system was employed to target and knock out the *MaACO1* gene in banana. The *MaACO1* gene encodes 1-aminocyclopropane-1-carboxylate oxidase (ACO), an enzyme crucial for the final step in ethylene biosynthesis. Specific guide RNAs (gRNAs) were designed to target the *MaACO1* gene, researchers were able to induce targeted mutations resulting in gene knockout. The edited banana demonstrated a significant reduction in ethylene production compared to the wild-type controls. This reduction was confirmed through gas chromatography, which quantified the ethylene levels in the edited plants. Additionally, phenotypic analysis of the edited plants showed delayed ripening, characterized by slower colour change and prolonged firmness.

V. CONCLUSION

The ripening process in bananas is a highly coordinated event controlled by a complex interplay of hormones, with ethylene being the central regulator. The molecular mechanisms underlying ethylene biosynthesis, signaling, and the subsequent activation of ripening-related genes provide critical insights for potential genetic interventions. CRISPR/Cas9 technology, by targeting key genes such as *MaACO1* and *MaACS1*, offers a promising approach to delay ripening and extend the shelf life of fruits. Understanding the molecular basis of ripening not only advances basic science but also paves the way for developing sustainable agricultural practices aimed at reducing post-harvest losses. The application of CRISPR-Cas9 technology for delaying banana ripening represents a groundbreaking advancement in agricultural biotechnology. By precisely targeting ripening-related genes, this approach offers a sustainable solution to post-harvest losses while improving fruit quality and marketability. While regulatory challenges and consumer acceptance remain hurdles, continued research and innovation in CRISPR technology hold great promise for revolutionizing the banana industry. As global food security concerns grow, CRISPR-mediated improvements in fruit crops will play a pivotal role in ensuring a more resilient and sustainable food supply chain.

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