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REVIEW

Phytoremediation and sequestration of soil metals using the CRISPR/Cas9 technology to modify plants: a review

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Abstract

Soil contamination by toxic metals is a major health issue that could be partly solved by using genetically-modified plants. For that, the recently developed technique of clustered regularly interspaced short palindromic repeats (CRISPR) has created a new dimension in genetic engineering. CRISPR was first found as a part of the adaptive immune system in bacteria and archaea, and further refined to generate targeted breaks in DNA in a broad range of organisms. Various DNA changes can take place during the cellular repair process. Many plants, including crops, have the potential to tolerate, stabilize, and transform both organic and metal contaminants and have been already modified using the CRISPR method. Furthermore, many genes necessary to increase the absorption and tolerance of metals have been identified. Thus, using CRISPR, target genes could be activated or repressed to optimize phytoremediation in plants. Here we review the CRISPR/Cas9 technology applied to phytoremediation and sequestration of metals in the soil environment. The availability of the genome sequence plays a critical role in the adaptation of the CRISPR-mediated genome editing to specific plants. CRISPR has demonstrated outstanding potential for genome editing. However, the outcome depends on the selected target site, Cas9/Cpf1 function, gRNA design, delivery systems, and the off-target effects that may restrict its efficacy.

Keywords CRISPR/Cas9 · Phytoremediation · Non-homologous end joining · Homology-directed repair · Cytidine base editors · Adenine base editors

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Introduction

Anthropogenic activities remain a leading cause of pollution with increased industrialization that lacks adequate planning to manage waste (Wuana and Okieimen 2011). Phytoremediation uses plants—phyto-to reduce emissions of contaminants into the environment and related toxic effects by clean production technologies and remediation measures. Phytoremediation is more cost-effective, environmentally friendly, and solar energy-driven than many other conventional remediation or mitigation methods such as solidification, soil washing, and permeable barriers (Kotrba et al. 2009).

However, the main concern of phytoremediation technology is the handling and disposal of contaminated plants. Moreover, this technology is confined to small-scale applications (Kafle et al. 2022). Plants can accumulate pollutants in different tissues (Dhankher et al. 2012), promoting their immobilization (phytosequestration). Sometimes they can also contribute to a reduction in pollutants toxicity by different mechanisms that can act simultaneously. Table 1 summarizes the main phytoremediation strategies that involve phytoextraction, phytodegradation, phytofiltration, phytovolatilization, and phytosequestration (Morel et al. 1999; Lichtfouse et al. 2012; Venegas-Rioseco et al. 2021).

The process of heavy metals sequestration makes plants tolerant to metal toxicity/accumulation in hyperaccumulating plants. Metal transporters/genes of organelles sequester heavy metals mainly to translocate their excess amount and reduce their toxicity (Jogawat et al. 2021). This vacuolar sequestration process is an important pathway that helps in increasing the removal capabilities of contaminants and thus reduce the amount of contaminated waste in the environment.

The phytoremediation capacity of commonly used macroplant species is generally limited by the type of contaminant (Abhilash et al. 2009; Basharat et al. 2018). Plants performing phytoremediation, known also as phytoremediators, also have several limits, including slow growth rate and low biomass production, metal selectivity, and different environmental growing conditions (Kärenlampi et al. 2000; Gratão et al. 2005). Therefore, multifaceted approaches, including genetic engineering and multi-omics, should help to obtain hyperaccumulating plants with high biomass production and a wide range of growth conditions (Mosa et al. 2016; Shriram et al. 2016).

Genome editing remains a potential tool for modifying the expression of the gene of interest (GOI) (Moradpour and Abdulah 2020). Before the advent of CRISPR, zinc-finger nucleases (ZFNs; Bibikova et al. 2002) and transcription activator-like endonucleases (TALENs; Christian

Table 1 Phytoremediation mechanisms in plants

Technique	Mechanism of action	Reported plants	References
Phytosequestration	Contaminants from soil or water remain entrapped in plant tissues only	*Agrostis castellana, Phyla nodiflora, Gentiana pennelliana, Lolium italicum, Festuca arundinacea, Anthyllis vulneraria*	Numan et al. (2018), Kumar and Verma (2018), Pastor et al. (2015) and Yoon et al. (2006)
Phytoextraction	Contaminants are adsorbed via root system but stored in aerial part which is further burnt to recycle the metal	*Youngia erythrocarpa, Thlaspi caerulescens* (synonym: *Noccaea caerulescens*), *Brachiaria decumbens, Alyssum vulneraria*	
Phytodegradation	Contaminants are degraded in situ by plant enzyme	*Myriophyllum aquaticum, Elodea canadensis, Spirodela oligorrhiza*	
Phytovolatilisation	Contaminants are converted to lesser toxic compound and released in the air	*Brassica juncea, Arabidopsis thaliana*	
Phytofiltration	Contaminants are taken up by root from ground water	*Manihot esculenta, B. juncea, Berkheya coddii, Eichhornia crassipes, Hydrocotyle umbellata, Lemna minor, Micranthemum umbrosum, Callitriche stagnalis, Potamogeton natans*	

et al. 2010) were used as editing tools to modify the genome at the transcriptional level (Chen and Gao 2015). ZFNs and TALENs employ the protein-based endonuclease Fok I by producing double-stranded breaks (DSBs) in the DNA for gene knockout (Gaj et al. 2013). DSBs at the targeted site by sequence-specific nuclease (SSN) were the primary strategy to introduce genetic changes in cells. In higher eukaryotes, the repair mechanism primarily follows non-homologous end joining pathways and, minorly, homology-directed repair (HDR). ZFNs and TALENs are difficult to use in practice due to their protein/DNA interaction-mediated mechanism determining specificity. In addition, these techniques need two different protein hybrid designs to locate infrequently existing regions flanking the target DNA (Li et al. 2011).

