

Opinion

Directed Evolution of Plant Processes: Towards a Green (r)Evolution?

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Directed evolution (DE) is a powerful approach for generating proteins with new chemical and physical properties. It mimics the principles of Darwinian evolution by imposing selective pressure on a large population of molecules harboring random genetic variation in DNA, such that sequences with specific desirable properties are generated and selected. We propose that combining DE and genome-editing (DE-GE) technologies represents a powerful tool to discover and integrate new traits into plants for agronomic and biotechnological gain. DE-GE has the potential to deliver a new green (r)evolution research platform that can provide novel solutions to major trait delivery aspirations for sustainable agriculture, climate-resilient crops, and improved food security and nutritional quality.

Directed Evolution: A Powerful Tool to Generate New Variability in Plant Metabolism

Directed evolution (DE; see [Glossary](#)) is a protein engineering method that mimics Darwinian evolution by inducing random mutations in DNA (similar to those occurring in nature) that can be useful for improving enzyme performance [1]. By DE, new proteins with enhanced and useful properties (e.g., thermostability, enantioselectivity, substrate specificity, solubility) can be generated without any need for prior knowledge of enzyme structural information [2].

The first successful application of DE by Frances Arnold merited the 2018 Nobel Prize for Chemistry, and indeed DE can be now considered as a driving technology to create new enzymatic variability in both chemical and biochemical applications, including the modification of plant enzymes towards developing a desired trait. We surmise that DE, in combination with **genome editing (GE)**, may rapidly deliver improved plant phenotypes and provide solutions to major global agronomic challenges. Examples include making plants more resistant to biotic and abiotic stresses, increasing crop yield, and enhancing food nutritional value.

DE typically works in two steps. The first step involves generating a randomly mutated DNA library of one or more genes for which a range of generic methods are available. The second step is more specialized towards the enzyme being evolved because it involves expressing a mutant library and selecting for enzymatic variants with the desired characteristics – preferably via a high-throughput screen (points 1–6 in [Figure 1](#), Key Figure). Numerous techniques are available to generate mutant DNA libraries [3–5]. Among the most commonly used are **error-prone PCR (ep-PCR)** [6,7] and **DNA shuffling** [8,9]. An alternative method called phage-assisted continuous evolution (PACE) is also very efficient and rapid [10]. The design and selectivity of the second, mutant-selection, step of DE applications is crucial because ‘you get what you screen for’. The screen should be enzyme-specific to ensure that it identifies functional changes specific to the target enzyme(s) [11,12]. Screens linked to organism survival or color-based cellular expression are typically preferred because they have the potential to enable high-throughput selection. Such applications are common in industrial biocatalysis [13] and in the production of chemicals and pharmaceuticals [14]. However, very few studies using DE have been carried out with plant enzymes [15–17], and *in vivo* trials are not available yet.

Combining DE and GE (DE-GE) towards a Green (r)Evolution?

GE comprises a collection of techniques that facilitate the site-directed modification of genomic DNA in many organisms [18,19]. Application of GE in plants has produced improved traits useful in agriculture [20–22] and plant breeding [23,24]. In particular, the application of CRISPR technology has revolutionized DNA modification in many organisms including crop plants [18,24–27]. The power of the CRISPR technology now allows a single nucleotide to be changed with a precision never achieved

Highlights

Coping with fast climate change, overpopulation, and increasing dietary requirements urgently demands resilient and productive plants.

Enzymes with desired, sometime promiscuous, activities can be generated by DE, allowing the introduction of new genetic variability into organisms to improve their fitness.

Targeting native genes for DE-mediated improvement can allow GE approaches, such as the SDN-2 modality of CRISPR, to integrate required nucleotide mutations back into a genome.

