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FOCUSED REVIEW

RNAi and genome editing of sugarcane: Progress and prospects

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SUMMARY

Sugarcane, which provides 80% of global table sugar and 40% of biofuel, presents unique breeding challenges due to its highly polyploid, heterozygous, and frequently aneuploid genome. Significant progress has been made in developing genetic resources, including the recently completed reference genome of the sugarcane cultivar R570 and pan-genomic resources from sorghum, a closely related diploid species. Biotechnological approaches including RNA interference (RNAi), overexpression of transgenes, and gene editing technologies offer promising avenues for accelerating sugarcane improvement. These methods have successfully targeted genes involved in important traits such as sucrose accumulation, lignin biosynthesis, biomass oil accumulation, and stress response. One of the main transformation methods—biolistic gene transfer or *Agrobacterium*-mediated transformation—coupled with efficient tissue culture protocols, is typically used for implementing these biotechnology approaches. Emerging technologies show promise for overcoming current limitations. The use of morphogenic genes can help address genotype constraints and improve transformation efficiency. Tissue culture-free technologies, such as spray-induced gene silencing, virus-induced gene silencing, or virus-induced gene editing, offer potential for accelerating functional genomics studies. Additionally, novel approaches including base and prime editing, orthogonal synthetic transcription factors, and synthetic directed evolution present opportunities for enhancing sugarcane traits. These advances collectively aim to improve sugarcane's efficiency as a crop for both sugar and biofuel production. This review aims to discuss the progress made in sugarcane methodologies, with a focus on RNAi and gene editing approaches, how RNAi can be used to inform functional gene targets, and future improvements and applications.

Keywords: RNA interference, VIGS, SIGS, VIGE, gene editing, TALEN, CRISPR, synthetic transcription factors, bioenergy, oilcane, biomass oil, lignin, sucrose, energycane.

INTRODUCTION

Sugarcane (*Saccharum* spp. hybrid) is cultivated across more than 110 countries on over 27 million hectares, providing 80% of global table sugar and 40% of global biofuel (OECD/FAO, 2023). Genetic improvement of sugarcane faces significant challenges due to its complex highly polyploid, heterozygous, and frequently aneuploid genome (2*n* = 100–120), which originated from interspecific hybridization between *Saccharum officinarum* (2*n* = 80) and *Saccharum spontaneum* (2*n* = 36–128) (Healey et al., 2024; Lovejot et al., 2017; Premachandran et al., 2011). Traditional breeding of new sugarcane cultivars is a lengthy process, typically requiring 12–15 years for parent identification, flowering synchronization, and progeny evaluation. The highly heterozygous nature of the genome necessitates vegetative propagation to maintain elite cultivar performance (Healey et al., 2024; Hoang et al., 2015; Raboin et al., 2008; Wang et al., 2022).

Biotechnology approaches offer tremendous opportunities to accelerate sugarcane improvement, but they require knowledge of candidate gene sequence and function (Mohan et al., 2022). While the large genome size (>10 Gb) and high frequency of sequence repeats initially hindered genome assembly efforts, several genetic

resources are now available. These include the recently published complete reference genome of cultivar R570, which represents unique DNA sequences across approximately 12 chromosome copies (Healey et al., 2024). Earlier resources include a mosaic monoploid genome of R570 (Garsmeur et al., 2018) and a representative gene space assembly of cultivar SP80-3280 (Souza et al., 2019). The close diploid relative, *Sorghum bicolor*, provides pan-genomic resources for translational genomics, with studies showing 95.5% sequence identity between a bacterial artificial chromosome (BAC) library of sugarcane cultivar R570 and the sorghum reference genome (Federico et al., 2022; Figueira et al., 2012; Wang et al., 2010). These resources have facilitated research into economically relevant traits such as sucrose accumulation, lignin biosynthesis, biomass oil accumulation, and stress tolerance (Khan, 2021; Laksana et al., 2024; Mehdi et al., 2024; Qin et al., 2021).

Modern sugarcane cultivars inherit 70–80% of their genome from *S. officinarum* and 20–25% from *S. spontaneum*, with 5–10% comprising recombinant chromosomes (Garsmeur et al., 2018; Healey et al., 2024; Mudge et al., 2009). Energycane cultivars, with a higher proportion of the *S. spontaneum* genome, exhibit increased biomass, tiller number, fiber content, and persistence, but reduced sugar content and stem diameter (Matsuoka et al., 2014). The expanding availability of genomic resources for this most complex of all crop genomes is enabling novel genetic modification technologies, including transgene overexpression, RNAi, and gene editing. However, challenges persist in transformation, target selection, high genetic redundancy, and mutant screening (Brant, May, et al., 2024; May et al., 2023). The highly polyploid nature of the sugarcane genome typically requires modification of all gene copies/alleles to generate a "loss of function" phenotype for the target locus. This requires highly efficient transformation and editing tools, as well as extensive screening of mutants (May et al., 2023). While the highly polyploid genome presents challenges for complete target locus modification, it also offers advantages for fine-tuning of the editing outcomes depending on the number of copies/alleles that are co-edited (Brant, Eid, et al., 2024; Eid et al., 2021).

RNAi studies, where gene silencing is achieved to similar or varying levels, can provide valuable insight into potential genomic targets for knockouts, as well as the desired extent of allelic co-editing in sugarcane.

SUGARCANE TRANSFORMATION

Transformation methods in sugarcane

The main methods for sugarcane transformation are biolistic gene transfer (BGT) and *Agrobacterium*-mediated transformation (AMT; Figure 1). While polyethylene glycol-mediated protoplast transformation has demonstrated success historically, its poor reproducibility and diminished agronomic performance of regenerated plants due to extended tissue culture periods have limited its practical application (Evans et al., 1980; Larkin, 1981; Srinivasan & Vasil, 1986).

BGT utilizes a gene gun to deliver DNA-coated micro-particles of tungsten or gold into cell cultures (Altpeter & Sandhu, 2010; Klein et al., 1987). While traditional protocols used large quantities of whole plasmid DNA, modern approaches employ minimal cassettes, resulting in lower copy numbers and improved transgene expression stability without undesired backbone DNA integration (Breitler et al., 2002; Fu et al., 2000; Lowe et al., 2009; Sandhu & Altpeter, 2008). BGT's advantages include the ability to simultaneously deliver multiple unlinked constructs with flexible host or tissue requirements due to its mechanical nature (Altpeter et al., 2005, 2016; Lacroix & Citovsky, 2020). However, the method can cause tissue and chromosome damage, along with transgene fragmentation when using high DNA and particle quantities (Liu et al., 2019). Therefore, careful optimizations of tissue culture conditions, DNA concentration and quality, biolistic parameters, and post-bombardment selection are necessary (Taparia, Gallo & Altpeter, 2012; Budeguer et al., 2021).

The first successful BGT demonstration in sugarcane by Bower and Birch in 1992, which was followed by optimization for herbicide resistance through *bar* gene expression (Gallo-Meagher & Irvine, 1996), established a foundation that continues to influence current research directions and applications.

AMT leverages *Agrobacterium tumefaciens'* natural ability to transfer DNA into host genomes. First demonstrated in sugarcane in 1998 with the *gusA* gene (Arencibia et al., 1998) and subsequently with the *bar* gene (Enríquez-Obregón et al., 1998), AMT typically produces lower transgene copy numbers and reduced fragmentation, facilitating regulatory approval (Jackson et al., 2013). However, the method shows limitations for co-delivering multiple constructs and exhibits high genotype dependency in monocot species (Rahman et al., 2024; Shrawat & Lörz, 2006).