Recently, the advances of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated9 (Cas9) tool demonstrated many advantages over the forerunners since Cas9 only requires simple cloning steps and has easy multiplexing and library building capacity (Sharma et al. 2017). The CRISPR system was first used in 2012 (Jinek et al. 2012), followed by the employ in mammalian cells (Cong et al. 2013; Mali et al. 2013a) and plants in 2013 (Li et al. 2013; Nekrasov et al. 2013; Shan et al. 2013) and has been becoming more advanced in the fields of plant sciences (Chen et al. 2019; Zhang et al. 2018a, b) succeeding the earlier technologies such as targeted mutagenesis (Ma et al. 2016; Schindele et al. 2018; Yin et al. 2017), base editing (Kim 2018), precise editing by HDR (Huang and Puchta 2019) and transcriptional regulation (Mahas et al. 2018). The CRISPR/Cas system (Bortesi and Fischer 2015) is more effective on monocotyledon crop plants due to their high GC (guanine-cytosine) content (Miao et al. 2013).

Recently, the applicability of CRISPR-assisted genome editing technology for phytoremediation of heavy metals/metalloids has been tested. CRISPR-mediated plant modifications help to withstand, immobilize, and stabilize various pollutants. The precision, cost-effectiveness, and promise of CRISPR-mediated genome editing offer exciting opportunities in many phytotechnologies such as phytoremediation (Venegas-Rioseco et al. 2021) (see Table 1). The present review aims to depict and critically evaluate the research reports published on CRISPR/Cas9 technology concerning phytoremediation, mainly addressed to sequestration of heavy metals with notes on biotechnological interventions and limitations of this system in plant genome engineering.

Bibliometry

A systematic literature review was carried out to comprehensively assess the literature concerning phytoremediation and the sequestration of heavy metals using CRISPR/Cas9. A search with the SCOPUS database, in all the paper fields, for "CRISPR" and "phytoremediation or sequestration" showed the existence of more than 2000 papers in this group. When the search was limited to "heavy metal", as pollutant source, the number of papers was 310. The attention of this review was devoted to mostly these papers.

The information of the papers extracted from the SCOPUS platform includes authors, document title, keywords, abstract, and references for the data analysis, synthesis, and interpretation. In particular, an analysis of all data from the bibliometric database, investigating the co-occurrence of text data, allows a cluster analysis of the literature (see Fig. 1). For this aim, data analysis was performed by "VOSviewer version 1.6.16," 2020. The study design, with the connected literature, was updated on September 12, 2021.

Figure 1a, based on the co-occurrence analysis of abstracts text data of bibliography, shows that the already available papers can be grouped in 2 clusters. The first one—66 items -, highlighted by red bubbles, is mainly devoted to technologies and their development, correlated, for example, with biotechnology and genomic applications. The second cluster—44 items—is represented by green bubbles and is mainly devoted to the metal remediation mechanisms.

Figure 1b shows the results of the co-occurrence network of keywords of the papers extracted from the SCOPUS search, revealing that these works can be grouped into 5 clusters. The first one—94 items -, represented by red bubbles, is mainly devoted to bioremediation, with great attention to non-human genetic engineering and genomic editing. The second cluster—74 items—highlighted by green bubbles is devoted to plant degradation of specific metals (connected with different plant tissues), with great attention to gene expressions and genotypes. The third cluster—54 items -, represented by blue bubbles, mainly concerns soil pollution, biochemistry, and chemical contamination. The fourth one—38 items—highlighted by yellow bubbles involves genome analysis, enzyme activities, and microbiology.

Finally, the last cluster—1 item -, is represented by a violet bubble, and is focused on the chemistry. Then, based on the cluster analysis results, the literature discussion was realized through reading the selected full-text papers. These papers were carefully analysed with regards to heavy metals' phytoremediation and sequestration ability. The review sections are organized to discuss the CRISPR/Cas9 system and plant genome engineering in Sect. 3, and the advancements in CRISPR/Cas9-mediated phytoremediation in Sect. 4. Finally, future directions are also presented in Sect. 5.