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in the past. CRISPR may be used in three site-directed nuclease (SDN) modalities. SDN-1 is the most commonly used modality because it silences a gene, thus producing a loss-of-function phenotype. SDN-2 is able to carry out targeted changes in the DNA using a template that allows a gain of function for the gene of interest. SDN-3 modality can be compared with classical transformation with *Agrobacterium tumefaciens* because it allows the insertion of an entire gene into the host DNA with the help of an adequate template (Boxes 1 and 2 for more details). Application of SDN-2 requires a thorough knowledge of how the individual genome mutations impact on the functionality of the enzyme/molecular target. Although systems-biology or phylogenetic modeling methods can be used to identify solutions for altering enzyme performance in a 'rational' manner, the DE approach typically delivers nonapparent, validated, mutagenic solutions for improving the catalytic fitness of enzymes. As such, DE can be viewed as a synthetic laboratory process that imitates the natural adaptive process of Darwinian evolution [2]. By this analogy, the selection of specific DNA substitutions by DE to improve enzyme function can be combined with GE-mediated insertion into a genome, thus establishing a versatile DE-GE analytical platform in plant bioengineering. A summary of the DE-GE platform is shown in Figure 1.

The first step in a DE-GE approach is to identify mutations that code for improved enzymatic function. This step involves the generation of a mutant library and the selection of genes encoding improved catalytic fitness, often using *Escherichia coli* to ensure high-throughput screening. The application of DE to complex biological organisms (e.g., plants) requires a higher standard of controls than when merely improving enzymes for use as catalysts of chemical reactions under strictly controlled conditions. In plants, the screen must discard mutated enzymes that have unfavorable feed-forward effects on cells and phenotypes. A fast line of selection could be based on transient expression in plant cells. Single-cell analyses are rapidly evolving techniques that promise rapid screening of desirable DNA sequences.

The nucleotide changes necessary to alter the amino acid sequence of the endogenous target plant enzyme are then introduced using CRISPR GE technology. The transformed plant tissue is propagated *in vitro* once the correct integration of the nucleotide modifications is confirmed (possible CRISPR delivery methods are given in Box 2). Characterization of phenotypes for the desired traits and for overall plant fitness is the last step. We provide below examples of plant enzymes that could be modified by DE-GE towards increasing photosynthesis, agricultural productivity, and yield [28], as well as to produce rare bioactive molecules, antioxidants, and other defense compounds [29,30].

DE-GE Applications to Improve Useful Plant Traits

A key determinant of flux through a metabolic pathway is the quantity and catalytic properties (including activity, reaction constants, and competition by alternative substrates) of each enzymatic step. Improvement of any of these parameters may be a winning strategy to enhance metabolic flux that could potentially generate a desired phenotype or deliver a desired trait. This is likely to be challenging to achieve, given that enzymes typically do not work in isolation, and instead operate in complex, overlapping metabolic pathways where improved enzyme traits may lose efficacy, for example, because of allosteric regulation or feedback inhibition that are typical of biological systems [31,32]. Despite this potential limitation, DE has been successfully applied in many cases to improve the production of particular metabolites [33]. Examples include improving the production of lycopene [29,34] and flavonoids [30], and engineering the carotenoid biosynthetic pathway [35–37]. DE could also be applied to improve production of rare molecules with important biological activities in plants. For example, Li *et al.* showed that improvement of catalytic efficiency of the limiting enzyme 10-deacetylbaicatin III-10- β -O-acetyltransferase (DBAT) increased the production of taxol, a key chemotherapeutic drug [38]. All these DE studies have been performed in model microorganisms such as *E. coli* [39,40] and have yet to be trialed in plants, possibly via a GE approach as hypothesized in Figure 2.

Climate change is increasingly and rapidly exacerbating drought and heat waves in temperate and subtropical areas of the world, strongly affecting agriculture yield [41]. This is heightening the global

Glossary

Ascorbate–glutathione cycle (AGC): a major ROS control system in plants. It includes a series of enzymes and metabolites that are involved in maintaining cellular redox homeostasis, and that crosstalk with other redox enzymes and hormonal signals. The activity of the AGC is linked to the resilience of plants to different stresses, and is involved in cellular differentiation and plant growth.

DE-GE: directed evolution-genome editing. We surmise that, combined, these two technologies constitute a research platform for identifying and integrating desirable traits into plants (detailed in Figure 1).

Directed evolution (DE): a gene mutation and selection technology based on Darwinian evolution to generate protein variability and select for enzymes with desired catalytic properties.

DNA shuffling: a widely applied technique for the generation of mutant gene libraries based on homologous recombination between genes with high DNA sequence identity. The technique was originally used to randomly recombine various mutants of a single gene, and was later used to recombine homologous genes from different species (family shuffling).