Comparative studies have revealed the relative strengths of both methods. Jackson et al. (2013) demonstrated comparable transformation efficiencies between AMT and low-DNA BGT in sugarcane genotype Q117, though BGT events exhibited superior field performance (Joyce et al., 2013). A comprehensive study by Wu et al. (2015) across 12 experiments with the CP88-1762 genotype found no significant differences between methods in transformation efficiency, single copy integration frequency, or transgene expression stability. While Jung and Altpeter (2016) observed higher editing efficiency with AMT

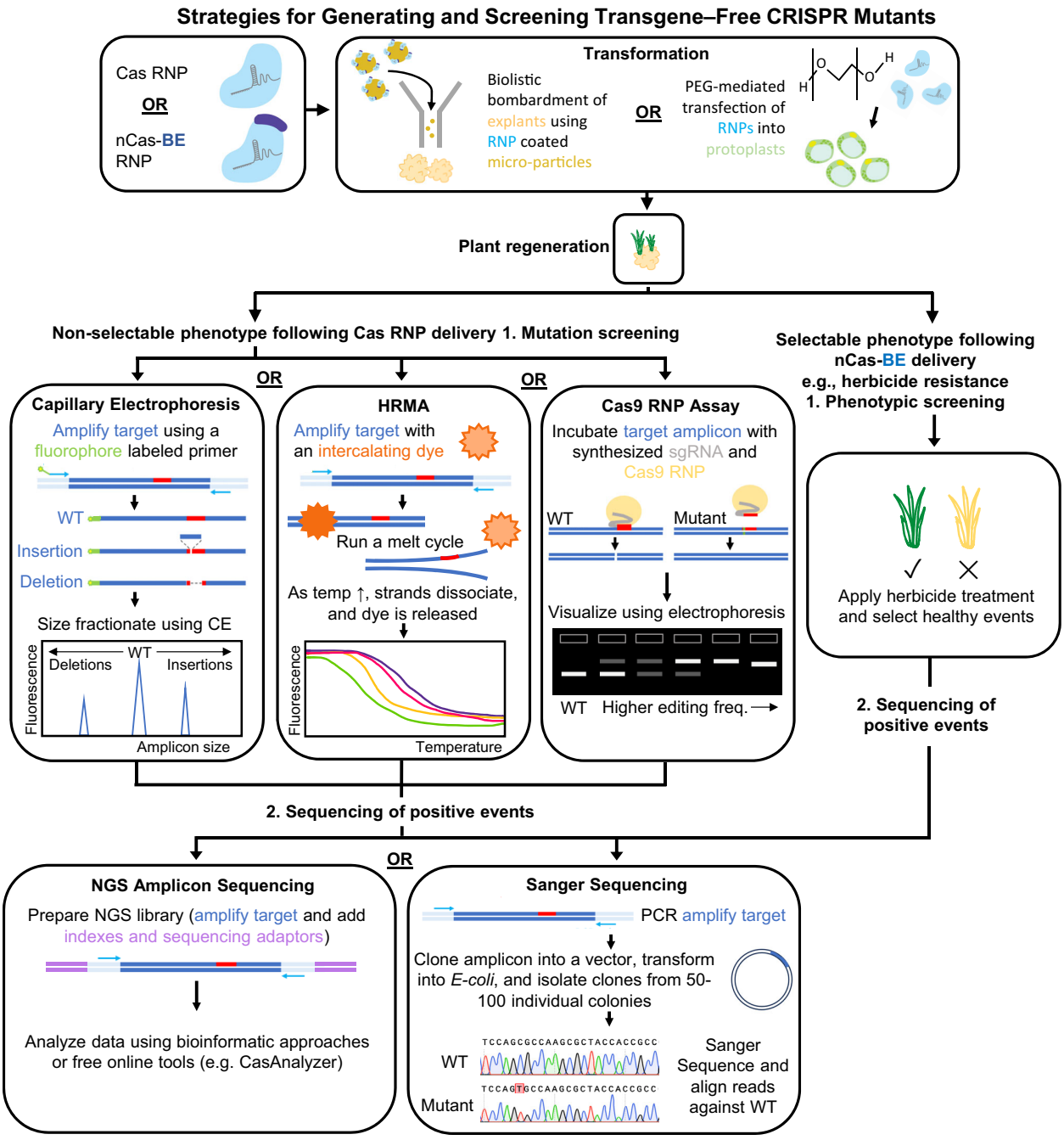

Figure 1. Strategies for generation, screening, and confirmation of transgene-free sugarcane plants.
Cas9 RNP and nCas-base editor RNP systems could be used alongside guide RNAs to generate transgene-free sugarcane plants. Biolistics and PEG-mediated transformation are the two available methods for RNP delivery, with biolistic transformation holding the benefit of fewer somaclonal variations and accelerated plant regeneration. Capillary electrophoresis, high-resolution melt analysis (HRMA), or Cas9 RNP assays could then be used to screen for mutants with non-selectable phenotypes, followed by Sanger Sequencing or Next Generation Sequencing (NGS) technologies to confirm mutation sequence and size. A selectable phenotype can be generated via nCas base editor-mediated precision nucleotide substitutions in genes like acetolactate synthase conferring a selectable herbicide-resistant phenotype. This will facilitate recovery of co-edited target genes and streamline the analysis of regenerated plants.

(74%) compared to BGT (30%) with transcription activator-like effector nucleases (TALEN), subsequent field trials again showed better agronomic performance of BGT events (Kannan et al., 2018).

Recent research has focused on addressing AMT's genotype limitations in sugarcane, with progress in optimization and expanded applications (Mohan et al., 2020).

Sugarcane tissue culture and regeneration of transgenic cells

Efficient sugarcane transformation depends on reliable tissue culture protocols that enable the production of totipotent, de-differentiated cells from meristematic explants capable of plant regeneration post-transformation. These cultures are initiated and maintained on media containing varying levels of phytohormones (Chen et al., 1988; Chengalrayan & Gallo-Meagher, 2001). Two primary routes exist for inducing embryogenesis: direct somatic embryogenesis (DSE), where embryos form directly from isolated tissues (Shah et al., 2009; Taparia, Fouad, et al., 2012; van der Vyver, 2010), and indirect somatic embryogenesis (ISE), involving callus formation before embryogenesis (Bower & Birch, 1992; Taparia, Gallo, & Altpeter, 2012).

While both methods have proven successful, ISE is generally preferred due to its higher transformation efficiency and lower escape rates, despite requiring longer culture periods (~18 vs. ~12 weeks for DSE) and increased risk of somaclonal variations (Kaeppler et al., 2000; Snyman et al., 2001). Regeneration efficiency depends on multiple factors, including explant type and quality, genotype, medium composition, phytohormone balance, culture duration, and environmental conditions. Most effective protocols utilize either immature inflorescences (Desai et al., 2004; Joshi et al., 2013; Liu, 1993; Wang, Javed, et al., 2024) or leaf whorl cross-sections (Brant, Eid, et al., 2024; Taparia, Gallo, & Altpeter, 2012; Ullah et al., 2016). Leaf whorls are often preferred due to year-round availability from maintained sugarcane stands, whereas immature inflorescences require specific seasonal or controlled growth conditions (Joshi et al., 2013). Alternative explants like basal or axillary meristems have shown lower transformation efficiencies (Enríquez-Obregón et al., 1998; Manickavasagam et al., 2004).

Compared to its monocot relatives, sugarcane's tissue culture and regeneration protocols are notably more reproducible and less genotype-dependent. Despite high sequence conservation with sorghum (Figueira et al., 2012; Wang et al., 2010), the latter remains more recalcitrant to tissue culture, with only one routinely transformable genotype, Tx430 (Parikh et al., 2021). Similar limitations exist in maize (Kausch et al., 2021), wheat (Lee et al., 2024), rye (Popelka et al., 2003), barley (Fang et al., 2002), and switchgrass (Xu et al., 2022), where tissue culture recalcitrance restricts experiments to few amenable genotypes (Long et al., 2022; Lowe et al., 2016).

A breakthrough came in 2016 when Lowe et al. demonstrated that overexpressing morphogenic genes *BABY BOOM* (*BBM*) and *WUSCHEL2* (*WUS2*) could overcome this recalcitrance in multiple crops, achieving up to 40% transformation efficiency in previously un-transformable genotypes. This approach has been refined for various crops and explant types (Johnson et al., 2023; Nelson-Vasilchik et al., 2022; Wang et al., 2023; Xu et al., 2022). Other successful morphogenic genes include *GROWTH-REGULATING FACTOR 4* and its cofactor *GRF-INTERACTING FACTOR 1* (Debernardi et al., 2020), as well as *GROWTH-REGULATING FACTOR 5*, *KNOTTED 1*, *WUSCHEL-RELATED HOMEOBOX 5*, *LEAFY COTYLEDON 1*, and *LEAFY COTYLEDON 2* (Lee & Wang, 2023; Yan et al., 2023). However, controlling the expression of morphogenic genes and their removal is typically necessary post-regeneration to prevent developmental abnormalities (Lowe et al., 2018) and ensure successful commercialization (Singh et al., 2013).

Modes for improving and advancing sugarcane transformation

While several elite sugarcane genotypes can be transformed with high efficiency, energycane cultivars remain largely recalcitrant to tissue culture (Luo et al., 2022). The time, resource, and labor-intensive nature of tissue culture and transformation necessitates continued exploration of novel technologies.

Recent advances in AMT of other monocotyledonous crops show promising potential for enhancing transformation efficiencies in sugarcane. Novel ternary vector systems incorporating additional accessory helper plasmids carrying extra virulence genes have been developed, expanding cargo capacity compared to superbinary vectors (Aliu et al., 2024). These systems have demonstrated up to 103% transformation efficiency in an elite maize inbred line (Anand et al., 2018; Zhang et al., 2020) and a 7.71-fold increase in sorghum when combined with *GRF4-GIF1* morphogenic genes (Li et al., 2024).