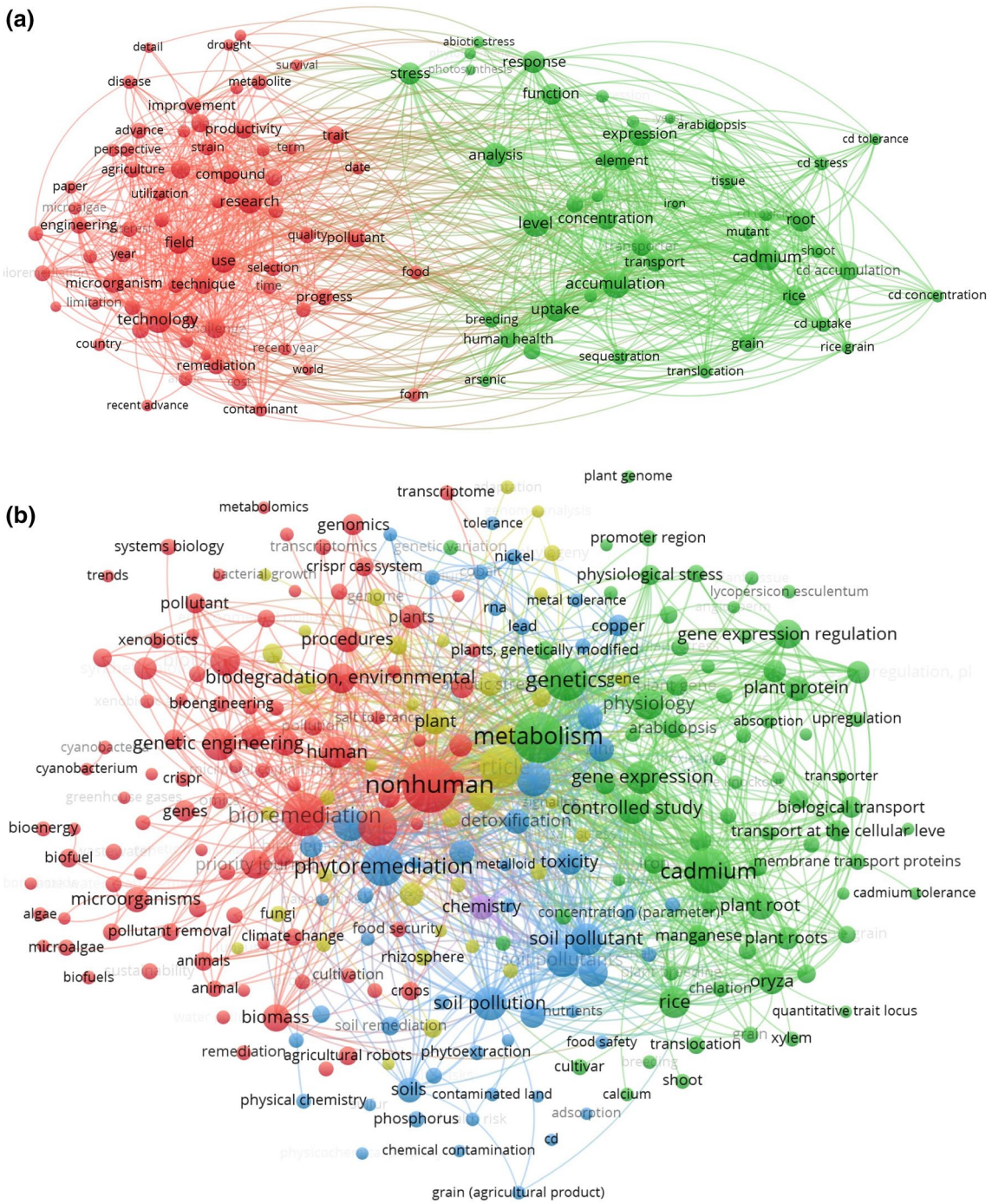

Fig. 1 Bibliometric cluster analysis results. The "co-word" analysis was realized to highlight emerging and popular themes for the investigated topic, based on the bibliography text data and keywords. The Figure shows a graphical representation of how frequently the selected variables, which are represented by a node, appear together. **a** The co-occurrence of text words in the abstracts, concerning "CRISPR", "heavy metals" and "phytoremediation (or sequestration)"; **b** The co-occurrence network of the keywords for the same papers selected for cluster analysis reported in **a**. The study was realized by using the SCOPUS database as a paper source, and the analysis was obtained by "VOSviewer version 1.6.16," 2020. The study design was updated on November 20, 2021. *CRISPR* clustered regularly interspaced short palindromic repeats

CRISPR/Cas9 and plant genome engineering

CRISPRs are short repeat DNA sequences separated by spacer regions. These are commonly found in bacteria and archaea. The CRISPRs, which are also known as CRISPR arrays, contain genes that encode Cas proteins used to classify diverse CRISPR systems depending on various factors, including phylogenetic, functional, and structural properties. CRISPR systems can be broadly classified into two types, class 1, where a single protein mediates the antiviral mechanism, and, otherwise, class 2 system (Makarova et al. 2015). Each class is further subdivided into multiple groups, class 1 has type I (Cas3), III (Cas10) and IV (Csf1) systems and class 2 systems have type II (Cas9), type V (Cas12a, Cas12b, Cas12c, Cas12d (also called as CasY), Cas12e (CasX), Cas12g, Cas12h, Cas12i, Cas14a, Cas14b, Cas14c) and type VI (Cas13a, Cas13b, Cas13c, Cas13d) (Burstein et al. 2017; Harrington et al. 2018; Liu et al. 2019; Shmakov et al. 2015; Yan et al. 2019). Each type is further divided into multiple subtypes depending on multiple factors, including the Cas protein types, and the classification system is constantly growing.

Cas 9 belongs to class 2, type II CRISPR system, and binds to a single-guide RNA [sgRNA; a fusion of crRNA and trans-activating crRNA (tracrRNA)]. Cas 9 then interacts with the target DNA sequence near a protospacer adjacent motif (PAM). *Streptococcus pyogenes* Cas9 (SpCas9) is the most diffused, using a simple PAM (NGG) for the target site recognition. This system is used in a wide range of organisms, including humans (*Homo sapiens*; hCas9) (Mali et al. 2013a, b), plants (pcoCas9 and Cas9p) (Li et al. 2013; Ma et al. 2015), *Arabidopsis thaliana* (AteCas9) (Fauser et al. 2014), maize (*Zea mays*; zCas9) (Wang et al. 2015; Lee et al. 2019) and soybean (*Glycine max*; GmCas9) (Michno et al. 2015). The Cas9 nickase (nCas9) is generated by introducing the point mutations D10A in the RuvCI domain or H840A in the HNH domain, which selectively cleaves the targeting or non-targeting strand, respectively (Cong et al. 2013; Mali et al. 2013a, b; Ran et al. 2013).

However, the nuclease activity is suppressed when both mutations are introduced, leading to a generation of catalytically inactive or dead Cas9 (dCas9) (Qi et al. 2013). Nevertheless, Cas9 nuclease, nCas9, and dCas9 have different genetic engineering uses. CRISPR provides excellent opportunities for plant genetic engineering despite the challenges generated due to the diversity. CRISPR/Cas9 will foster progress in areas where multiple genetic factors are involved, such as complex genetic architecture and ploidy levels. CRISPR/Cas9 is also useful to discover the genes and interrelated traits in plant species (Table 2).

Generation of transgene-free edited plants

The CRISPR-mediated transgenic plants should be free from transgene so that they do not appear invasive to native species, limit ecological consequences and improve public acceptance. So, CRISPR transgenes are removed mainly by attaching a fluorescent marker or herbicide susceptibility to CRISPR encoded genes (Gao et al. 2016; Lu et al. 2017). However, this approach is unsuitable for plants with vegetative growth, plants with longer life cycles, self-incompatible and polyploid plants. The mutated plants are therefore generated without integrating a transgene at all.

The CRISPR machinery is either expressed in the cellular system (Iaffaldano et al. 2016; Zhang et al. 2016) or the CRISPR/Cas RNPs (ribonucleoproteins) are delivered to plant protoplasts via polyethylene glycol mediated transformation in *Arabidopsis*, wild tobacco (*Nicotiana attenuata*), lettuce (*Lactuca sativa*), rice (Kim et al. 2017; Woo et al. 2015), apple (*Malus pumila*), grape (*Vitis vinifera*) (Malnoy et al. 2016), *Petunia*×*hybrid* (Subburaj et al. 2016) and soybean (Kim et al. 2017).