Error-prone PCR (ep-PCR): a common method to create a randomly mutated combinatorial DNA library. The library is generated by PCR using an error-prone Taq DNA polymerase or by using conditions that reduce the fidelity of Taq DNA polymerase (such as the presence of manganese ions or nucleotide analogs) to promote misincorporation of nucleotides.

Genome editing (GE): methods to introduce precise modifications into a genome. Traditional methods include zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), and the more recently discovered clustered regularly interspaced short palindromic repeats (CRISPR) method provides superior gene-editing precision and efficiency that has revolutionized numerous research fields, including plant transformation.

Isoprene: the most abundant volatile organic compound emitted

demand for climate-resilient crops. All environmental and many biotic stimuli generate oxidative stress in plants. The production of **reactive oxygen species (ROS)** is controlled by the biosynthesis of detoxifying enzymes and metabolites with overlapping functions. This signaling pathway allows plant cells to continuously optimize their metabolism in response to changing environmental or developmental conditions [42]. The **ascorbate–glutathione cycle (AGC)** is one of the best-understood systems involved in ROS control [43,44]. *In vivo* studies with transgenic plants demonstrated that overexpression of AGC genes improves resistance to many stresses [45,46]. Plant varieties with higher stress tolerance also have increased AGC levels [47,48]. A key regulator of the cycle is ascorbate peroxidase (APX), an enzyme with multiple isoforms that is ubiquitously expressed in almost all cellular compartments. APX has a much higher affinity for H₂O₂ than catalase, but is rapidly inactivated when H₂O₂ overcomes a threshold value, in particular when ascorbate levels are reduced under oxidative stress [43,49]. APX undergoes post-translational modifications at specific cysteines which alter its catalytic properties in different ways, probably depending on cellular oxidative status [50,51]. Applying DE to engineering a plant APX with reduced sensitivity to H₂O₂ may provide a solution to enhancing AGC function in stressed plants, possibly using screening systems that have been applied for other peroxidases [52,53]. These include a color-based screen that exploits the capacity of many peroxidases to use exogenous electron donors to generate chromogenic molecules after oxidation [52]. Improving chloroplast APX isoenzyme activity would likely have a pervasive effect on the productivity of plants exposed to high oxidative stress and ROS production which otherwise impair the efficiency of photosynthesis [54]. However, possible negative effects of altering APX activity might include unwanted consequences for the activity of other enzymes involved in maintaining chloroplast redox state [55]. H₂O₂ signaling could also be weakened by excessive constitutively expressed ROS scavenging activity; in particular, under stress-free conditions, impairment of ROS-dependent retrograde pathways from chloroplast to nucleus could affect plant productivity [42,54].

Another possible application of the DE-GE approach is engineering the biosynthesis of isoprenoids. Carotenoids and xanthophylls are important protective antioxidants and have already been a workable target of DE [37]. **Isoprene** is made in plants by an affiliated reaction of the methylerythritol phosphate (MEP) pathway that generates carotenoids and other bioactive compounds (e.g., cytokinin and abscisic acid hormones). Isoprene functions as an antioxidant and membrane strengthener to help plants to cope with oxidative and thermal stress [56–58]. However, the production of isoprene comes at a carbon cost to plants, especially under stress conditions [56], in addition to contributing to air pollution. Isoprene biosynthesis is catalyzed by isoprene synthase (IspS), which is largely controlled by substrate and energy availability [59]. Changing the properties of IspS may be relatively easy because the enzyme controls the single step that diverts the precursor (isopentenyl pyrophosphate) away from polymerization into nonvolatile isoprenoids towards the synthesis of isoprene. IspS was lost by plants multiple times during evolution [60], and today only ~20% of plants produce isoprene [61]. Modulating isoprene production in modern crops may help them cope with warmer and drier growing conditions, and could sustain or even enhance productivity [56,62]. Comparative analysis of IspS crystal structure from *Populus × canescens* with IspS and terpene synthase sequences from rosid species identified four conserved catalytic amino acids [60]. Of these, F310 seems to influence active-site size and modulate substrate specificity [63], making amino acids around F310 in IspS a favorable target for initial DE studies. The ease with which isoprene can be detected by accurate and ultrafast technologies [64] suggest that the development of a DE screen with suitable throughput should be feasible, and indeed DE approaches for improving IspS properties and enhancing isoprene production have already been successfully demonstrated in microorganisms [65,66].