Further improvements include the development of thymidine auxotrophic *Agrobacterium* strains (Aliu et al., 2024). Ranch et al. (2012) addressed common overgrowth issues by generating the LBA4404Thy- strain through *THYMIDYLATE SYNTHASE* gene mutation or removal. This allows reduction of required antibiotic loads post-cocultivation, when medium supplementation with thymine is discontinued to suppress *Agrobacterium* overgrowth. This advancement has been implemented in various strains including AGL1, EHA101, EHA105, and EHA105D, and has also been explored with methionine auxotrophy (Aliu et al., 2024; Prías-Blanco et al., 2022; Tamzil et al., 2021).

SUGARCANE GENOME MODIFCATION

Over expression transgenesis

In terms of genome modification, overexpression (OE) transgenesis has successfully enhanced sugarcane's biotic and abiotic stress tolerance. Recent comprehensive reviews by Budeguer et al. (2021) and Dinesh Babu et al. (2022) detail these improvements. Sugarcane's vegetative propagation capability provides significant

advantages for multi-gene OE studies and gene stacking through re-transformation, as it prevents transgene segregation and maintains initial genetic backgrounds (Altpeter & Karan, 2018). The absence of viable pollen and seed production under typical growing conditions enhances transgenic plant containment and biosafety during field testing (Altpeter & Oraby, 2010).

Given sugarcane's importance as a biofuel feedstock, significant research has focused on reducing lignocellulosic biomass recalcitrance to saccharification for bio-ethanol production (reviewed by Budeguer et al., 2021; Dinesh Babu et al., 2022) and metabolic engineering for increased biomass oil or biomass production. Notable success has been achieved in triacylglycerol (TAG) hyper-accumulation through overexpression of lipogenic genes like *WRINKLED1* (*WRI1*), *OLEOSIN* (*OLE*), and *DIACYLGLYCEROL ACYLTRANSFERASE* (*DGAT*), combined with TAG hydrolysis suppression via RNAi (Cao, Kannan, et al., 2023; Cao, Luo, et al., 2023; Luo et al., 2022; Parajuli et al., 2020; Zale et al., 2016). These "oilcane" plants have demonstrated impressive field performance, yielding 0.38 MT/ha of vegetative lipid with 88.7% recovery efficiency, approaching soybean oil yield levels (Cao, Kannan, et al., 2023; Kannan, Liu, et al., 2022; Maitra et al., 2024).

RNA antisense (RNAa) and RNAi

Gene silencing through RNA antisense (RNAa) and RNAi technologies are evolutionarily conserved mechanisms spanning from algae to animals (Agrawal et al., 2003; Dana et al., 2017). RNAa technology utilizes single-stranded RNA to trigger degradation of mRNA prior to its translation (Santini et al., 2022; Tilahun et al., 2021). When a plasmid containing a DNA antisense fragment is introduced into a cell, the antisense RNA binds to its complementary "native sense" RNA strand, forming a duplex. This complex is recognized by RNAse H enzyme, which degrades the mRNA-antisense RNA duplex in the nucleus (Abhary & Rezk, 2016; Bird & Ray, 1991; Frizzi & Huang, 2010; Santini et al., 2022).

The first commercial application of RNAa in plants was the "FlavrSavr" tomato, which used an antisense copy of *POLYGLACTURONASE* (*PG*) to reduce cell wall degradation during ripening (Frizzi & Huang, 2010). Applications of antisense technology in sugarcane include down-regulation of *PYROPHOSPHATE: FRUCTOSE 6-PHOSPHATE 1-PHOSPHOTRANSFERASE*, enhancing sucrose accumulation in immature internodes (Groenewald & Botha, 2008). Wang et al. (2009) demonstrated an antisense fragment's ability to inhibit the ACC OXIDASE enzyme, which negatively impacted plant development through dwarfism and delayed development. RNAa was also used by Rossouw et al. (2010) to reduce neutral invertase activity in sugarcane, resulting in an increase in sucrose synthase activity, as well as an increased sucrose-to-hexose ratio.

RNAi, in comparison, is more efficient by delivering double-stranded RNA directly to cells or using RNAi hairpin constructs (Fire et al., 1998; Waterhouse et al., 1998). RNAi plays crucial roles in epigenetic regulation of endogenous genes and preserving genome integrity by eliminating foreign nucleic acids from viruses and transposable elements (Brant & Budak, 2018; Holoch & Moazed, 2015; Kim & Rossi, 2008; Obbard et al., 2009). RNAi has been adapted for targeted gene silencing in functional genomics and crop improvement (Dana et al., 2017; Koeppe et al., 2023; Napoli et al., 1990).

Gene suppression through RNAi can occur via post-transcriptional gene silencing (PTGS) through RNA destabilization or translation inhibition, or via transcriptional gene silencing (TGS) through DNA methylation (Liu et al., 2020). The RNAi mechanism involves the processing of double-stranded RNA (dsRNA) by DICER and ARGONAUTE proteins. In the cytoplasm, DICER cleaves the dsRNA into 20–30 nucleotide sequences. An ARGONAUTE protein then binds to the short interfering RNA (siRNA), forming an RNA-Induced Silencing Complex (RISC). This complex separates the RNA strands and uses the antisense strand to locate and bind to complementary mRNA. Endonucleolytic cleavage of the target mRNA sequence by the catalytically active ARGONAUTE protein requires a near-perfect complementary match between the guide strand and target mRNA sequence. Alternatively, a partial sequence match between the guide strand and target mRNA leads to translational repression (Pratt & MacRae, 2009).

RNAi technology has been successfully applied to various polyploid plant species, including banana (Dang et al., 2014; Shekhawat et al., 2012), potato (Hameed et al., 2017; Sun et al., 2016), and wheat (Christensen et al., 2004; Loukoianov et al., 2005; Schweizer et al., 2000; Yan et al., 2004).

For RNAi in plants, hairpin constructs (hpRNA) typically require sense/antisense arm lengths of approximately 100 base pairs with high target region homology. However, these can be much longer without compromising efficiency. This is longer than the 21–23 bp siRNA's due to processing needs for DICER (Smith et al., 2000; Wesley et al., 2001). Using an intron as a spacer between sense and antisense arms can elevate silencing efficiency to nearly 100%, likely due to lariat formation during splicing that enhances RNA duplex formation (Wesley et al., 2001).

RNA-DEPENDENT RNA POLYMERASE amplifies dsRNA in plants, generating secondary small RNAs for systemic RNAi spread (Baulcombe, 2007). This makes RNAi a powerful tool for delivery strategies that do not require stable transgene integration into the host genome (Liu et al., 2020).

However, researchers have reported unintended silencing of off-target genes during RNAi applications

(Grimm, 2011; Senthil-Kumar & Mysore, 2011; Xu et al., 2006). To minimize these effects and maximize on-target gene silencing, specialized software tools have been developed to improve precision and effectiveness (Ahmed et al., 2020).

RNAi in sugarcane

RNAi technology has presented significant opportunities for sugarcane improvement and functional genomics. While the primary challenge of RNAi in sugarcane revolves around identifying conserved target regions capable of silencing multiple gene copies and alleles, recent publication of a reference genome has greatly streamlined this approach (Garcia et al., 2022; Healey et al., 2024; Lawrence & Pikaard, 2003).

Several studies have demonstrated RNAi's potential in sugarcane (Table 1). Osabe et al. (2009) successfully downregulated the *PHYTOENE DESATURASE* (*PDS*) gene, creating photobleached plants by targeting five different alleles using a 498 bp RNAi hairpin construct. This resulted in four independent transgenic lines with reduced *PDS* expression.

Research focusing on biofuel production has been particularly promising. Studies targeting lignin biosynthesis genes like *CAFFEIC ACID O-METHYLTRANSFERASE* (*COMT*), *4-COUMARATE: COENZYME A LIGASE* (*4CL*), *or FERULATE 5-HYDROXYLASE* (*F5H*) reported reduced sugarcane recalcitrance in biofuel production by increasing the fermentable sugar yields from lignocellulosic biomass (Jung et al., 2012, 2013). The *COMT* RNAi construct, targeting a highly conserved region with 95–100% nucleotide identity across multiple sequences, achieved a 97% decrease in gene expression (Jung et al., 2012). Field trials demonstrated up to 12% total lignin reduction and increased saccharification efficiency between 28 and 32% (Jung et al., 2013). Transgenic sugarcane with RNAi suppression of *4CL* displayed a reduction of up to 16.5% of total lignin content and improved saccharification efficiencies of 52–76% compared to wild-type controls in the field (Jung et al., 2016). These results highlighted for the first time how RNAi can be applied to add value to lignocellulosic feedstocks in biochemical processing for bioethanol production. Bewg et al. (2016) confirmed that the RNAi suppression of the lignin biosynthetic genes *COMT* and *FERULATE 5-HYDROXYLASE* (*F5H*), in contrast to *CAFFEOYL-COA O-METHYLTRANSFERASE* (*CCoAOMT*) suppression, improved cell wall saccharification without reduction in soluble sugar production.