However, protoplast-mediated plant regeneration can cause somaclonal variations (Li et al. 2019). RNPs are delivered into plant tissues and plant zygotes by particle bombardment (Liang et al. 2017; Svitashev et al. 2016; Toda et al. 2019). RNP reduces the random DNA integration and off-targeting effects by reducing the exposure time of genomic DNA to CRISPR reagents. In recent advances, edited T1 rice plants have been made with a transgene killer CRISPR system (He et al. 2018).

Off-target effects

The off-target effect of Cas proteins is a major challenge in gene therapies. DSBs caused by Cas9 can result in genome rearrangements and large deletions (Kosicki et al. 2018). Whole-genome sequencing has been carried out to find the off-target in Arabidopsis, rice, and cotton (Feng et al. 2014; Tang et al. 2018; Li et al. 2019).

Most of the mutations in transgenic plants have been due to somaclonal variation since Cas9 and Cas12a have high specificity. Whole-genome sequencing suggested that CBEs (cytosine base editors that change target G-C into A-T) instead of ABEs (adenine base editors that change A-T to G-C) promote genome-wide off-target effects. For example, in more than one cytosine presence in the catalytic window, non-target cytosine-to-uracil conversion takes place (Jin et al. 2019). Modified base editors have significantly reduced their RNA editing activity (Rees et al.2019; Grünewald et al. 2019). To minimize off-targets, paired nCas9 (that reduces off-target mutation) is often used (Ran et al. 2013). Modified high fidelity SpCas9, such as SpCas9-HF1-4 (Kleinstiver et al. 2016), SpCas9 (K855A)

Table 2 Genes involved in phytoremediation

Genes/proteins	Mechanism of action	References
Toluene-o-monooxygenase (TOM)	Plays crucial role in removal of trichloroethylene	Mahendra and Alvarez-Cohen (2006) and Aburto-Medina et al. (2017)
Toluene 4-monooxygenases (T4MO)	Removal of nonaromatic nitrosodimethylamine (NDMA)	Scott et al. (2008), Li et al. (2013), Bouhajja et al. (2017) and Yamaguchi et al. (2018)
Polyphosphate kinase (PPK)	Removal of uranium	Ibanez et al. (2015), Daghan (2019) and Kaur et al. (2019)
Mercuric resistance operon regulatory protein (MerR)	Provides resistance against Hg(II)	Checcucci et al. (2017), Wei et al. (2018) and Singh et al. (2019)
Metallothionein-like protein (MTL)	Degradation of lignin and polyaromatic hydrocarbons	Peng et al. (2017) and Khan et al. (2018)
Biphenyl-polychlorinated biphenyl (bph) operon	Degradation of polychlorinated biphenyls and biphenyls	Jiang et al. (2018) and Kour et al. (2019)
Organophosphorus hydrolase (OPH)	Deduction organophosphorus	Scott et al. (2008)
lux	Helps in bioluminescence by specific aromatic compounds like naphthalene	Ariani et al. (2015), Stolarikova-Vaculıkova et al. (2015) and Rome et al. (2016)
Cytochrome P450CAM	Oxidation of hexane and 3-methylpentane	Azab et al. (2016), Legault et al. (2017) and Daudzai et al. (2018)
Ortho-dechlorination gene	Degradation of chlorobenzoic acids	Chakraborty and Das (2016)
Chlorobenzoate dehalogenase (CBAD)	2,4-dinitrotoluene	Agullo et al. (2019) and Kumar and Pannu (2018)
Polynucleotide phosphorylase (pnp) gene operon	Degradation of paraoxon	Ye et al. (2015) and Gupta and Kumar (2017)
Xylose operon regulatory protein (XylR)	Reduction of atrazine	Wu et al. (2015) and Zhao and Huang (2018)
Arylsulfatase B/C (*arsB/C*)	Removal of arsenate	Mergeay et al. (2003)
Vitreoscilla globin (vgb)	Increases growth	Khleifat et al. (2006) and Aburto-Medina et al. (2017)
AtHMA4	Restricts Cd transport from the roots to the shoots	Siemianowski et al. (2014)
Copper resistant protein C (CopC)	Hyperaccumulation of Cu in the shoots	Rodriguez-Llorente et al. (2012)
ATP-binding cassette (ABC)	Enhanced accumulation of Cd and Pb in the shoots	Bhuiyan et al. (2011)
HvNAS1	Enhanced accumulation of Ni reducing Ni-toxicity	Kim et al. (2005)
Phytochelatin synthase (PCS)	Higher accumulation of Cd and Pb, more tolerant to Cd	Huang et al. (2012) and Chen et al. (2015)
Glutamyl cysteine synthetase (GCS)	Enhances PCS activity, higher production of phytochelatins in relation to Cd tolerance	Zhao et al. (2014)

and eSpCas9 (1.1) (Slaymaker et al. 2016), HypaCas9 (Chen et al. 2017), evoCas9 (Casini et al. 2018), sniper-Cas9 (Lee et al. 2018) and HiFi Cas9 (R691A) has shown to decrease Cas binding and increasing editing specificity. The eSpCas9 (1.0 and 1.1) and SpCas9–HF1 in rice have maintained on-target editing activity with enhanced specificity when using the T-RNA–sgRNA processing system (Zhang et al. 2017).

Two other Cas9 variants, eHF1-Cas9 and eHypa-Cas9, are also reported in rice (Liang et al. 2018). Compared to wild-type, Cas9xCas9 has higher targeting specificity in rice (Zhong et al. 2019), although many high-fidelity SpCas9s may not be used for plant genome editing due to intrinsically lower nuclease activities (Zhang et al. 2017; Zhang et al. 2018a, b).

Temperature sensitivity

Cas9 and Cas12a function optimally at a higher temperature in mammalian cells compared to plants (Xiang et al. 2017; Moreno-Mateos et al. 2017; LeBlanc et al. 2018; Malzahn et al. 2019). However, an increase in Cas9 and Cas12a efficiency has been shown upon elevation of temperature in rice, *Arabidopsis,* and maize (Malzahn et al. 2019; LeBlanc et al. 2018). Cas9 exhibited optimal nuclease activity in human cells at 37–39 °C (Xiang et al. 2017), and improved Cas9 editing was observed in plants after heat stress at 37 °C (LeBlanc et al. 2018).