Reinstating the production of anthocyanins is a further potential application of DE-GE. Although anthocyanins are known to benefit human health [67], they are not produced by many crop species, including tomatoes. The loss of anthocyanin production in tomatoes possibly originated during domestication of this plant [68]. Transgenic approaches to create a (black) tomato with high flavonoid content have included inserting constitutive promoters or transcription factors aimed at producing anthocyanin both in peel and pulp [69]. Classical breeding generated the Sun Black tomato variety in which the black peel is rich in anthocyanins but the pulp remains red and poor in flavonoids [70]. Production of target flavonoids might be enhanced by improving the performance of enzymes in

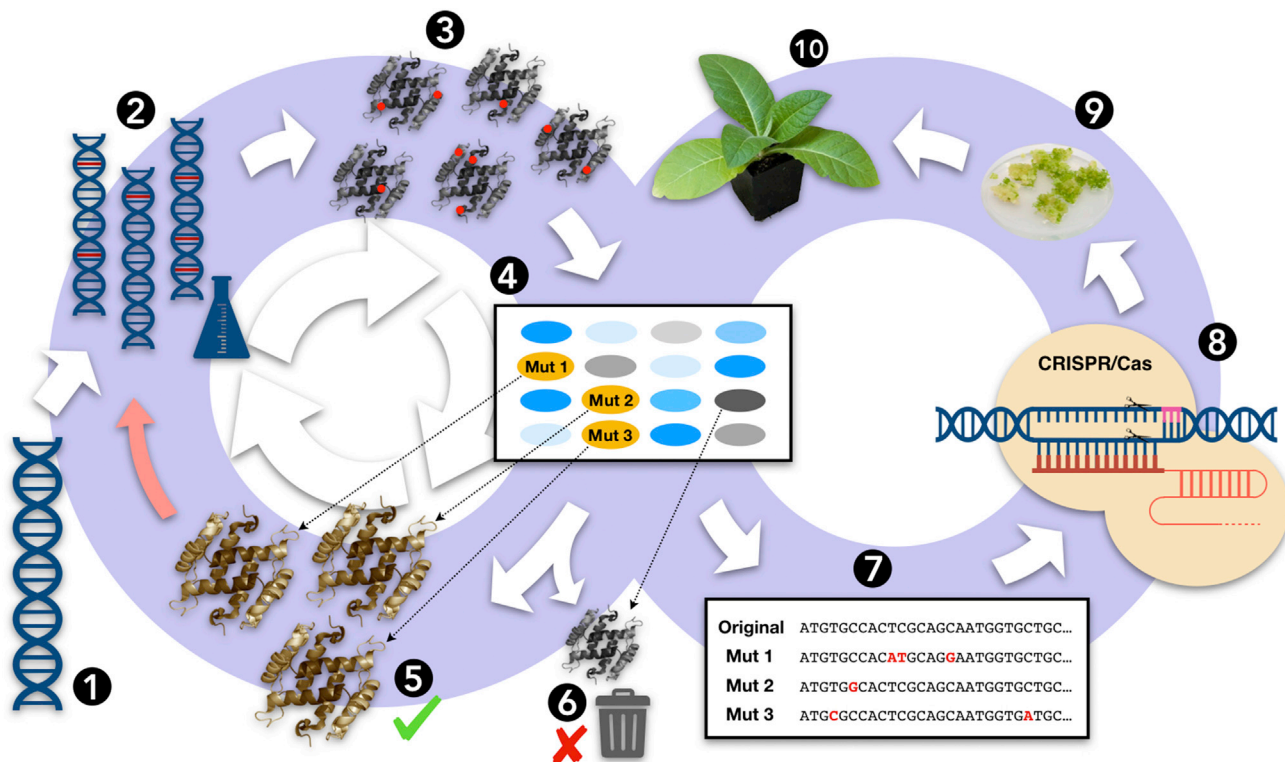
by vegetation. It is believed to act as a membrane stabilizer and antioxidant, and has important interactions with atmospheric pollutants. Isoprene is the simplest and most volatile component of the large family of plant isoprenoids synthesized by two biochemical pathways in chloroplasts [the methylerythritol phosphate (MEP) pathway] and the cytosol [the mevalonic acid (MVA) pathway].