Viral resistance has been another significant application. Guo et al. (2015) used an RNAi hairpin to target the coat protein of sorghum mosaic virus, achieving 87.5% resistance. Similarly, Aslam et al. (2018) developed an RNAi construct against the sugarcane mosaic virus, reducing coat protein expression by 80–90% in different genotypes. Widyaningrum et al. (2021) further demonstrated that promoter selection significantly impacts resistance, with the *Zea mays Ubiquitin* promoter generating 82.3% resistant lines compared to 57.6% with the *CaMV*-promoter.

Glassop et al. (2017) investigated the role of SUCROSE TRANSPORTER 1 (SUT1) protein in the transport of sucrose in sugarcane plants. This group identified six *SUT1* variants and designed a hairpin construct to target the 3′-UTR adjacent sequence in the *SUT1* native copies. No correlation between the silencing of *SUT1* and the accumulation of sucrose was observed (Glassop et al., 2017).

Most recently, Tang et al. (2024) simultaneously targeted three *TONOPLAST SUGAR TRANSPORTER* genes, *TST1*, *TST2b-1A*, and *TST2b-1C*, reducing stem sucrose content by 80% and highlighting these genes' critical role in sugar accumulation.

Metabolic pathway engineering has also benefited from RNAi approaches. Zale et al. (2016) and Parajuli et al. (2020) combined RNAi with gene overexpression to manipulate biomass oil accumulation. By suppressing genes like *PEROXISOMAL ABC TRANSPORTER1* (*PAX1*), *ADP-GLUCOSE PYROPHOSPHORYLASE* (*AGPase*), *SUGAR DEPENDENT1* (*SDP1*), and/or *TRIGALACTOSYL DIACYLGLYCEROL1* (*TGD1*) while overexpressing key metabolic genes, triacylglycerol accumulation increased in leaf tissue up to 400-fold and in stem tissue up to 72-fold compared to wild-type (WT) plants (Parajuli et al., 2020; Zale et al., 2016). Zale et al. (2016) described high levels of *PXA1* (20% of WT) and *AGPase* (1% of WT) silencing in the highest TAG accumulating line (Zale et al., 2016).

Hormone regulation represents another innovative application. Neris et al. (2022) silenced ethylene production genes *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASES 1; 2; 3* (*ScACS1*, *ScACS2*, *ScACS3*), resulting in increased growth, faster lateral germination, and significant physiological changes. Transgenic lines showed a 31% height increase and a 193.6% increase in leaf area compared to wild type.

These diverse applications underscore RNAi's versatility in sugarcane improvement. By enabling targeted gene suppression across various metabolic pathways, viral resistance, and developmental processes, RNAi offers a powerful tool for crop enhancement. The technique's ability to silence multiple gene copies simultaneously makes it particularly valuable for polyploid crops like sugarcane.

The research demonstrates that RNAi can be effectively used for sugarcane improvement and characterization of candidate genes. These outcomes can be used to highlight targets for improving agronomic traits with targeted mutagenesis by gene editing, particularly if used in combination with traditional gene over-expression.

Genome editing technologies

Site-specific nucleases (SSN) have revolutionized genome modification by introducing double or single-strand breaks

Table 1 Use of RNAi and RNA-antisense (RNAa) technology in sugarcane

	Target gene name	Length sense/ antisense (bp)	No. of transgenic lines: generated/with suppression/with phenotype	Target gene suppression compared to WT	Observed phenotype	Field test	References
RNAi	*PDS (PHYTOENE DESATURASE)*	498	4/3/3	NR	Photobleaching	N	Osabe et al. (2009)
	COMT (CAFFEIC ACID O-METHYLTRANSFERASE)	346	38/3/3	67–97% ↓	Reduced lignification by 3.9 −14%	N	Jung et al. (2012)
		346	38/4/4	80–91% ↓	Reduced lignification by 5.5 −12%	Y	Jung et al. (2013)
	SbMV-CP (SORGHUM MOSAIC VIRUS-COAT PROTEIN)	423	16/14/14	NR (No virus detected by RT-PCR)	Improved resistance to SrMV	N	Guo et al. (2015)
	COMT (CAFFEIC ACID O-METHYLTRANSFERASE)	400	8/3/1	21–32% ↓*	Reduced lignin content by 9.5%	N	Bewg et al. (2016)
	FSH (FERULATE 5-HYDROXYLASE)	400	9/3/1	4–84% ↓*	Improved glucose release		
	CCoAOMT (CAFFEOYL-COA O-METHYLTRANSFERASE)	400	9/3/1	83–97% ↓*			
	PXA1 (PEROXISOMAL ABC TRANSPORTER 1) + *AGPase (ADP-GLUCOSE PYROPHOSPHORYLASE)*	*PXA1:* 200 *AGPase*: 205	61/11/10	*PXA1:* 27–86% ↓ *AGPase*: 97–99% ↓	Increased TAG accumulation (stem = 11- to 43-fold) (leaves = 37- to 95-fold) in combination with OE of lipogenic genes	N	Zale et al. (2016)
	Sh4CL1 (4-COUMARATE: COENZYME A LIGASE)	212	60/8/7	0–97% ↓	Reduced lignification by 10 −17%	Y	Jung et al. (2016)
	ShSUT1 (SUCROSE TRANSPORTER1)	384	20/20/5	52–92% ↓	Sugar content variations (not significant)	Y	Glassop et al. (2017)
	ScMV-CP (SURGARCANE MOSAIC VIRUS-COAT PROTEIN)	21 (shRNA)	21/6/6	20–90% ↓	Variable SCMV resistance	N	Aslam et al. (2018)
		21 (shRNA)	18/6/6	10–80% ↓			
RNAi	*SDP1 (SUGAR DEPENDENT 1)* + *TGD1 (TRIGALACTOSYL DIACYLGLYCEROL 1)*	*SDP1:* 278 *TGD1:* 244	Both: 31/5/5 *TGD1* only: 92/8/8	*SDP1:* 75–85% ↓ *TGD1:* 5% ↓*	Increased TAG accumulation up to 8.1% DW in leaves and 4.3% DW in stems when combined with OE of lipogenic genes	N	Parajuli et al. (2020)
	ACS1 (ACC SYNTHASE 1) + *ACS2 (ACC SYNTHASE 2)* + *ACS3 (ACC SYNTHASE 3)*	*ACS1:* 402 *ACS2:* 476 *ACS3:* 466	30/10/10	*ACS1:* 40–90% ↓ *ACS2:* 250–700% ↑ *ACS3:* 50–95% ↓	Reduced ethylene emission	N	Neris et al. (2022)
	V-ATPase E (VACUOLAR H + *-ATPASE)*	334	52/52//5	0–80% ↓	Higher larval mortality after feeding on transgenic plants	N	Mohan et al. (2021)

(continued)

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Table 1. (continued)

	Target gene name	Length sense/ antisense (bp)	No. of transgenic lines: generated/with suppression/with phenotype	Target gene suppression compared to WT	Observed phenotype	Field test	References
	ScMV-CP (SURGARCANE MOSAIC VIRUS-COAT PROTEIN)	997 (CaMVp)	46/26/15	NR (CP protein absent in immunoblot)	Resistance to SCMV	N	Widyaningrum et al. (2021)
		997 (Ubip)	19/17/14				
	ScPDS-amiRNA *(PHYTOENE DESATURASE- artificial microRNA)*	21 (shRNA)	26/26/25	4.5–8.5 fold ↓	Photobleaching	N	Garcia et al. (2022)
	SDP1 (SUGAR DEPENDENT 1) + *TGD1 (TRIGALACTOSYL DIACYLGLYCEROL 1)*	*SDP1*: 287 *TGD1:* 244	31/8/3	*SDP1:* 70–95% ↓ *TGD1:* 0–18% ↓	Increased TAG accumulation to 0.65–1.52% DW when combined with OE of lipogenic genes	N	Luo et al. (2022)
	GUX2 (GLUCURONYLTRANSFERASE 2)	498	NR/9/9	85–97% ↓	Decreased recalcitrance to steam pretreatments	N	Gallinari et al. (2024)
	TST1, TST2b-1A and *TST2b-1C (TONOPLAST SUGAR TRANSPORTER*s)	*TST1:* 214 *TST2b-1A:* 110 *TST2b-1C: 119*	8/8/8	85–100% ↓	80% decrease in stem sucrose content		Tang et al. (2024)
RNAa	*PFP (PROPHOSPHATE: FRUCTOSE 6-PHOSPHATE 1-PHOSPHOTRANSFERASE)*	1200	10/10/8	0–70% ↓	Increased sucrose	Y	Groenewald and Botha (2008)
	ACO (1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE)	1000	19/2/2	NR	Dwarfed plants	N	Wang et al. (2009)
	Neutral invertase (*NI*)	1800	NR/2/2	0–52% ↓	Reduced vigor and dwarfed in tissue culture, altered sugar	N	Rossouw et al. (2010)

N, no; No., number; NR, not reported; Y, yes.
*RNAi lines that showed an increase in gene expression compared to wild type are not reported here.

at targeted DNA locations. These molecular scissors include meganucleases (MNs), zinc-finger nucleases (ZFN), TALENs, and clustered regularly interspaced short palindromic repeats (CRISPR).

