The Advancement of Phytoremediation Using Gene Overexpression, CRISPR-Cas9, and Gene Stacking Technologies

Madison Kendall

[redacted]

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Introduction

The anthropogenic activities required to support a growing world population, such as mining, industry, technology, and agriculture, are causing the contamination of soils by heavy metals (Tchounwou et al., 2012). Heavy metal contamination is concerning due to their persistence in the environment, as well as their ability to bioaccumulate in tissues when consumed and their ability to magnify with increasing trophic levels (Sayqal & Ahmed, 2021). Some of the heavy metals that cause the most concern are cadmium, chromium, lead, arsenic, and mercury, due to their high level of toxicity and their known or probable classification as human carcinogens (Tchounwou et al., 2012). With approximately 5 million soil sites contaminated with heavy metals worldwide, the health and quality of life of humans and other organisms are at risk (Li et al., 2019, as cited in Henao & Ghneim-Herrera, 2021). Several remediation methods exist for removing heavy metal contamination from soils, including physically removing the soils from the area, thermal desorption, and chemical and electrokinetic remediation, though bioremediation using plants has quickly become one of the more promising techniques due to it being cost-effective, aesthetically pleasing, efficient, and environmentally friendly (Awa & Hadibarata, 2020; Li et al., 2019). Due to their evolved ability to efficiently accumulate low concentrations of heavy metals, certain heavy metal-absorbing plants are being planted on contaminated sites, after which they are harvested and burned (Baird & Cann, 2012).

Phytoremediation can occur via many processes, all of which are plant-dependent. According to Kafle et al., and as depicted in Figure 1, phytoremediation mechanisms include rhizodegradation, phytodegradation, phytovolatilization, phytoextraction, phytostabilization, phytofiltration, and phytodesalination (2022). While each mechanism varies slightly, they each involve the uptake of heavy metals by the roots of a plant, after which the toxicants are metabolized, precipitated, combined with other biomolecules, or undergo evapotranspiration into the atmosphere (Kafle et al., 2022).

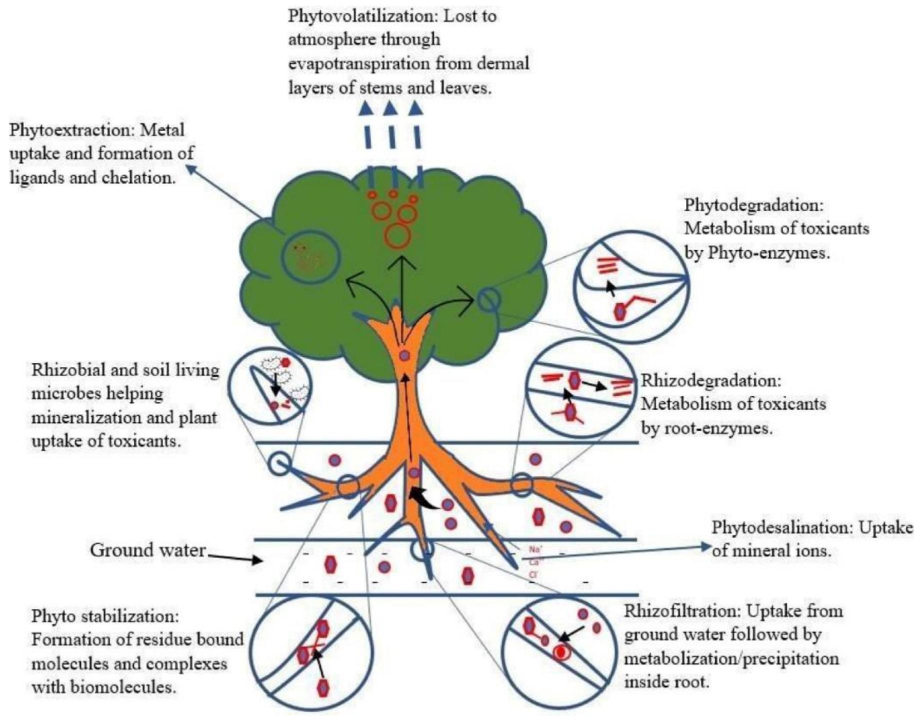

Figure 1. *Note.* From "Phytoremediation: Mechanisms, plant selection, and enhancement by natural and synthetic agents," by Kafle et al., 2022, *Environmental Advances*, 8, https://doi.org/10.1016/j.envadv.2022.100203