Reactive oxygen species (ROS): highly reactive molecules derived from the partial reduction of molecular oxygen that have important roles in cell signaling and homeostasis. ROS are formed as a natural product of oxygen metabolism, but during stress conditions their levels can increase dramatically, causing significant oxidative damage to cellular structures and metabolism.

Rubisco: ribulose-1,5-bisphosphate carboxylase/oxygenase, the photosynthetic enzyme that catalyzes the CO₂-fixation step of Calvin–Benson cycle. It is the most abundant enzyme on Earth and accounts for 20–50% of total soluble proteins in leaves. Owing to its slow carboxylation rate (K_{ca}) and competitive inhibition by oxygen, the rate of photosynthesis is often limited by Rubisco activity.

Key Figure

The Trail to Improving Plants by Directed Evolution-Genome Editing (DE-GE).



Trends in Plant Science

Figure 1. (1) The gene that encodes the enzyme to be improved is selected. (2) Mutagenesis of the selected gene is achieved *in vitro* [e.g., using error-prone (ep)-PCR or DNA shuffling] and a library of mutated genes is created. (3) Mutated gene libraries (nucleotide substitutions are represented by red dots) are transformed into a microorganism (e.g., *Escherichia coli*) and the mutated enzymes are expressed. (4) A (preferably high-throughput) screen for the desired catalytic phenotype is used to identify the beneficial mutant enzymes, and (5) these are subjected to further cycles of random mutagenesis and screening to increase enzyme performance, whereas (6) enzymes with reduced fitness are discarded. (7) Selected enzymes are sequenced to identify the altered nucleotides (red letter in the figure). (8) Nucleotide changes encoding desired enzyme amino acid substitutions are introduced into the plant genome using the site-directed nuclease (SDN)-2 mode of CRISPR/Cas, and (9) plants are propagated *in vitro* to generate (10) mature transformed plants for molecular, biochemical, and physiological (growth chamber, field) analysis.

the flavonoid biosynthetic pathway. In particular, chalcone isomerase (CHI) is considered to be a bottleneck enzyme in tomato because anthocyanin biosynthesis is blocked at the level of naringenin chalcone, the substrate of CHI, because of poor expression of the *CHI* gene [70,71]. However, wild species of *Lycopersicon pennellii* v. *puberulum* express the *CHI* gene in both peel and pulp [68]. This suggests that DE-GE applied to CHI of these tomato hybrids could be used to potentiate the enzyme by using an established DE screen [72]. Classical breeding could then be used to insert the evolved *CHI* gene into tomato cultivars of agronomic interest with the aim of producing a super-food containing significant amounts of anthocyanins in the peel and pulp.

Extensive efforts are being made to increase global agricultural production to feed the estimated 9 billion people by 2050 [73]. Among the transgenic strategies being pursued, improving the efficiency of photosynthesis is a goal that more recently has had success [28,74]. A historic target for

Box 1. The CRISPR/Cas Complex and Its Uses

The CRISPR complex comprises a CRISPR-associated protein (Cas) endonuclease that can introduce a site-specific double-strand break (DSB) in the target DNA. Numerous enzymes belong to the Cas family [24], but the most widely used is Cas9, a *Streptococcus pyogenes* endonuclease [26]. All Cas endonucleases use a guide sequence formed by a RNA duplex: the CRISPR RNA (crRNA) determines the DNA target site by Watson–Crick base-pairing and a trans-activating crRNA (tracrRNA) binds the duplex to the Cas enzyme. In other words, this RNA duplex forms a single guide RNA (sgRNA) [85–87]. In Figure 1A the protospacer adjacent motif (PAM) sequence (Box 2) and the genomic DNA are shown in pink and blue, respectively. The Cas enzyme cuts the target sequence near the PAM, and repair takes place according to one of the three possible SDNs (Figure 1B).

