CRISPR tools for plant metabolic engineering: achievements and perspectives

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Abstract

The plant kingdom represents the biggest source of feedstock, food and added-value compounds. Engineering plant metabolic pathways to increase the phytochemical production or improve the nutraceutical value of crops is challenging because of the intricate interaction networks that link multiple genes, enzymatic steps and metabolites, even when pathways are fully elucidated. The development of clustered regularly interspaced short palindromic repeats–CRISPR associated (CRISPR–Cas) technologies has helped to overcome limitations in metabolic engineering, providing efficient and versatile tools for multigene editing. CRISPR approaches in plants were shown to have a remarkable efficiency in genome editing of different species to improve agronomic and metabolic traits. Here, we give an overview of the different achievements and perspectives of CRISPR technology in plant metabolic engineering.

Clustered regularly interspaced short palindromic repeats–Cas-mediated editing of coding sequences

Clustered regularly interspaced short palindromic repeats–CRISPR associated (CRISPR–Cas) is revolutionizing the (biotech) world and new discoveries are constantly pushing the boundaries of its application. Nevertheless so far the most widely adopted technique to efficiently modify protein-encoding genes has been the Cas9-targeted induction of loss-of-function mutations by exploiting the error-prone nonhomologous end-joining DNA repair pathway. Numerous are the successful examples of biofortified crops with an enhanced nutraceutical value or superior content of specialized metabolites [1-4], showing that targeted gene disruption allows to impart qualitative/quantitative changes in proteins, carbohydrates, fatty acids, volatiles and pigment composition. This could be achieved by streamlining metabolic fluxes [5,6], reducing competition for substrates [5,7] and relieving pathways from negative regulators [8,9].

One of the most desirable applications is the induction of homology-directed repair (HDR) mechanisms to precisely excise parts of DNA and replace them with DNA from a donor template. Some technical aspects of genetic transformation (e.g. cotransfection of large nucleases and the abundance of DNA donor template) that previously hampered the large-scale exploitation of HDR in plants, have been overcome by the application of different Cas proteins and DNA delivery systems. For instance, the combination of Cas12a with highly replicating small-sized geminiviral replicon systems as DNA template carrier was reported to greatly enhance HDR in tomato [10]. Similarly, to circumvent the low efficiency of HDR to engineer protein-encoding sequences by point mutations, a catalytically inactive Cas9 protein, named “dead” Cas9 (dCas9) has been linked to a base deaminase, enabling dCas9 to still bind DNA without cutting it, while the deaminase induces C to T or A to G transitions [11,12]. Though no examples have been described so far for metabolic engineering, this strategy was used in tomato to create a salt-tolerant allele of the *high-affinity K+ transporter1* by single amino acid substitution [10] or to confer herbicide-resistance in potato by point mutation of the *ACETOLACTATE SYNTHASE1* gene [13].

Differently from HDR, which requires precise in-frame substitutions, CRISPR-based targeted knock-in [14] offers more flexibility for *de novo* insertion of complex traits or cassettes thanks to the possibility

to exploit intergenic regions, thus avoiding possible adverse effects linked to random T-DNA insertions [15]. By using biolistics, an optimized CRISPR–Cas9-based method was successfully applied to rapidly obtain a "new" golden rice by introducing a 5.2-kb carotenogenesis cassette into selected safe harbor regions that would ensure genome integrity, high transcription and thus the production of β-carotene in rice endosperm [16].