As of 2019, 500 types of plants were identified as hyperaccumulators – in other words, plants with the natural ability of removing large amounts of heavy metals from contaminated sites (Li et al., 2019). Advances in bioengineering have also made it possible to enhance the ability of many types of plants, not just hyperaccumulators, to phytoextract heavy metals. This paper will discuss recent advances in gene overexpression, CRISPR-Cas9, and gene stacking technologies in improving the phytoremediation abilities of plants in removing heavy metals from contaminated soil sites.

Gene Overexpression

According to Venegas-Rioseco et al., one of the ways to enhance heavy metal absorption and accumulation in plants is to overexpress metal-binding proteins or metal transporters (2021). An example of overexpressing metal transporters is seen in the experiment published in 2013 by Shim et al., where poplars were genetically modified to express the yeast ScYCF1 gene which codes for a toxic metal vacuolar transporter (Venegas-Rioseco et al., 2021). These poplars were then grown on soil contaminated with heavy metals, after which they showed reduced symptoms of cadmium toxicity and ended up absorbing and retaining more cadmium when compared to wild, unaltered poplars (Venegas-Rioseco et al., 2021).

When it comes to overexpressing metal-binding proteins, metallothioneins are the target, due to their role in maintaining metal homeostasis, detoxification, and scavenging reactive oxygen species in microorganisms, plants, and animals (Xia et al., 2012, as cited in Venegas-Rioseco et al., 2021). The overexpression of metallothionein encoding genes in “poplar, tobacco, and Arabidopsis” has resulted in their increased ability and capacity to both tolerate and accumulate cadmium, copper, and zinc (Turchi et al., 2012, Lv et al., 2013, and Xia et al., 2012, as cited in Basharat et al., 2018). Another protein that could be overexpressed to improve phytoremediation capabilities in plants is phytochelatin, which play roles in detoxifying and tolerating heavy metals in plants (Guo et al., 2008, as cited in Venegas-Rioseco et al., 2021). A study published by Zhu et al. in 2021 isolated a phytochelatin gene called BnPCS1 which carried a promoter region that had elements involved in abiotic stress response (Venegas-Rioseco et al., 2021). Zhu et al. found that BnPCS1 was induced when in the presence of cadmium, and overexpressing this gene resulted in higher cadmium accumulation compared to unaltered wild plants under the same conditions (Zhu et al., 2021, as cited in Venegas-Rioseco et al., 2021).

Some issues remain in the usage of gene overexpression to develop hyperaccumulators. For example, overexpressing genes that promote the accumulation of a given metal may also cause hypersensitivity to that metal in the plant (Basharat et al., 2018). This was seen in the overexpression of the plasma membrane protein NtCBP4 in a transgenic tobacco plant which increased its ability to accumulate lead, though resulted in the plant’s increased sensitivity to the metal (Arazi et al., 1999, as cited in Basharat et al., 2018). Another issue that remains is the legal and normative obstacles that arise when implementing genetically-modified organisms (Venegas-Rioseco et al., 2021). However, the positive results seen in overexpressing genes of natural accumulators of plants are promising for future exploration.