On the other hand, Cas12a nucleases showed optimal activities at around 28–29 °C in plants, and 22–29 °C is

the most suited temperature range for most plants. Further knowledge on the effects of heat treatment will help to facilitate a more robust editing toolkit in plants. However, the temperature sensitivity of Cas12a nucleases should not hinder applying CRISPR-based genome editing in various other plants (Malzahn et al. 2019).

Genome editing in polyploid plants

Many crops are either triploids [citrus, banana (*Musa acuminata*, *M. balbisiana,* and *Musa*×*paradisiaca*), seedless watermelon (*Citrullus lanatus*) and some varieties of apples]; tetraploids [*Triticum durum*, cotton, potato, canola (*Brassica napus*), rapeseed, peanut (*Arachis hypogaea*), tobacco, *Panicum virgatum* and some varieties of apple], hexaploids [*Camelina sativa*, bread wheat and oats (*Avena sativa*)] or octoploids [sugar cane (*Saccharum officinarum*) and strawberry (*Fragaria*×*ananassa*)].

As gene knockout provides low output in polyploid species compared to diploid species, there is a need for a highly active Cas nuclease and an efficient expression system. This has been successfully achieved in many polyploid plant species (Ryder et al. 2017; Shan et al. 2018; Andersson et al. 2017; Braatz et al. 2017; Gao et al. 2017; Jiang et al. 2017; Liu et al. 2018; Morineau et al. 2017; Wang et al. 2014; Wang et al. 2018; Zhang et al. 2016; Zhang et al. 2019). So far, genome editing has improved oil quality and disease resistance in crop plants (Jiang et al. 2017; Wang et al. 2014; Jia et al. 2017).

Floral dip transformation-mediated germline editing

The generation of germline-edited plants remains challenging for plants such as *Arabidopsis* since the *Agrobacterium*-mediated floral dip method to deliver the CRISPR transgene has low editing efficiency in germlines (Lee et al. 2019). *Agrobacterium* delivers the CRISPR-carrying T-DNA to the egg cells, but germline edits are only possible when CRISPR makes modifications after the *Agrobacterium* infection but before the embryogenic cell division; otherwise, the results can be considered as chimeric plants.

Egg cell-specific promoters for Cas9 expression can overcome this challenge by limiting or boosting genome editing in germinal cells. Such promoters are EC1.2 (Lee et al. 2019), the sporogenesis expression promoter SPOROCYTELESS (Mao et al. 2016) and meiosis I-specific promoter (Eid et al. 2016), as well as CDC45, DMC1, SOP11, YAO, and RPS5A promoters (Yan et al. 2015; Tsutsui and Higashiyama 2017). The use of preselected transgenic lines with high germline expression of Cas9 in *Arabidopsis* helped to achieve HDR (Miki et al. 2018). However, similar problems persist in other crops such as *Camelina* (Jiang et al. 2017). Different goals, which were achieved by CRISPR in plant genetic engineering, are summarized in Fig. 2.

Advancements in CRISPR/Cas9-mediated phytoremediation

Many phytoremediators have been sequenced partially or completely, as *Thlaspi caerulescens* (synonym: *Noccaea caerulescens*; hyperaccumulator for Cd, Zn, and Ni), *Arabidopsis halleri* (hyperaccumulator for Cd and Zn), *Hirschfeldia incana* (capable for withstanding Pb), *Pteris vittata*, and *Brassica juncea* (Basharat et al. 2018; Mandàkovà et al. 2015; Auguy et al. 2016; Briskine et al. 2017). The availability of the genome sequence plays a fundamental role in the adaptation of the CRISPR-mediated genome editing to specific plants.

Phytoremediation provides an efficient, environmentally non-destructive, and cost-effective remediation method. Table 3 lists the application of different biotechnological tools for phytoremediation. Engineering genes can genetically manipulate the efficacy of phytoremediation for metal uptake, transport, and sequestration. Genes such as metal chelator, metal transporter, metallothionein, and phytochelatin have been transferred to plants to improve metal uptake and sequestration.

For example, the CRISPR-mediated control of the metal transporter gene OsNRAMP5 resulted in low Cd-accumulation in indica rice without compromising yield (Tang et al. 2017). In addition, transgenic plants have been developed with the ability to detoxify, to tolerate or to accumulate toxic metals and metalloids (Eapen and D'Souza 2005) as increased tolerance to toxic metals can lead to hyperaccumulation thereby promoting phytoremediation (Huang et al. 2019).

Finally, the phytoremediation of toxic metal mainly focuses on the increased synthesis of metal ligands like metallothioneins, phytochelatins, metal transport proteins like CDF, HMA, and ZIP families, plant growth hormones like CKs and GAs, and root exudates like siderophores. For example, overexpressing the NAS1 gene in Arabidopsis and tobacco plants increased tolerance against metals like Cd, Cu, Fe, Ni, and Zn and uptake of metals like Mn and Ni. Increased capacity of Cd, Cu, and Zn accumulation was observed during overexpression of metallothioneins encoding genes (such as MTA1, MT1, and MT2) in tobacco and Arabidopsis (Xia et al. 2012; Sebastian et al. 2019). The overexpression of ATP Sulfurylase gene (*APS*) and Selenocysteine Methyltransferase gene (*SMT*) in *B. juncea* led to increased tolerance towards Se (LeDuc et al. 2006). Similarly, expressing the two metallothionein genes *BcMT1* and *BcMT2* from *B. campestris* increased the tolerance to Cd and Cu in *A. thaliana* (Lv et al. 2013).