CRISPR-mediated base editing can be particularly useful for those enzyme families for which the link between active sites and substrates/products have been already elucidated so that point mutations could increase enzyme kinetics or specificity towards specific substrates/products, or relieve intrinsic regulatory mechanisms such as feed-back and feed-forward inhibition of enzyme activity. For instance, the S728F mutation of the ß-amyrin synthase SAD1, an oxidosqualene cyclase that commits linear sterols to the production of pentacyclic triterpene in *Avena strigosa*, was demonstrated to be sufficient to modify its substrate affinity from oxidosqualene to dioxidosqualene, abolishing the production of pentacyclic (ß-amyrin type) triterpenes and leading to the accumulation of tetracyclic (dammarane type) products [7]. Similar results were also obtained in *Arabidopsis thaliana* (Arabidopsis) and other plant species where point mutations on key residues of oxidosqualene cyclases could expand or narrow down the product diversity [7,17-19]. The impact of point mutations clearly goes beyond enzymes and triterpenes. Studies on trait segregation in melon revealed that a single nucleotide polymorphism in a highly conserved residue of the *ORANGE* (*CmOr*) gene is responsible for ß-carotene accumulation in the flesh of the fruit and it is linked with orange and white/green fruit phenotypes [20]. Characterization of *BoOR*, the cauliflower ortholog, demonstrated its involvement in controlling the stability and turnover of phytoene synthase, a rate-limiting enzyme of carotenoid biosynthesis [21].

Overall, these examples demonstrate that protein characterization combined with targeted editing empowers our capacities to diversify the nature of precursors and products of biosynthetic pathways to produce novel and diverse compounds and, eventually superior plant traits.

Clustered regularly interspaced short palindromic repeats-based editing of *cis*-regulatory elements

The main goal of engineering the regulation of metabolic pathways is to fine-tune the expression of different enzymes to optimize metabolic fluxes or the accumulation of toxic intermediates [22]. In plants, mutations in *cis*-regulatory elements or 5' untranslated regions (UTRs) can severely affect gene expression patterns, hence possibly generating new variations of phenotypic and metabolic traits [23,24]. CRISPR–Cas-based approaches were successfully employed in tomato to edit promoters of genes related with fruit size and ascorbic acid biosynthesis, obtaining improved tomatoes with bigger fruits, higher ascorbic acid levels and thus antioxidant properties [25].A remarkable accumulation of

vitamin C in lettuce was achieved through editing of the 5'UTR of the *GDP-L-GALACTOSE PHOSPHORYLASE* (*LsGGP2*) gene [26], increasing its tolerance to oxidative stress. In rice, editing of the promoter and 5'UTR of the *WAXY* (*Wx*) gene, encoding an enzyme controlling the biosynthesis of amylose in endosperm, enabled producing new rice variants with a reduced starch content and improved grain quality [27].

The control of gene expression can also be reached without altering DNA sequences. For instance, modified dCas proteins can be used to positively or negatively modulate the expression levels of target genes depending on the effector domain fused to the dCas protein, with either an activating (CRISPRa) or inhibiting (CRISPRi) activity, respectively [28]. Accordingly, a new generation of CRISPR/dCas9 tools was developed through optimization of domain recruitment (Figure 1A). Studies in several plant species, such as in Arabidopsis, *Nicotiana benthamiana*, rice and wheat, revealed that these new strategies improved the activation rates without compromising target specificity [29-32]. More recently, the CRISPR-Act3.0 system [33] was presented as the latest, highly efficient, Cas9-based targeted transcriptional activator that maximizes the recruitment of activation domains without the need to recognize the protospacer adjacent motif (PAM) (Figure 1A), hence combining enhanced transcription with increased flexibility of target site selection.

These new CRISPR-based transcriptional regulators represent a versatile toolkit for metabolic engineering. Their potential was already tested in Arabidopsis to increase the expression of *PRODUCTION OF ANTHOCYANIN PIGMENT1*, a transcription factor controlling anthocyanin production [29], and in rice to simultaneously activate multiple genes involved in proanthocyanin and β-carotene biosynthesis [33]. Concomitant targeted activation of multiple genes was also attained in *N. benthamiana*, employing a second-generation, optimized CRISPRa system named dCasEV2.1 [34]. In this study, the metabolic flux through the flavonoid pathway was efficiently tailored by selectively activating four different combinations of multiple enzymes, leading to custom-made accumulation of flavonol or flavanone compounds. This work, together with others focusing on alkaloid or phenylpropanoid biosynthesis [35,36], demonstrates how complex biosynthetic pathways can be modulated to tailor metabolic fluxes and as such the abundance of desired metabolites. Differently from classical approaches that involve overexpression of transcription factors [37], CRISPR systems allow precise and selective transcriptional regulation of multiple targets (Figure 1B). Lastly, another method allowing the fine control of recombinant metabolic pathways relies on the assembly of specific synthetic promoters containing prefixed DNA sequences that are recognized by the CRISPR-based transcriptional machinery [38,39]. This strategy can be used to balance the production of each enzyme of a particular pathway, avoiding possible toxic intermediates, and maximizing its productivity (Figure