SDN refers to an enzyme that cuts DNA at a specific target site in the nucleotide sequence. SDN-1: once a DSB is triggered by Cas, the cell can repair the damage by non-homologous end-joining (NHEJ), but the repair is imprecise and generates random mutations at the endonuclease target site (red insert), frequently leading to inactivation and silencing of the target gene and consequently its loss of function [21]. SDN-2 uses the homology-directed repair (HDR) mechanism of the host cell, inserting a short DNA template together with the Cas enzyme. This template, without being integrated in the host genome, acts as a guide during DSB repair, causing one or more targeted nucleotide changes (green insert) and a gain of function in the target gene [21]. SDN-3 also employs a DNA template that guides DSB repair, but this contains the entire DNA sequence of a complete gene that is copied and inserted into the host DNA via HDR. The outcome will be a new gene sequence incorporated into the target site (yellow insert), resulting in the production of a cisgenic, intragenic, or transgenic plant, depending on the origin and characteristics of the inserted gene [18].

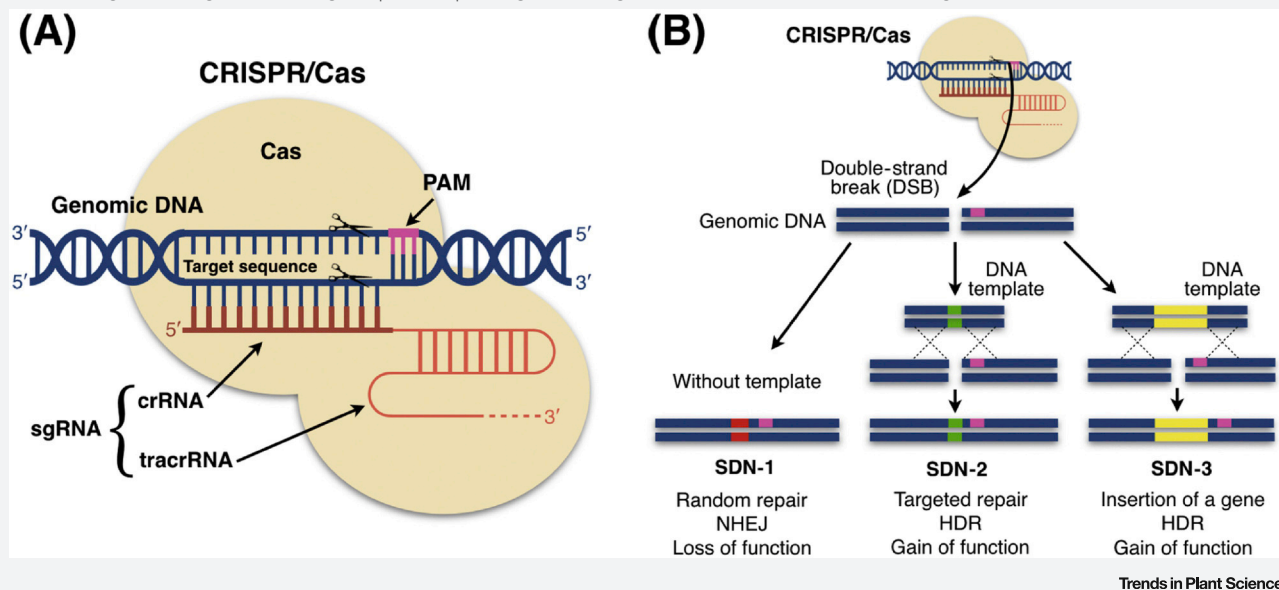


Figure 1. Introducing the CRISPR/Cas Complex (A) and the Three CRISPR-Induced Gene Modifications (B).

improvement of photosynthesis has been the CO₂-fixing enzyme Rubisco whose catalytic inefficiency requires plants to produce large amounts of the enzyme to support adequate rates of photosynthesis. Improving Rubisco by rational design has proven to be particularly frustrating [75,76] despite extensive knowledge of Rubisco structure and function [77]. Recent success in finally identifying amino acid changes that improve the carboxylase properties of nonplant Rubiscos using DE [78,79] suggest that DE-GE may help in enhancing plant Rubisco performance [80]. The nuclear *rbcS* gene encoding the S subunit of Rubisco, and/or the *rca* gene(s) encoding Rubisco activase, a metabolic regulatory enzyme required to maintain Rubisco activity [75,81], could be suitable targets to improve Rubisco catalysis and sustain activity, thereby enhancing photosynthesis, growth, and yield [74].