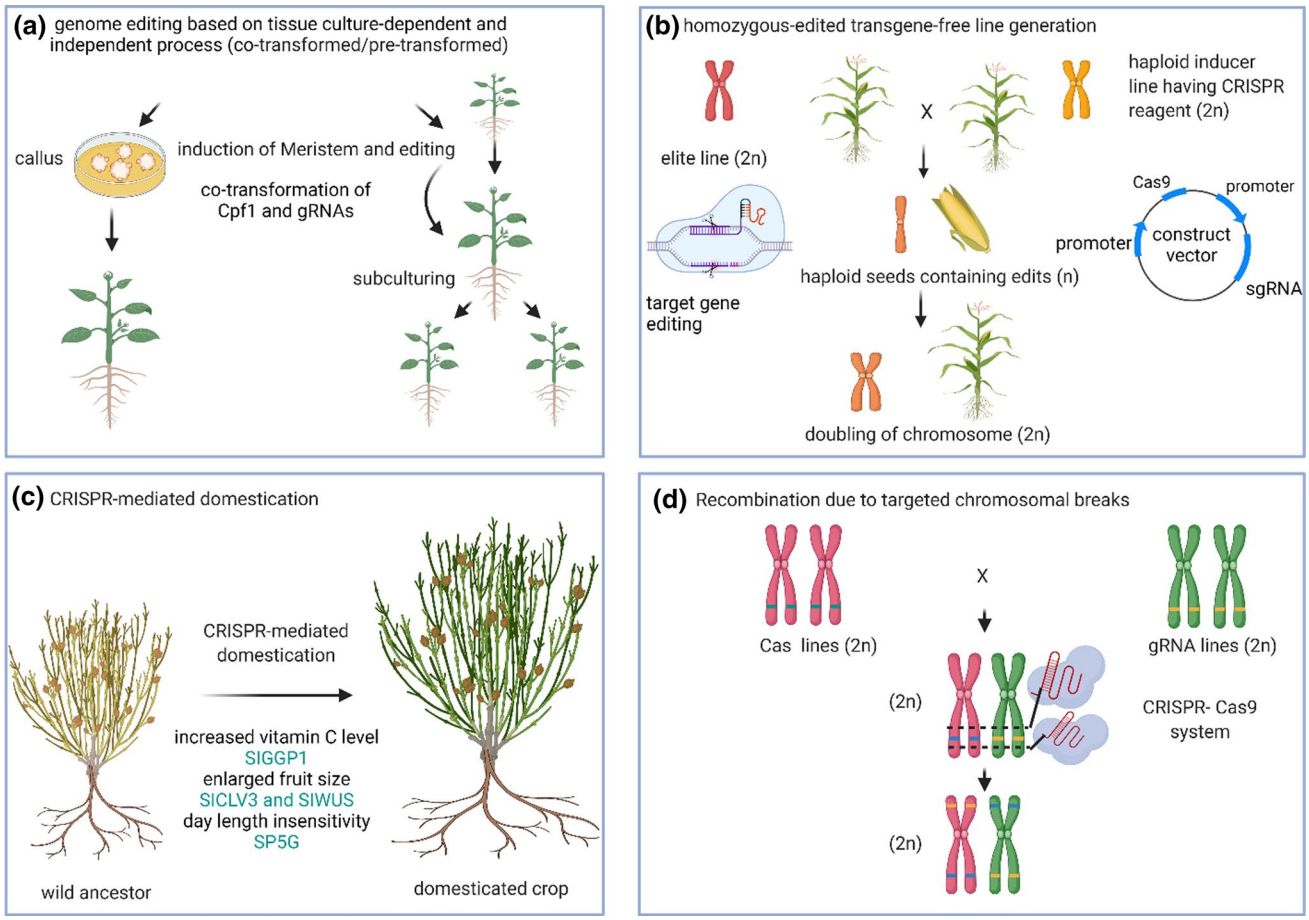

Fig. 2 Multifaceted applications of CRISPR-mediated genetic modifications in plants: **a** in tissue-culture dependent and independent process, where Cpf1 or Cas12a (CRISPR associated protein 12a) is an RNA guided endonuclease and Cpf1 is co-transformed with guide RNA (gRNA) in the callus culture to propagate transgenic plants **b** in homozygous-edited transgene-free line generation, where an elite line i.e. with the desired trait is crossed with an inducer plant having CRISPR construct. This results in haploid seeds which are made diploid by mainly self- crossing. **c** In domestication where CRISPR editing is used to augment a metabolite level like vitamin C or increased fruit size or day length insensitivity and in **d** in targeted chromosomal breaks to induce recombination. *CRISPR* clustered regularly interspaced short palindromic repeats

Table 3 Different biotechnological tools involved in phytoremediation process

Name of the editing tool	Mechanism of action	References
CRISPR-Cas9	It is a DNA endonuclease that mediates its action in a RNA-guided manner and targets specific sequences in the genome	Ebbs et al. (1997), Feng et al. (2018), Tang et al. (2017), Habibi et al. (2017), Agnihotri and Seth (2019), Kaur et al. (2019) and Pandey and Singh (2019)
CRMAGE	CRISPR-Cas9 in combination with the Lambda (l) and is engineered with the MAGE technique	Ronda et al. (2016) and Mukherjee (2017)
MuGENT	Genome integration with mutants that consists of high efficiency	Agnihotri and Seth (2019)
TALENs	Nonspecific exonuclease targets DNA-binding domain for modifications	Basharat et al. (2018) and Mahfouz et al. (2014)
ZFNs	Genome editing by DNA-binding proteins at targeted sites by forming commencing double-strand breaks in the DNA	Miller et al. (2007), Gabriel et al. (2011) and Noman et al. (2016)

The transformation of highly toxic mercury Hg^{2+} to Hg^0 is carried out using genetically modified *Liriodendron tulipifera* (yellow poplar). Besides this, in tissue culture, this plant can grow in higher mercury concentrations (Rugh et al. 1998). Explosives like TNT were shown to be cleaned up by *Vetiveria zizanioides* with a success rate of 97% (Das et al. 2010) and by *Nicotiana tabacum*, which produces *nsfI* nitroreductase enzyme in the roots (Hannink et al. 2007). Similarly, *Populus deltoides* plants were found to be involved in converting hexahydro-1,3,5-trinitro-1,3,5-triazine to metabolic components (Just et al. 2004). Rhizodegradation is a process where bacteria and mycorrhizae degrade toxins. Rhizomes of *Typha latifolia* were found to degrade terbuthylazine (TER) (Papadopoulos et al. 2019). Polycyclic hydrocarbons are carcinogenic and mutagenic and are often accumulated in soil and plant parts (Bryselbout et al. 2000). *Rhizophora mangle* mangrove with plant growth-promoting rhizobacteria (*Pseudomonas aeruginosa* and *Bacillus* sp.) was found to degrade carcinogenic and mutagenic polycyclic aromatic hydrocarbons (Harvey et al. 2002; Sampaio et al. 2019; Nedjimi 2021).