2). Currently, a collection of synthetic promoters inducible by CRISPR has been made available [38,39], offering a chance to optimize expression of recombinant pathways in plants.

Clustered regularly interspaced short palindromic repeats–deadCas9 for epigenetics

Large-scale transcriptome and epigenome studies have opened new perspectives for the implementation of CRISPR technologies to modulate gene expression by tweaking epigenetic regulation [40]. Epigenetic regulation results from DNA and histone modifications, such as DNA methylation and acetylation of lysine residues in histones, which influence gene expression by modulating chromatin compactness and thus DNA accessibility by the transcriptional machinery. In this context, CRISPR–dCas9 fused to the catalytic domain of chromatin-remodeling enzymes can alter DNA and histone modifications at specific locations guided by the gRNA [41].

CRISPR–dCas9 epigenetics has been applied to alter flowering time [31] and to improve drought stress tolerance [42] of Arabidopsis. Although no examples have yet been reported for plant metabolic engineering, this strategy has great potential, because metabolic pathways, such as those of anthocyanins in apple [43], indole compounds in tea [44] or chalcone in tomato [45], are frequently under epigenetic control. Epigenetic marks can specify cell, tissue or organ identity by switching on/off specific sets of genes. This applies also to metabolic regulons that are frequently expressed by specific plant cell types or tissues, because the pleiotropic, constitutive production of bioactive metabolites might cause toxic or detrimental effects to the plant's fitness [46]. CRISPR–dCas9 technologies could be used for targeted modification of the epigenetic landscape, offering great benefits to increase metabolite production as compared with conventional strategies such as hormonal elicitation or overexpression of genes encoding enzymes or transcriptional regulators. For example, saponins in quinoa are ubiquitously produced in all parts of the plant as defense compounds [47]. Especially seeds, which are consumed as food and feed, contain high levels of these toxic compounds. To extract or eliminate these compounds, several labor-intensive industrial processes need to be used [47]. CRISPR–dCas9 epigenetics could be used to alter saponin production in a tissue-specific manner, for instance by silencing it in seeds, streamlining processing for food production, while retaining saponin accumulation in aerial parts to safeguard adequate defense responses. Moreover, these technologies can be used to restrain the increasing DNA methylation levels typical of *in vitro*-maintained plant cell cultures, allowing stable and sustainable production of valuable natural compounds over multiple generations [48].

It is important to note that the need to cope with a potential substantial level of off-targeting and native epigenetic regulation constitutes hurdles when using these technologies [31]. Many

developmental processes are under epigenetic control and altering the epigenetic landscape, even though specifically, may cause pleiotropic effects. Moreover, the endogenous epigenetic machinery might reverse targeted modifications. This should all be well-considered when designing CRISPR–dCas9 constructs for epigenetic regulation.

Future perspectives

In this review, we presented how CRISPR–Cas tools have amazing applications and potential for plant metabolic engineering. In addition to what has been achieved so far, many tools are still being developed and tested in plants to enhance the precision and complexity of CRISPR outputs.