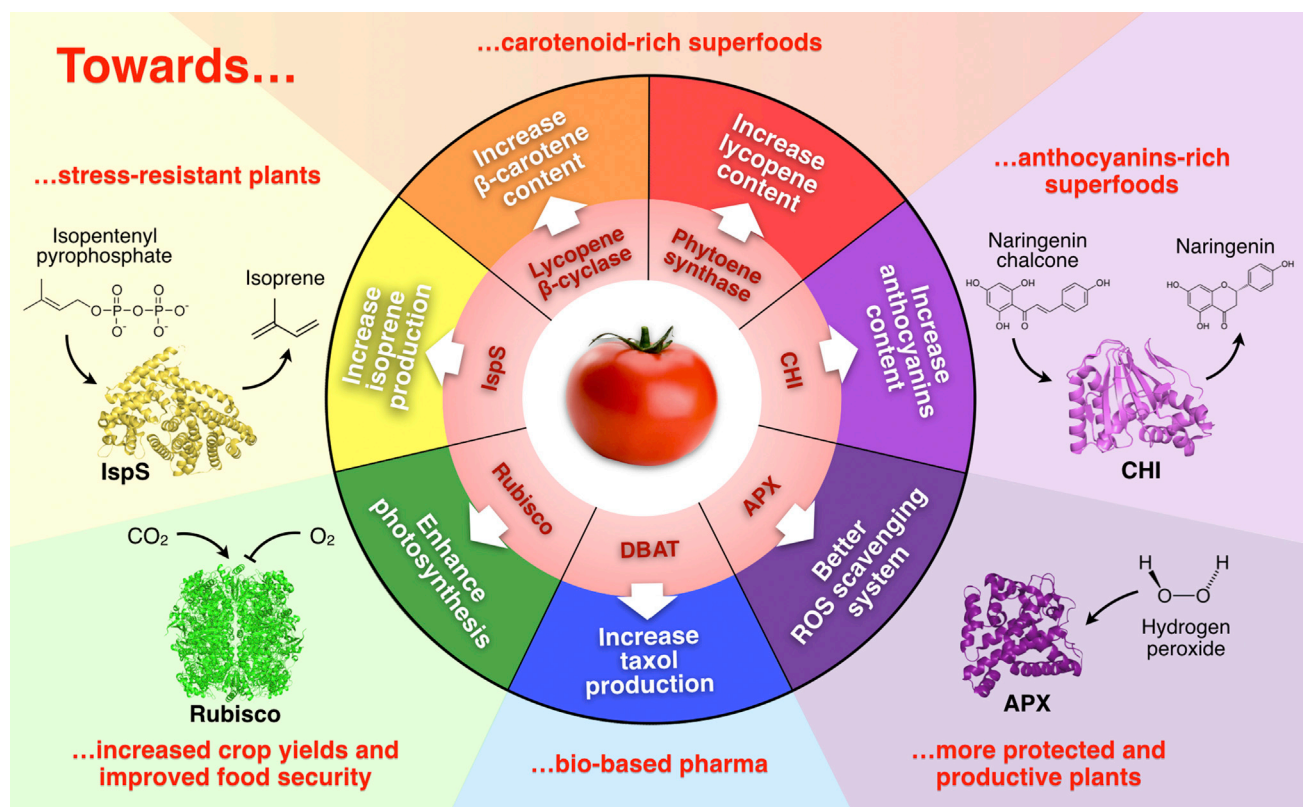
Concluding Remarks and Future Perspectives

By simulating Darwinian evolution, DE is able to generate genetic variation in a short time, while GE is an efficient tool to accurately introduce precise changes into DNA. We have provided examples of how applying a DE-GE approach to targeted enzymes might potentially produce climate-resilient crops with improved yield and enhanced nutritional value.

Box 2. Considerations on CRISPR Applications and on Delivery of DE-Generated Changes into Host Plants

A key limitation of CRISPR/Cas technology is that a PAM must be present at the target site, otherwise the Cas enzyme is unable to cut. Each enzyme is specific to one or more PAM sequences. DE has been used to solve this limitation by creating numerous variants of Cas that recognize different PAM sequences [88–90]. DE has also allowed further improvements in CRISPR technology, increasing its specificity and minimizing off-target mutations [91]. CRISPR/Cas also allows multiplex gene editing by the simultaneous expression of two or more sgRNAs [23].