MNs, originating from naturally occurring homing endonucleases, recognize and cleave specific 14–40 bp DNA sequences but are limited by specificity and catalytic activity (Smith et al., 2006). ZFNs combine 4–6 engineered ZF protein domains with a Fok1 endonuclease. ZFN design remains challenging due to potential low on-target precision and high off-target activity (Petolino, 2015).

TALENs utilize highly modular TAL effector motifs combined with a Fok1 catalytic domain, offering greater design flexibility and targeting specificity than ZFNs (Khan et al., 2017). CRISPR distinguishes itself by employing RNA-guided DNA cleavage through customizable 20 bp guide RNAs (gRNAs) directed by Cas endonucleases. This approach provides enhanced target flexibility, simplified system design, and high multiplex targeting potential (Knott & Doudna, 2018; Stella et al., 2017).

The CRISPR toolbox has been expanded to include precision tools like base editors, prime editors, and CRISPR-associated transposons, enabling specific nucleotide substitutions and insertions (Molla et al., 2021; Tou et al., 2023).

Following DNA cleavage with SSNs, cellular repair mechanisms such as non-homologous end joining (NHEJ) or homology-directed repair (HDR) are initiated. NHEJ may induce random mutations (indels), where HDR provides a more precise template-mediated repair of cleaved DNA (Manova & Gruszka, 2015; Nisa et al., 2019).

These technologies have revolutionized plant biotechnology and significantly advanced functional genomics and improvement of crops, including polyploids (May et al., 2023).

Application of TALENs in sugarcane

The complexity of the sugarcane genome has limited site-specific nuclease (SSN) applications, with no reports using meganucleases or zinc-finger nucleases. Jung and Altpeter (2016) pioneered gene editing in sugarcane using TALENs to target the lignin biosynthesis gene *COMT*, building on previous RNAi research demonstrating lignin reduction potential (Table 2; Jung et al., 2012, 2013).

In their groundbreaking study, two *COMT* variants (*COMTa* and *COMTb*) were identified in the CP88-1762 WT genotype. TALEN sites were designed in a conserved region of the first exon, with plant transformation performed using AMT or BGT methods. This resulted in 39 and 27 transgenic lines, respectively.

Mutation assessment through capillary electrophoresis and pyrosequencing revealed that up to 99% of WT *COMT* sequences were converted to mutant versions,

Table 2 Genome editing using TALENs in sugarcane

Target gene name	Transform. method	No. of transgenic lines: generated/ with edits/with phenotype	No. or % of co-edited gene copies/ alleles	Observed phenotype	Field test	Stably edited progeny	References
COMT (CAFFEIC ACID O-METHYLTRANSFERASE)	*Agrobacterium* Biolistics	39/29/6 27/8/7	Up to 99.1% Up to 93.8%	Lignin reduction (11–32%) in lines with high mutation frequencies	N	Y	Jung and Altpeter (2016)
	Biolistics & *Agrobacterium*	NR/8/8	Up to 107 of 109	Reduction of lignin (19.7%) and S/G lignin monomer ratio, 43.8% improved saccharification efficiency	Y	Y	Kannan et al. (2018)
	Biolistics	NR/1/1	107 of 109	Combining *COMT*-edited biomass with engineered yeast strain increased ethanol yields by 148%; *COMT* editing alone increased ethanol yield by 42%	Y	Y	Ko et al. (2018)

N, no; No., number; NR, not reported; Transform., transformation; Y, yes.

resulting in a 29–32% reduction in lignin content. The S lignin monomer content decreased while hemicellulose content increased (Jung & Altpeter, 2016).

A subsequent study by Kannan et al. (2018) provided further validation, confirming that 107 of 109 *COMT* copies/alleles were edited in the sugarcane line CB6. This mutated line maintained the same biomass yield as non-modified sugarcane but demonstrated a 19.5% lignin content reduction and a significantly altered S/G lignin monomer ratio, elevating biomass saccharification efficiency by 38%.

The research expanded with Ko et al. (2018), who evaluated ethanol production using gene-edited biomass. When combined with a recombinant xylose-utilizing yeast strain, bio-ethanol production increased by 148% compared to non-modified biomass. Even with a non-modified yeast strain, the edited biomass elevated bio-ethanol production by 42%, demonstrating the potential of engineering both feedstock and microorganisms to dramatically improve bioethanol yield from lignocellulosic biomass.

Application of CRISPR in sugarcane

The first reported use of CRISPR-Cas9 in sugarcane was a proof-of-concept study demonstrating the excision of a 5′-*loxP* fragment using Cas9 directed to the site by a matching sgRNA (Table 3; Zhao et al., 2021). This research compared *Cre/lox* site-specific recombination, which allows transgene removal by inciting recombination between *loxP* sites with CRISPR/Cas-homologous recombination (HR; Gilbertson, 2003). Interestingly, homology arms as short as 30 bp could initiate error-free repair by CRISPR/Cas-induced HR. However, the *Cre* system was found to be more efficient than CRISPR/Cas-HR excision (Zhao et al., 2021).

Eid et al. (2021) achieved the first CRISPR-mediated mutagenesis of a native gene in sugarcane by targeting the *MAGNESIUM CHELATASE SUBUNIT I* (*MgCh*). This produced a chlorophyll-deficient phenotype with leaf colors ranging from green to yellow, depending on the number of co-edited *MgCh* copies/alleles. Sanger sequencing identified 49 edited copies/alleles, with chlorophyll reduction varying from 20 to 87%. The severity of chlorophyll depletion correlated directly with the number of edited alleles, providing a visual phenotype for optimizing genome editing technologies. Initial optimizations included heat treatments to elevate the editing efficiency of Cas9.

Oz et al. (2021) demonstrated efficient and reproducible targeting of three *ACETOLACTATE SYNTHASE* (*ALS*) copies conferring herbicide tolerance. Using template-mediated HDR, they introduced two amino acid substitutions (W574L and S653I) across 11 transgenic lines. Single mutations were also observed, either as W574L or S653I in 25 or 18 lines, respectively (Oz et al., 2021).

In 2024, researchers from Thailand used CRISPR/Cas9 to reduce sugarcane lignin content by targeting the *SoLIM* transcription factor. Three gRNAs produced edited lines with 9.74–51.46% reduced lignin content, potentially enhancing the plant's suitability for bioethanol production. Agronomic performance of the events was not reported (Laksana et al., 2024).

Sugarcane breeding could potentially be accelerated through the adoption of *in vivo* haploid induction technology by targeted mutation of specific genes including *CENTROMERIC HISTONE3* (*CENH3*), *PEROXIDASE* (*POD65*), *PHOSPHOLIPASE D3* (*PLD3*), *KOKOPELLI* (*KPL*), *DOMAIN OF UNKNOWN FUNCTION 679 membrane protein* (*DMP*), and *MATRILINEAL/PHOSPHOLIPASE A1/NOT LIKE DAD* (*MTL/PLA1/NLD*). Targeted loss of function co-editing of 4 sugarcane *MATRILINEAL* gene (*ScMTL*) copies/alleles, led to a haploid induction rate of 0.59–0.96%. Higher haploid induction rates may be achieved by co-editing of a larger number of *ScMTL* copies (Guo et al., 2024).

A groundbreaking study by Brant, Eid, et al. (2024) reported the first field trials of CRISPR-edited sugarcane. The researchers modified leaf inclination angle (LIA) by targeting *LIGULELESS 1*. This holds great potential for enhancing crop yield through increased light capture in the tall sugarcane canopy. Next-generation sequencing revealed co-mutation frequencies of 7.4–100% across 40 *LG1* copies/alleles. An intermediately edited line with 12% co-mutation and 54% LIA reduction showed an 18% increase in biomass yield. This demonstrates the advantage of the polyploid genome context for fine-tuning the editing outcome, allowing elevated crop performance. The authors also confirmed the absence of off-target mutations (Brant, Eid, et al., 2024).