To reach maximum performance of plants as production platform for natural compounds, complex regulatory systems need to be modified to increase the metabolite flux, while minimizing growth defects and maintaining the response to dynamic environments [49]. To cope with this, conditional dCas9–gRNA constructs in combination with genetic circuits [50], anti-CRISPR molecules [51] or the use of miRNA-regulated gRNAs [52] represent the tools at the forefront to reach fine-tuned, controllable outputs. Recently, biosensor-based technologies, such as copper- or light-inducible CRISPR systems [53,54], were shown to have the potential to combine inducibility, optogenetics and CRISPR to redirect the metabolic flux from primary to specialized metabolism. Examples from microbiology can be inspiring to aim at conditional CRISPR systems, in which metabolite-sensing responsive elements are exploited to trigger heterologous biosynthetic pathways only when cells reach a specific nutritional status. Moreover, new technologies and insights into the protein–metabolite interactome [55] will likely allow the identification of new metabolite-interacting domains that could be exploited for the production of chimeric metabolite-sensing Cas variants that would become active only upon sensing of a specific metabolite.

Another frontier of plant engineering is the development of T-DNA-free delivery methods, not only to minimize off-target effects but also to circumvent the tight regulatory framework for transgenic organisms. Viral delivery systems with a high cargo capacity [56], as well as ribonucleoprotein complexes of nucleases delivered via particle bombardment or other means [57-59], are valuable tools here. Likewise, protoplast transfection for CRISPR–Cas9 vector delivery has been implemented to engineer bitterness metabolite levels in transgene-free *Cichorium intybus* [59].

Biofortified crops are emerging as a means to counter global malnutrition, especially in those areas where people have limited access to a nutritious and diverse diet [60]. CRISPR–Cas9 technology was used to insert or boost carotenoid pathways in agronomically relevant crops to combat vitamin A

deficiency [16,61] and to prevent enzymatic browning linked to phenolic oxidation [57] of fruits and vegetables, thus extending shelf life [62] and reducing food waste. In essence, CRISPR–Cas has made plant genome editing affordable to virtually any molecular biology lab and it could empower scientists and institutions to adapt local plant varieties to the needs of specific agricultural and economical settings [63].

In conclusion, the CRISPR toolkit is continuously expanding together with our knowledge about the molecular mechanisms, actors and networks steering plant metabolism. This, together with powerful deep learning systems, such as AlphaFold [64], may give scientists a chance to predict *a priori* the metabolic outcome(s) of specific mutations. New CRISPR tools and metabolic knowledge may lead to a new green revolution, in which not only yield is safeguarded but also sustainability, resilience and nutritional quality of agricultural products.

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An impressive example of applied CRISPR-based genetic engineering to reduce the gluten accumulation in wheat through the simultaneous mutation of 35 α-gliadin encoding genes, generating a transgene-free variant with low immunoreactivity. This is one of the first examples of the application of the CRISPR technology to change the protein composition of a crop to avoid autoimmune response in coeliacs.