However, tweaking metal accumulation can result in the development of hypersensitivity. For example, overexpression of the plasma membrane protein in tobacco plants caused an increased capacity for accumulation of Pb but also resulted in an increased sensitivity of the plant to Pb. Similarly, overexpression of the merC gene caused both increased accumulation and hypersensitivity towards Hg in Arabidopsis and tobacco plants (Fasani et al. 2018).

In addition to editing plant genes to increase phytoremediation, the plant–microbe interaction should not be neglected. Microbe's interaction provides plants the tolerance to adverse conditions by promoting phytohormone production (by fungi such as *Laccaria bicolor*, *Tuber borchii,* and *T. melanosporum* in *Cistus incanus* (Boivin et al. 2016), siderophore production (for example, by the members of Vibrionaceae (Thode et al. 2018), root nodule formation and nitrogen fixation (by the nodule endophytic *Bacillus megaterium* strain from *Medicago polymorpha* (Chinnaswamy et al. 2018).

Earlier, several studies have indicated the potential of plant–microbe interactions in removing heavy metals from contaminated and polluted sites. Novel and efficacious microbes and their promising applications in the plant rhizosphere can further be utilized in the phytoremediation of different soil contaminants (Mandal et al. 2016).

CRISPR has opened the way of phytoremediation for many plants, like maize and poplar, which were considered capable but not yet investigated due to the complex architecture of their genome. Recent advances have suggested modifying maize plants by CRISPR despite the complex genome and high ploidy levels (Agarwal et al. 2018). Since maize is a fast-growing crop with a high yield of biomass and the potential of accumulating metals, the sustainability of the phytoremediation must be further investigated in this case. Poplar is also of potential candidate for CRISPER due to the available intensive root system that penetrates deep into the soil and accumulates contaminants (Baldantoni et al. 2014).

Furthermore, computational biology and multi-omics approaches, such as genomics, proteomics, metagenomics, and transcriptomics, are essential to obtain target genes. The constraint-based mathematical modelling system (Zhu et al. 2016), flux balance analysis for metabolic pathway assessment (Cheung et al. 2015), and genome integration assessment via omics tools (Yoshida et al. 2015; Do Amaral and Souza 2017) have been used to explain the physiology and molecular manifestations of the edited plants. Figure 3 represents the applications of multi-omics and genetic engineering in CRISPR-mediated phytoremediation.

Perspective

CRISPR-assisted genome editing holds immense promise for exploring plant genomes to facilitate phytoremediation. Editing the target genes, modulating their expression, manipulating the pathway(s) and pollutant homeostasis networks involved in hyperaccumulation, degradation, or tolerance can be wide-ranging and far-reaching for a pollution-free environment using phytoremediation. Recombinant DNA technology and synthetic biology-based approaches pave newer ways to confer desired phenotypes in the organism of interest. Besides, the progress in plant genome editing can provide a new dimension in improving plants for phytoremediation. From single-base alteration to mega alteration and copy number variation, genome editing provides tools to advanced phenotype generation. The gRNA-Cas9 facilitates the targeting of multiple sequences simultaneously, allowing the simultaneous manipulation of multiple traits involved in enhanced plant growth and biomass production, abiotic and biotic stress tolerance, and metal accumulation. Since the discovery, the CRISPR-Cas9 system has revolutionized genetic engineering, providing a convincing and prospective approach for transgenic plant generation.

Table 3 presents the different biotechnological tools in relation to phytoremediation. To date, most of the CRISPR-based studies are performed in mammalian cells, making laborious to easily replicate these results in plants. Further improvement of the CRISPR system needs the optimization of the sgRNA scaffold with a good binding affinity for Cas9. Plants with complex genomes, such as wheat and sugarcane, have not yet been studied using CRISPR. The generation of more competent Cas proteins and their optimization would open new areas in genetic engineering. Further, screening of transgenic plants in actual environmental conditions is necessary.