These studies illustrate the tremendous potential of CRISPR-Cas9 in sugarcane genetic modification, offering precise tools for enhancing agricultural traits and understanding complex genetic mechanisms.

dCas9: Transcriptional modulation

Catalytically inactivated "dead" Cas (dCas) nucleases can bind DNA without cleaving, enabling gene expression regulation in multiple plant species by linking to activation, repressor, methylation, or demethylation domains (Brocken et al., 2018; Gjaltema & Rots, 2020; Nakamura et al., 2021).

In sugarcane, dCas9 systems offer potential for modulating expression of native genes or transgenes. Hooghvorst and Altpeter (2023) demonstrated gene suppression by fusing dCas9 with three copies of the SRDX repression domain, targeting the *MgCh* gene. Notably, targeting the 5′UTR achieved the highest average gene repression at 74%, followed by exon 1 at 60% and exon 3 at 47%. This remains the sole report of dCas application in sugarcane to date.

Table 3 Genome editing using CRISPR technologies in sugarcane

Target name	Transform. method	Cas enzyme/ mechanism	No. of gRNAs	No. of transgenic lines: generated/ with edits/with phenotype	No. or % of co-edited gene copies/alleles	Observed phenotype	Field test	Stably edited progeny	References
loxP site from pYZLoxGus plasmid	Biolistics	Cas9/NHEJ	1	NR/21/21	NR	Error-free homologous recombination restored reporter gene activity	N	NR	Zhao et al. (2021)
MgCh (MAGNESIUM CHELATASE SUBUNIT I)	Biolistics	Cas9/NHEJ	2	52/9/9	Up to 49 of 59 (83.1%)	20–87% chlorophyll reduction in leaves (yellow appearance)	N	NR	Eid et al. (2021)
ALS (ACETOLACTATE SYNTHASE)	Biolistics	Cas9/HDR	2	146/54/54	3	Nicosulfuron herbicide resistance (Accent® DuPont)	N	Y	Oz et al. (2021)
MgCh (MAGNESIUM CHELATASE SUBUNIT I)	Biolistics	dCas9/Repression	5	88/NR/44	NR	Target gene suppressed by up to 89%. Leaf greenness/chlorophyll content reduced.	N	NR	Hooghvorst and Altpeter (2023)
SoLIM (LIMONENE SYNTHASE GENE)	*Agrobacterium*	Cas9/NHEJ	3	50/20/20	NR	Lignin content reduced by 9.74–51.46%	N	NR	Laksana et al. (2024)
LG1 (LIGULELESS1)	Biolistics	Cas9/NHEJ	3	78/16/4	Up to 40 of 40 (100%)	25–94% reduction in leaf inclination angle enhanced light penetration into the canopy, increased biomass by 18% (field) to 39% (greenhouse), increased tillering	Y	Y	Brant, Eid, et al. (2024)
MTL (MATRILINEAL)	*Agrobacterium*	Cas9/NHEJ	2	30/NR/1	Up to 4 of 6	Decreased anther length and pollen germination rate. Haploid induction in progeny.	N	Y	Guo et al. (2024)

dCas9, dead Cas9; HDR, homology-directed repair; N, no; No., number; NHEJ, non-homologous end joining; NR, not reported; Transform., transformation; Y, yes.

FUTURE PROSPECTS

Novel technologies

Transient delivery of gene-editing tools through methods like AMT, BGT, or PEG-mediated protoplast transfection has shown promise for creating transgene-free edits in various crops. In sugarcane, while these approaches have been employed to study gene functions (Gao et al., 2013; Smith et al., 1992; Wu et al., 2021), their application for generating heritable, transgene-free edits remains unexplored. Recent innovations in *Agrobacterium*, such as mutations in the virD2 gene, enable T-DNA delivery into the nucleus with significantly reduced genomic integration, potentially paving the way for transgene-free editing (Gelvin & Lee, 2024).

BGT offers higher transient expression than AMT and is suitable for a broader range of genotypes. To enhance reproducibility, Miller et al. (2021) developed a double-barrel biolistic device coupled with cell-counting software, allowing simultaneous comparison of samples. This innovation has been employed to optimize BGT parameters and evaluate multiple single-guide RNAs, showing its utility in refining gene-editing approaches (Jiang et al., 2023; Miller et al., 2021, 2022).

Transient RNAi techniques, such as spray-induced gene silencing (SIGS) and virus-induced gene silencing (VIGS), have also been explored to identify gene-editing targets. SIGS involves applying a fine layer of dsRNA onto plant surfaces. Once taken up by the plant cell, it is processed into siRNAs to silence genes without stable transformation (Figure 2; Hoang et al., 2022). This method has shown potential in both monocot and dicot species for target confirmation, pest, and pathogen protection, though challenges like improving the cellular uptake of dsRNAs remain (Hoang et al., 2022; Wang & Jin, 2017). Enhancements, such as using siRNAs as structural motifs to synthesize RNA nanoparticles have improved RNAi efficiency and demonstrate the method's evolving capabilities (Zhao et al., 2024).

VIGS, an RNAi variant, uses viral vectors as a functional genomics method to silence target genes in plants (Figure 2; Rossner et al., 2022). This approach has been successfully implemented in monocots using vectors derived from viruses like barley stripe mosaic virus, brome mosaic virus, and foxtail mosaic virus (Kant & Dasgupta, 2019). Sugarcane-specific studies are lacking so far, but the successful use of sugarcane mosaic virus in maize suggests potential applications (Chung et al., 2021). Viral vectors are modified by inserting gene fragments into non-essential regions of the viral genome to ensure that the virus can still replicate and move within the host plant. The target gene fragment can be inserted in either sense or antisense orientation or as a hairpin. A recent report on VIGS in rice indicated the highest silencing efficiency for anti-sense orientation, followed by hairpin and then sense orientation (Kant & Dasgupta, 2017). VIGS systems have size limitations for insertion of recombinant DNA due to vector stability, replication efficiency, and systemic movement. The ideal insert size is typically in the range of 200–400 bp. Various inoculation methods can be used, including biolistic delivery, rubbing, spraying, and injection techniques (Kant & Dasgupta, 2019).

Despite limitations in vector stability and insert size, VIGS remains a versatile tool for transient gene silencing.

While CRISPR/Cas research in sugarcane remains in the early stages, the remarkable results obtained from recent publications incite high expectations for application of this technology in the years to come.

Advances in precision gene editing, such as base editing (BE) and prime editing, hold significant promise for sugarcane improvement. Traditional editing methods are limited by the imprecision of non-homologous end joining (NHEJ) and the inefficiency of homology-directed repair (HDR) (Molla et al., 2021). BE enables precise base conversions without double-strand breaks by fusing a deaminase with a catalytically inactive or nickase Cas protein. Cytosine base editors (CBE) convert cytosine to uracil, while adenine base editors (ABE) convert adenine to inosine, achieving targeted base changes with high specificity (Hua et al., 2020; Komor et al., 2016). These technologies have been successfully applied to various crops, including rice, soybean, and wheat (Cai et al., 2020; Ren, Sretenovic, Liu, Zhong, et al., 2021; Zhang et al., 2019).

Prime editing offers even greater precision and flexibility by combining a Cas nickase with a reverse transcriptase and an extended prime editing guide RNA (pegRNA). This allows DNA insertions or substitutions without requiring large DNA templates, which often integrate randomly. While most studies on prime editing have focused on diploid species, early success in wheat and potatoes indicates its potential for polyploid crops like sugarcane (Ni et al., 2023; Veillet et al., 2020). Although neither BE nor prime editing has been demonstrated in sugarcane, they hold tremendous potential for enhancing editing precision and efficiency.