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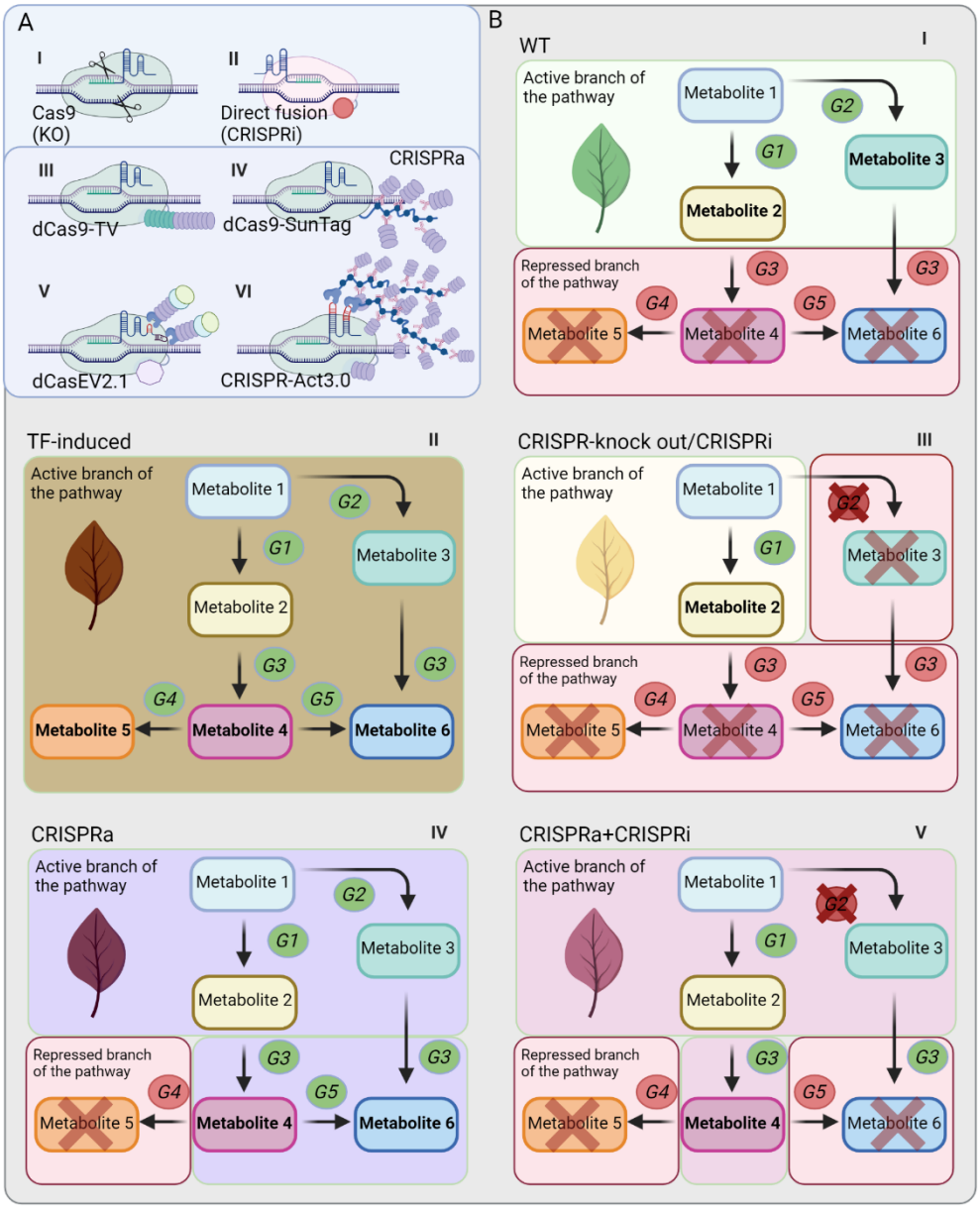

Figure 1. CRISPR-based strategies for pathway regulation applied for plant metabolic engineering. A) Schematization of CRISPR-based regulatory complexes. **I)** Cas9-mediated knockout (KO). RNA guided Cas9 nuclease is used to target double-strand breaks (DSBs) that frequently result in gene disruption. **II)** CRISPRi with the direct fusion of a repressive domain to the C-terminus of dCas12a. This