The CRISPR complex can be inserted (delivered) into the host genome in at least three distinct ways, all of which have different implications and acceptances. (i) Stable transformation. Through genetic engineering it is possible to insert the genes coding for Cas endonuclease and guide RNA (gRNA) within the host plant genome by exploiting a vector, for example, *A. tumefaciens* [20,23]. These two genes are then expressed by the transformed plant where they exert their action. Once the desired modification has been achieved, the genes are easily eliminated by crossing techniques when plants are grown to maturity, which implies that the receiving plant will not host foreign genes (but see conclusions about different legislations regulating GMOs worldwide). (ii) Transient transformation. A plasmid DNA encoding the two genes (Cas and gRNA) is inserted into the host cell without being integrated into the plant genome. The plasmid stays within the cell only for a time sufficient to introduce the modification. (iii) Direct delivery. Cas protein and the gRNA molecule are inserted directly into the nucleus, where they exert their action, inducing the desired mutation without being integrated in the plant genome [23]. Because the CRISPR complex is degraded in the recipient cells after a short time, the cell remains in contact with the mutant agent only for a limited time. Direct delivery is therefore a promising strategy to reduce off-target mutations [92,93].



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Figure 2. Plant Pathways Where Directed Evolution-Genome Editing (DE-GE) May Be Usefully Applied.

The figure summarizes examples of how DE-GE could help to increase the yield of useful secondary metabolites or to improve primary metabolism in plants, as detailed in the text. Working on lycopene β -cyclase [37], phytoene synthase [29], and chalcone isomerase (CHI) may help to increase plant production of β -carotene, lycopene, and anthocyanins, respectively. Increasing the catalytic activity of isoprene synthase (IspS) and 10-deacetylbaicatin III-10- β -O-acetyltransferase (DBAT) [38] may trigger isoprene and taxol production, respectively. Enhancing the affinity/resistance of ascorbate peroxidase (APX) to hydrogen peroxide will increase plant resilience to stress, and Rubisco with improved carboxylation properties is predicted to increase agricultural yield [76]. High-throughput DE screening for peroxidases, IspS, CHI, and Rubisco is described in [52,65,72,78], respectively.

Although DE technically only accelerates evolution (unless a mutagenic agent is used to induce DNA variation), GE produces genetically modified organisms (GMOs) that are subject to different regulations in different countries (see Outstanding Questions). For example, the US Department of Agriculture (USDA) allows full marketability of plants modified using CRISPR without adding foreign genes (as is the case of SDN-1 and SDN-2) because they are comparable to plants obtained by spontaneous mutation and conventional breeding [82,83]. However, the European Court of Justice asserts that all GE plants (as well as plants produced by mutagenesis) should be treated as GMOs [84]. Growth of DE-GE plants would therefore be restricted in European countries under current legislation, which precludes their use in agriculture. While the GMO debate continues globally, the speed of population growth, changing dietary styles, and climate change impact on the demands on agriculture. Satisfying the basic requirements of sufficient nutrient food for all requires immediate action. Gains obtained by conventional breeding do not appear to be able to keep pace with growing global demand. Because the genetic diversity among commercial crop varieties has steadily been reduced, traits introduced by GE may first need to be integrated into ancestral crop varieties and then cross-bred into modern elite crop cultivars. Years after the green revolution was made possible by the inspired work of breeders such as Nazareno Strampelli and Norman Borlaug, DE may lead to a new Green (r) Evolution able to satisfy current and future needs in terms of food security and sustainability.

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Outstanding Questions

Most DE studies are targeted for deployment in microorganisms or synthetic *in vitro* applications. How well can plant enzymes modified by DE be integrated back into plants by GE technologies?

Should the organisms used to screen a DE gene library, and the resulting GE plants incorporating desired nucleotide changes, be considered GMOs?

To what extent are enzyme improvements selected by DE translatable back into metabolically complex organisms such as plants?

How do associated shifts in metabolic and cellular homeostasis influence plant performance (e.g., stress resilience, growth, and productivity)?

How feasible is it to improve the catalytic properties of plant enzymes by DE using available catalytic screens? Are there alternative screening strategies to consider/develop, especially with regard to ensuring they are high-throughput?

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