Traditional gene-editing methods involving AMT or BGT often result in random DNA integration, raising concerns about off-target effects and insertional mutagenesis (Tsanova et al., 2021; Zhang et al., 2021). DNA-free approaches using ribonucleoprotein complexes (RNPs) present an attractive alternative. RNPs, comprising pre-assembled Cas proteins and guide RNAs, are delivered transiently, reducing the risk of genomic integration and streamlining regulatory approval (Menz et al., 2020; Woo et al., 2015). Techniques such as PEG-mediated protoplast transformation and BGT have been used to deliver RNPs, achieving successful edits in crops like maize, wheat, and potato (Liang et al., 2017; Makhotenko et al., 2019;

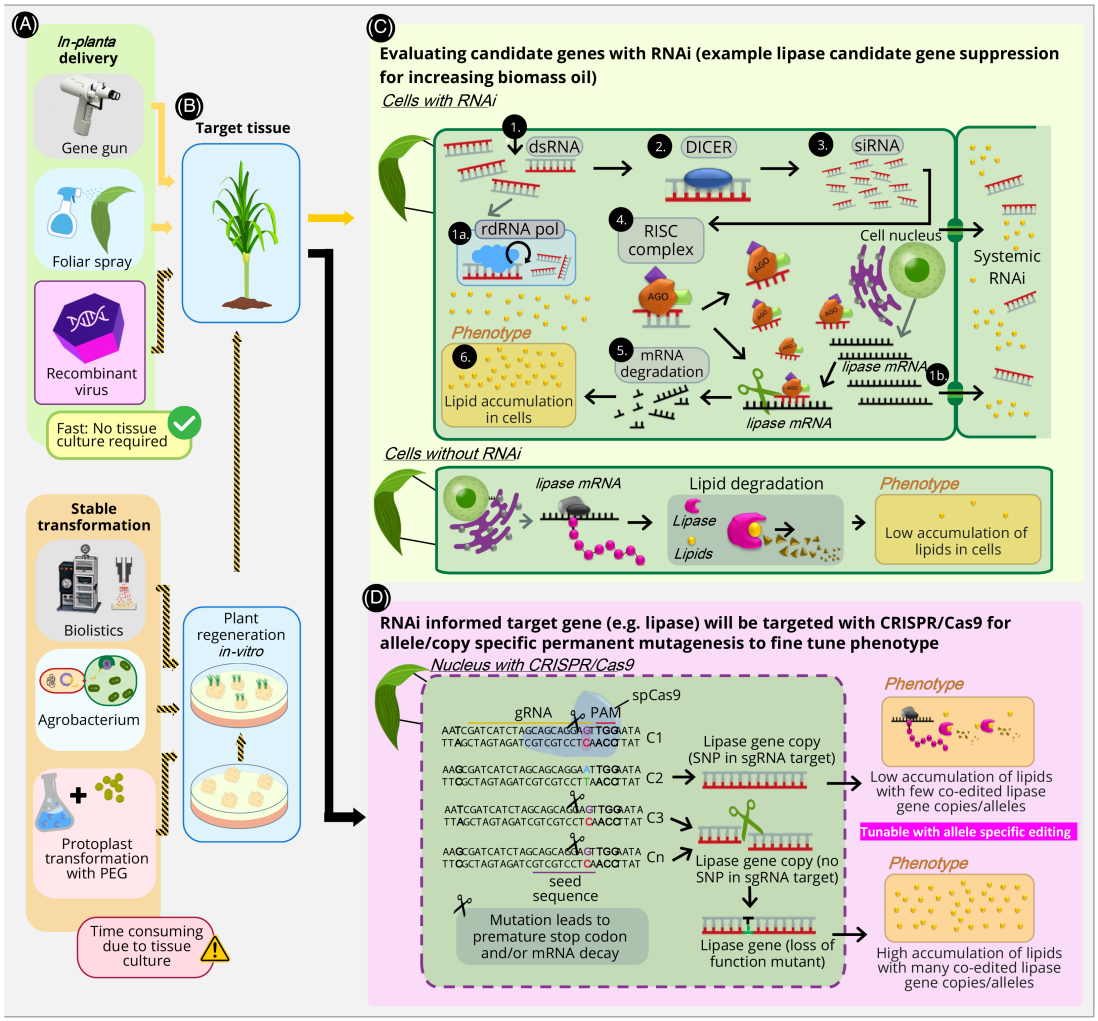

Figure 2. RNAi delivery to inform gene editing targets (for example: selecting a lipase target gene for enhancing biomass oil accumulation).
(A) Stable transformation involving plant tissue culture and *in planta* delivery. Tissue culture free methods for RNAi (orange lines) or gene editing constructs (black lines) or both RNAi and gene editing constructs (lines with orange and black stripes).
(B) Type of tissue for transfer of double-stranded RNA, RNAi expression constructs, or gene editing constructs. For *in planta* methods, reagents are directly applied to plants while stable transformation typically uses callus cultures and their selection and regeneration to plants which is a time-consuming process.
(C) RNAi to identify suitable candidate genes (for example, lipase gene suppression to boost biomass oil accumulation). RNAi technology is used to suppress a candidate gene (such as a specific lipase candidate gene, to enhance lipid accumulation in leaves). The process involves delivering double-stranded RNA (dsRNA) into cells, which triggers the RNAi pathway as follows: (1) dsRNA delivery via multiple methods (spray, biolistic, viral, or stable transformation) leading to the presence of dsRNA in the plant cell. (1a) RNA-dependent RNA Polymerase (rdRNA pol): inside the plant cell, the dsRNA can be amplified by the enzyme RNA-dependent RNA polymerase (rdRNA pol). This boosts the gene-silencing efficiency by providing additional dsRNA molecules for systemic transfer. (1b) Cell-to-cell movement of dsRNA via plasmodesmata: The introduced dsRNA can spread from the initial cell to neighboring cells through plasmodesmata. This leads to systemic spread of the RNAi signal across multiple cells. (2–3) Processing of dsRNA by DICER generates to produce small interfering RNAs (siRNAs). (4) The RNA-induced silencing complex (RISC), assembles at siRNA's and unwinds the double-stranded molecule to use one strand in a homology search for the matching target mRNA (for example, a specific lipase mRNA) followed by degradation of the completely homologous target mRNA (step 5). This reduces the production of lipase. (6) Phenotype: suppression of lipase activity leads to lipid accumulation within the leaf cells. The bottom section contrasts this with control leaf cells where RNAi is not applied, allowing normal lipase function and resulting in lipid degradation and low lipid accumulation.
(D) CRISPR-Cas9 gene editing for mutagenesis of the target gene informed by RNAi (for example, lipase gene knockout). In the highly polyploid sugarcane CRISPR-Cas9 gene-editing can co-edit variable numbers of copies and alleles of the same target gene. Design of sgRNA's can intentionally include or avoid single nucleotide polymorphisms (SNPs) in the seed sequence of some of the copies/alleles to prevent or support cleavage of all multiple alleles of the lipase gene, respectively. In this example, a single sgRNA is designed to bind a conserved region across most copies/alleles of the lipase gene, aiming to induce mutations in many copies/alleles and reduce overall gene function significantly. However, one of the copies/alleles contains an SNP in the seed sequence of the sgRNA target. The sgRNA-CRISPR-Cas9 complex will most likely not cut that specific allele retaining some basic expression level of the targeted lipase. The ability to fine-tune editing outcomes by copy/allele-specific design of sgRNAs is a unique opportunity in the genome of highly polyploid species. Sugarcane typically has 10 or more copies/alleles of each target gene. RNAi can inform the desired level of suppression of the target gene. However, achieving a complete loss of function phenotype by gene editing, if desired is far more challenging than in a diploid species.

Svitashev et al., 2016). However, transgene-free editing with RNP delivery typically does not allow to create a selectable phenotype, making the identification of edited events a daunting task (Molinari et al., 2024; Prado et al., 2024). To reduce costs associated with sequencing of a very large number of regenerated events, mutation screening methods such as capillary electrophoresis (CE), Cas9 RNP assays, and high-resolution melt analysis (HRMA) have been compared in sugarcane. CE was the superior approach (Figure 1; Brant, May, et al., 2024).

Alternatively, the development of RNP-based base editors offers the opportunity to generate selectable phenotypes, such as herbicide resistance through precise nucleotide substitutions in the *ALS* gene (Oz et al., 2021). This approach could simplify the identification of edited sugarcane plants. Given sugarcane's clonal propagation and high heterozygosity, RNP delivery could streamline the production of commercially viable, genetically modified varieties.

The CRISPR toolkit has been expanded to include diverse Cas nucleases with varying properties, such as PAM recognition and cleavage activity. For example, Cas12a recognizes a different PAM sequence and generates staggered DNA breaks, while engineered nucleases like Cas-NG and RYCas9 have reduced PAM requirements (Paul & Montoya, 2020; Ren et al., 2019; Ren, Sretenovic, Liu, Tang, et al., 2021). Although only Cas9 has been used in sugarcane, introducing alternative nucleases could enhance editing flexibility. Smaller nucleases also hold potential for delivery via plant viruses, enabling tissue culture-free editing and rapid target screening (Khakhar & Voytas, 2021; Liu et al., 2024). Meanwhile, transgenic plants expressing Cas proteins can facilitate gRNA delivery through viral vectors to expedite editing trials (Banakar et al., 2022; Baysal et al., 2024).

Fusing orthogonal dCas proteins to transcription activators and repressors will allow simultaneous activation and repression of different sets of genes (Pan et al., 2021; Vazquez-Vilar et al., 2023). Multigene, metabolic engineering strategies in sugarcane will benefit from these versatile tools.