strategy exploits the use of a catalytically inactive Cas protein that is driven to the target location by a gRNA and instead of inducing DSBs cause repression of transcription of the target gene. **III)** dCas9-TV for transcriptional activation (CRISPRa), here consisting of six copies of the TAL activation domain fused in tandem to eight copies of the VP16 (Viral Protein 16) activation domain [32]. This system allows strong transcriptional activation of single/multiple target genes in both plants and mammalian systems. **IV)** dCas9-Suntag for transcriptional activation or epigenetic regulation [65]. This strategy consists of adding a multiepitope tail to the C-terminus of dCas9, which is recognized by a single-chain antibody fused to a transcriptional activation or an epigenetic regulation domain causing upregulation or DNA methylation at target sites, respectively. **V)** dCasEV2.1 for transcriptional activation [34]. This system employs a modified gRNA scaffold (gRNA2.1) that includes two RNA aptamers at the 3' end of the scaffold, which are recognized by the coat protein of the phage MS2 (MCP) fused to the VPR (VP65, P65 and Rta) activation domain. This strategy also includes a direct fusion of the plant activator domain EDLL at the C-terminus. **VI)** CRISPR-Act3.0 for gene activation [33]. This strategy employs a modified gRNA scaffold that includes two RNA aptamers inside the scaffold loops which are recognized by MCP. The MCP is fused to the multi-epitope tail, which is recognized by a single-chain antibody fused to VP64, the latter being a transcriptional activator composed of four copies of VP16. **B)** Schematic representation of a hypothetic metabolic pathway to depict how the different CRISPR-based strategies can steer the metabolic flux and impact the final accumulation of pigment (depicted as a colored leaf). Circles represent different genes (*GX*) controlling different metabolic steps of the pathway; the green or red colors indicate whether they are activated or repressed, respectively. The metabolites that accumulate predominantly are highlighted in bold. **I)** Wild-type (WT) state of the pathway, the products are metabolites 2 and 3. **II)** Upregulation of genes *G3, G4* and *G5* by transcription factors, leading to the accumulation of metabolites 4, 5 and 6. **III)** Targeted CRISPR knockout/CRISPRi of gene *G2*, causing accumulation of metabolite 2. **IV)** Targeted activation/CRISPRa of genes *G3* and *G5* that would steer the metabolic flux towards the production of metabolites 4 and 6. **V)** CRISPRa combined with CRISPRi for simultaneous activation of genes *G3* and *G5* and repression of gene *G2*, causing accumulation of metabolite 4.

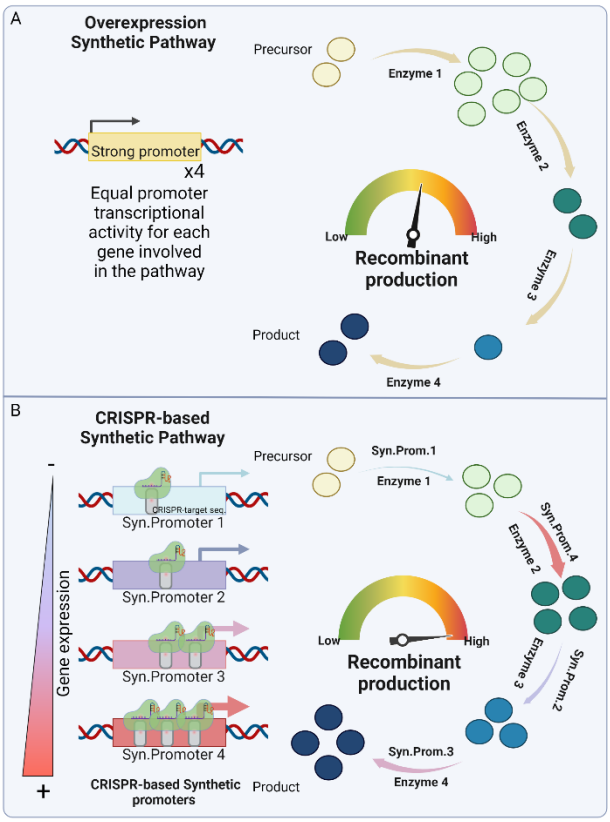

Figure 2. Two possible synthetic metabolic pathways. A) Conventional overexpression strategy for the enhanced production of a target metabolite, employing strong promoters with equal transcriptional activity driving the expression of each enzyme involved in the pathway. **B)** CRISPR-based approach to balance the recombinant production in a synthetic pathway, employing a collection of CRISPR-inducible artificial promoters with different transcriptional strengths that optimally regulate the expression of the different enzymes involved in the pathway, thus minimizing inefficient accumulation of precursors and maximizing product accumulation. Metabolites are depicted as circles of different colors, while the size of arrows represents the strength of expression for each of the different enzymes.