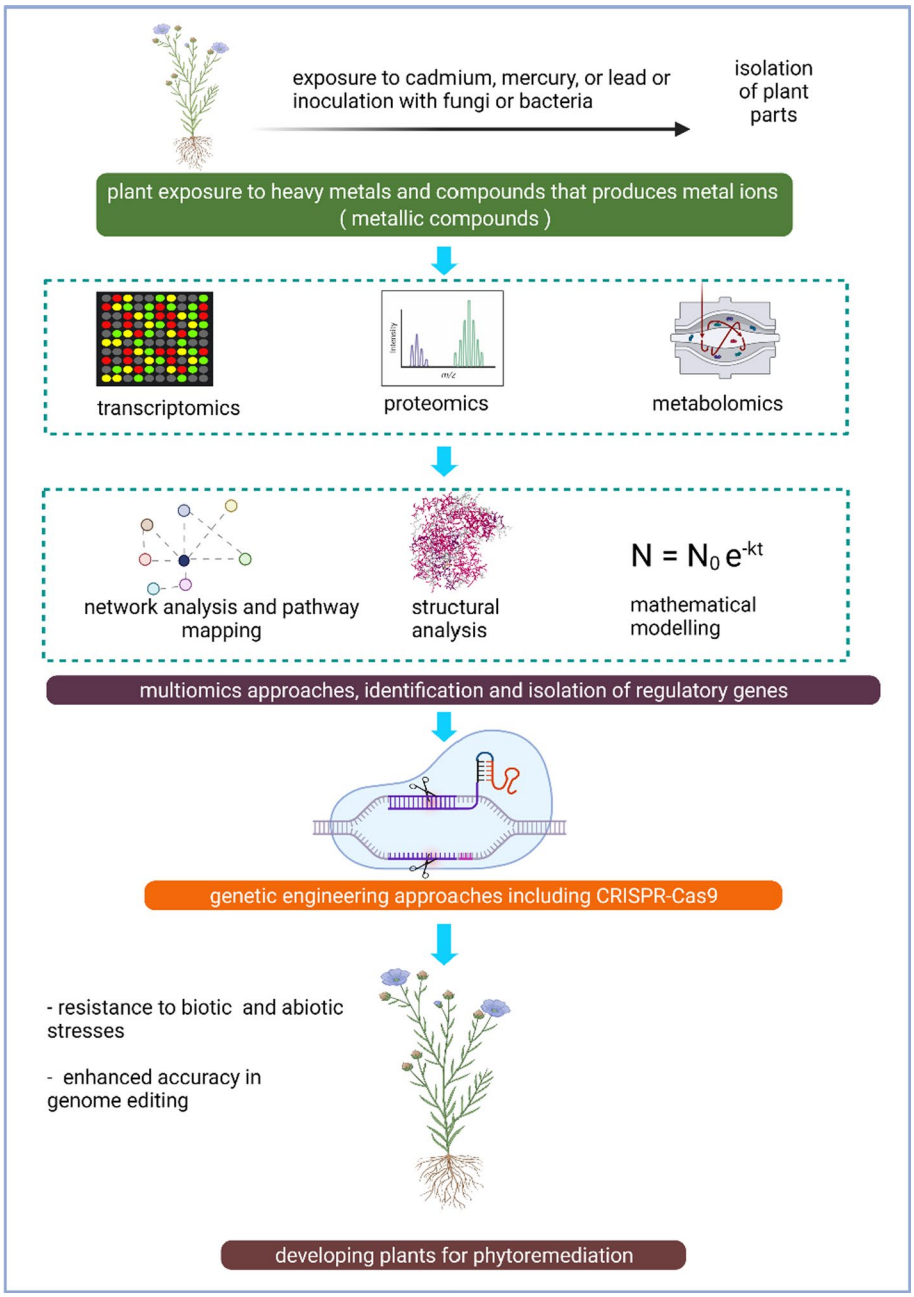

Fig. 3 CRISPR-mediated phytoremediation: applications of multi-omics including genomics, transcriptomics, network analysis and mathematical modelling in identification, isolation, and genetic engineering of regulatory genes. *CRISPR* clustered regularly interspaced short palindromic repeats

Nevertheless, challenges to utilizing CRISPR in plant genome editing remain in target specificity, delivery system, and genetic makeup of the plant. Although CRISPR has shown convincing potential in increasing the remediation rate, further advancements are required to overcome the challenges. Progress may include the immediate transfection of Cas9 alongside gRNAs into the plant protoplasts, T-DNA-conveyed gRNA_Cas9, and plant retrieval from single-cells (Mikami et al. 2015). The binding ability of Cas9 protein also helps in controlling the expression at the level of transcription by the regulation mode of the transcription factors (TFs) (Miglani 2017), that can function precisely over 1000-fold range (Piatek et al. 2015; Lowder et al. 2015).

In bacteria and Chlorophyta, Cas9 has exhibited toxicity, hence compromising growth. However, no report on Cas9 toxicity has been noted in higher plants. However, DSBs and Cas9 are known to cause genome instability in various systems, limiting the application of CRISPR/Cas9. In Streptomyces, DSBs result in large-scale genome deletions and rearrangements in the 6–12 Mb linear chromosome (Hoff et al. 2018). DSB-free, highly accurate single-nucleotide resolution-based CRISPR-Base Editing System, CRISPRi, and deaminase-based DNA base editors have been efficiently

employed to avoid such a scenario tool for genome engineering (Tong et al. 2020).

In addition, in various polyploid plants, the CRISPR/Cas editing strategy is limited because of their high copy numbers, large genome size, and lack of annotated genomes to prepare the appropriate sgRNAs. However, the CRISPR/Cas9 toolkit demonstrates many benefits compared to the conventional editing strategies as CRISPR/Cas9 can simultaneously target several genes without demonstrating any linkage drag (Montecillo et al. 2020; Wada et al. 2020; Zhou et al. 2020).

However, many regulatory and ethical issues have been raised lately regarding plants' CRISPR/Cas9 editing. In July 2018, the European Court of Justice stated that organisms obtained as results of directed mutagenesis are to be reckoned as genetically modified organisms within the meaning of Directive 2001/18. Michael M. Landa, J.D., Director of the US Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition, stated in 2014 that FDA is "confident that the genetically engineered (GE) foods in the US marketplace today are as safe as their conventional counterparts." The FDA further declared that manufacturers are responsible for assuring the GE food products' safety, and the FDA is not responsible for testing their products. Therefore, regulatory and ethical considerations of novel genome engineering techniques, including CRISPR, must evaluate their likely yet unrealized hazards and uncontrolled applications (Gelinsky and Hilbeck 2018). Recently, Robin Fears, director of the European Academies Science Advisory Council's Biosciences Program, criticized the current EU regulations regarding the use of CRISPR/Cas and advocated broader use of this technique by saying, "There is a societal cost of not using new genome editing techniques or being slow in adoption."

Conclusion

The present review article summarizes the potential of CRISPR-Cas genome editing in phytoremediation aiming on breakthrough advancements and future perspectives in the field. A wide variety of plants were considered for genetic manipulation with the availability of codon-optimized Cas9 versions for both monocots and dicots. Many plant genes have been edited with different techniques to improve different steps and boost phytoremediation. CRISPR has emerged as a great genome-editing toolkit, improving day by day and making editing possible even in plants with challenging genomes. However, the latest research also investigates the possible role of plant–microbe interactions and the involvement of microbial genes as regulators of different aspects of phytoremediation.

Chronic exposure to heavy metals and human diseases are potentially interlinked since many metals and metalloids are carcinogenic with a long half-life and are non-degradable. The ever-changing environment and climate and the plants' resilience and subsequent adaptations are crucial factors in phytoremediation practice. Engineering plant genomes for metal hyperaccumulation could provide new opportunities to eliminate such problems. Although CRISPR has demonstrated a huge potential for genome editing, the outcome depends on the target site selection, Cas9/Cpf1 function, gRNA design, delivery systems, and the off-target effects that may restrict the efficacy. Further research is required to use these advanced genome-editing tools for precise and targeted modifications in order to facilitate phytoremediation to secure sustainable development.

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Declarations

Conflict of interest The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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