Sugarcane breeding faces challenges due to the limited genetic diversity of the parental germplasm used in breeding programs and its complex polyploid genome. Synthetic *in planta*-directed evolution (SDE), which combines iterative editing cycles with selection for desirable traits can be very beneficial in this context (Kababji et al., 2024; Rao et al., 2021). For instance, SDE has been used in Arabidopsis and rice to develop variants with novel mutations conferring herbicide tolerance (Butt et al., 2022; Dong et al., 2024; Wang, Pan, et al., 2024). Sugarcane's genetic redundancy makes it an ideal candidate for SDE, as unmutated alleles can preserve essential functions while mutated alleles acquire new traits.

In conclusion, transient delivery systems, advanced editing technologies, and innovative strategies like orthogonal synthetic transcription factors and SDE hold tremendous potential to accelerate sugarcane genetic improvement. These approaches promise to overcome traditional breeding limitations and enable precise, efficient, and complex modifications in this commercially important crop.

Important target traits for sugarcane improvement

Improving sugarcane biomass production under various stress conditions is crucial for yield stability in predicted climate change scenarios. Genetic improvement strategies must address responses to pests, diseases, drought, flooding, salinity, heat, cold, ozone, shading, high wind speed, and nutrient-deficient soils (Kumar et al., 2024; Shabbir et al., 2021; Surya Krishna et al., 2023). Promising approaches to improve sugar and biomass yields include suppressing flowering, altering plant architecture, and improving photosynthetic efficiency (Brant, Eid, et al., 2024; Kannan, Nguyen, et al., 2022; Salesse-Smith et al., 2025).

For improving sucrose accumulation a few genes were so far explored by RNAi and RNAa (Glassop et al., 2017; Groenewald & Botha, 2008; Mehdi et al., 2024; Rossouw et al., 2010; Sachdeva et al., 2011; Tang et al., 2024) and overexpression approaches with limited success (Khan et al., 2023). The sucrose metabolism involves several compartments for synthesis translocation, carbon partitioning, accumulation, and degradation (Groenewald & Botha, 2008; Ma et al., 2000; Qin et al., 2021). Investigating this intricate process may be accelerated by using orthogonal dCas transcription activators and repressors under inducible promoters (Pan et al., 2021). A detailed description and table summarizing major sugarcane genes and molecules involved in sucrose metabolism is provided by Khan et al. (2023). Engineering the duration of stem maturation can also be explored (Khan et al., 2023; Qin et al., 2021).

Recent research has highlighted the role of 23 microRNAs in sucrose accumulation by regulating carbon-related transcription factors (Banerjee et al., 2022). Future investigations may focus on editing microRNA binding sites to better understand this complex trait. A comprehensive approach combining transgenic, breeding, and gene editing strategies may be necessary to significantly increase sucrose content (Jackson, 2005; Khan et al., 2023).

Biomass oil accumulation represents an emerging target, with significant progress already made in sugarcane and energycane (Cao, Luo, et al., 2023; Maitra et al., 2024; Parajuli et al., 2020). Refining this strategy requires a deeper understanding of regulatory elements that direct transgene expression to late stem development (Wang et al., 2021).

The potential for engineering additional co-products in sugarcane or energycane supports the emerging bioeconomy. By manipulating shikimate or isoprenoid pathways, these high-biomass crops can yield valuable biochemicals and biofuels for industrial applications (Lin & Eudes, 2020). Such strategies aim to reduce dependence on petroleum-derived chemicals and fuels, offering a more sustainable approach to industrial production.

Regulatory aspects of gene edited sugarcane

Genetic modification and commercialization of transgenic crop species remains a complex and controversial global topic, with significant developments in regulatory approaches and biotechnological advancements over recent years. The global landscape of genetically modified crop cultivation has expanded substantially, marked by several notable milestones (Beker et al., 2016; Bhajan et al., 2022; Mathur et al., 2017). In 2018, Brazil achieved a significant breakthrough by approving the world's first GM sugarcane for feed and food cultivation, with varieties CTC20BT and CTC9001BT demonstrating resistance to sugarcane borer (*Diatraea saccharalis*) and adaptability to diverse environmental conditions (CTNBio, 2019; Lajolo et al., 2021).

Indonesia similarly advanced its agricultural biotechnology sector by approving a GM drought-resistant sugarcane variety, NXI-4T, with regulatory approvals based on the premise that downstream processing for sucrose production effectively degrades DNA (Cheavegatti-Gianotto et al., 2011; Gain, 2018).

Argentina emerged as a global leader in regulatory guidelines for gene-edited crops. Its regulatory agency CONABIA was recognized by the Food and Agriculture Organization (FAO) as "The Only Global Reference Center for Biosafety" in 2014, a designation renewed in 2023 for an additional 4 years (Ministry of Economy of Argentina, 2022). The Argentinian regulatory model distinguishes between crops with stable genome insertion of foreign DNA and biotech products that are transgene-free, which are considered to be equivalent to conventionally bred crops (Fernández Ríos et al., 2024; Schmidt et al., 2020; Beker et al., 2016). This approach has been widely adopted by numerous countries, including Brazil, Chile, Colombia, Guatemala, Honduras, Paraguay, Ecuador, Philippines, Kenya, and Nigeria, where most gene-editing products are eligible for regulatory exemption (Fernández Ríos et al., 2024; Jenkins et al., 2023).

Regulatory frameworks vary internationally, with countries like Canada evaluating transgenic and gene-edited plant products or those generated by conventional mutagenesis based on their novelty rather than development methods.

In the United States, the regulation and oversight of gene editing in plants is facilitated by the U.S. Coordinated Framework for Regulation of Biotechnology. The US Department of Agriculture (USDA), the US Environmental Protection Agency (EPA), and the US Food and Drug Administration (FDA) collectively regulate the marketing and environmental release of gene-edited products. The United States has evolved its regulatory stance, with the USDA implementing a revision in May 2020 (SECURE rule) that allowed regulatory exemptions for a very small subset of edits, including a single edit in a diploid or haplotype. Therefore, for most transgene-free, gene-edited events, the same regulatory review process was required as for transgenic crops (National Archives, 2024a). A recent amendment to these USDA regulations (November 13th, 2024) would have allowed up to 12 edits, made simultaneously or sequentially in all polyploids, without consultation. One edit was defined as edits to 2 copies of one locus, and it did not matter if the loci were homologous or homoeologous. The type of edit eligible for the exemptions would have included insertions and deletions occurring during cellular DNA break repair, as well as a single nucleotide substitution in the absence of an externally provided repair template (National Archives, 2024b).

On December 2, 2024, the United States District Court issued a summary judgment order in the case of National Family Farm Coalition versus Tom Vilsack (case no. 3:21-cv-05695) vacating the entire SECURE rule (issued in May 2020). However, the summary judgment maintained the validity of the confirmation of exemptions, the regulatory status reviews, and permits issued under the SECURE rule. The court decision returns the USDA APHIS regulation that existed prior to May 2020. Under those regulations, APHIS had established the "Am I regulated" process, used by many developers of gene-edited and transgenic plants, to determine the regulated status of modified organisms. Following inquiry, a non-regulated status is granted if no "plant pest" donor organism, recipient organism, vectors, or vector agents were used in the generation of transgenic or gene-edited organism. This rule is now in place again and favors the use of biolistic transformation over *Agrobacterium*-mediated gene transfer since the latter is considered a plant pest vector.

The EPA regulates substances that are pesticidal or are plant regulators that are introduced into genetically modified plants. These are considered a "Plant Incorporated Protectant" (PIP) and are treated and regulated like a pesticide. In 2023, the EPA amended its regulations to include two exemption categories for PIPs, one for loss of function PIPs and one for PIPs created through genetic engineering from a sexually compatible plant. EPA established processes for developers to notify and confer with EPA on these types of exempted PIPs (Mendelsohn et al., 2023).

The FDA recently issued guidance for industry describing how developers of gene-edited plants can voluntarily engage with the FDA. Consultation with the FDA is

strongly encouraged if there is any potential for allergenic proteins to be present (FDA, 2024).

Other countries such as Australia, Japan, England, and India have developed regulatory systems that provide exemptions for transgene-free genome edits causing loss of function through natural DNA repair mechanisms, regardless of plant ploidy or gene copy number (Jenkins et al., 2023).

The emerging global consensus underscores the current importance of developing strategies for transgene-free editing, which not only reduces regulatory complexities and associated costs but also accelerates cultivar development. As biotechnological capabilities continue to advance, many regulatory frameworks are adapting to balance innovation, safety, and agricultural productivity.

AUTHOR CONTRIBUTIONS

FA acquired funding, conceived the idea, directed the manuscript content and structure, and edited the manuscript. EB and EZ-S wrote and contributed equally to the manuscript and should be considered joint first authors. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors have not declared a conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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