CRISPR-Cas9

According to a review conducted in 2018 by Basharat et al., the sequencing and manipulation of genetic sequences have allowed for the establishment of CRISPR systems that can be used to improve phytoremediation by targeting the plant mechanisms involved in pollutant "accumulation, complexation, volatilization, and degradation". One area of phytoremediation improvement under exploration is the usage of CRISPR-mediated gene expression to increase the synthesis of metal ligands, which can in some cases act to stabilize heavy metals (Basharat et al., 2018; Yan et al., 2020). Other areas of modification that could result in improved phytoremediation include metal transport proteins, plant growth hormones, and root exudates (Basharat et al., 2018). Certain plants modified using CRISPR systems have already been seen to have increased heavy metal uptake abilities. For example, Arabidopsis and tobacco plants have received the NAS1 gene and have shown greater tolerance towards heavy metals including cadmium and copper, as well as increased uptake of metals such as manganese and nickel, a quality provided for by the NA synthase enzyme coded for by NAS1 (Kim et al., 2005, as cited in Basharat et al., 2018).

It's suggested that the CRISPR technique that could produce the greatest results in improving phytoremediation capabilities in plants is the implementation of "gRNA-guided dCas9 to modulate gene expression" Basharat et al., 2018). This mechanism, known either as CRISPRi (interference) or CRISPRa (activation), involves fusing transcription factors with dCas9 that can be used to enhance or repress transcription carried out in plant genomes by RNA polymerase (Basharat et al., 2018). According to Gilbert et al. in 2014, CRISPRi and CRISPRa can affect gene expression "over a 1000-fold range" (Basharat et al., 2018). For example, CRISPR has been used to reduce cadmium uptake in rice by removing

OsNramp5, a metal transporter gene – this suggests that the reverse could be achieved by amplifying this gene to increase uptake (Tang et al., 2017, as cited in Basharat et al., 2018).

One of the challenges facing the usage of CRISPR to create hyperaccumulators for phytoremediation is that plants modified using CRISPR should be free of the transgene involved “so that they do not appear invasive to native species, limit ecological consequences and improve public acceptance” (Bhattacharyya et al., 2022). This is done either by removing the transgenes via fluorescent marker or herbicide, or the generation of CRISPR-affected plants without integrating a transgene – this is done by either expressing the CRISPR mechanism in the cellular system or by delivering the CRISPR/Cas ribonucleoproteins via a “polyethylene glycol mediated transformation” (Bhattacharyya et al., 2022). Other challenges in using CRISPR to modify plants to become phytoremediators, and in some cases, hyperaccumulators, include “target specificity, delivery system and genetic makeup of the plant” (Bhattacharyya et al., 2022). Additional challenges include regulatory and ethical pushback from governing bodies, such as the European Court of Justice and the FDA, which both suggested that the implications of using CRISPR to modify plants to become hyperaccumulators should also be considered against the possibility of unrealized hazards and uncontrolled applications from using the technology (Gelinsky & Hillbeck, 2018, as cited in Bhattacharyya et al., 2022). That notwithstanding, CRISPR is still a relatively new technology that has shown promising applications in the realm of phytoremediation and may yet become a mainstream method of helping plants remove heavy metals from contaminated soils once application, ethical, and regulatory challenges are overcome.

Gene Stacking

Gene stacking is the process of introducing more than one gene into a plant, either sequentially or simultaneously, which has the effect of imparting many traits or characteristics to the recipient genome (as opposed to one trait, as is seen in single-gene technology) (Sheryar et al., 2019). It has many crossovers with gene overexpression and CRISPR technologies, often using CRISPR to insert the new genes and then overexpressing them to ensure maximum performance from their phytoremediation qualities.

In a study published in 2008 by Guo et al., the simultaneous stacking and overexpression of AsPCS1 from garlic and GSH1 from baker's yeast in *A. thaliana* produced a plant that had a significantly higher tolerance and accumulation potential to cadmium and arsenic than the wild, unaltered form of the plant (Venegas-Rioseco et al., 2021). Similar results were seen in a gene stacking experiment carried out by Zhao et al. published in 2014, where species that were altered with a single gene (either PaPCS or PaGCS) had a lower tolerance and a lower accumulation potential for cadmium than species that were altered using both genes, resulting in a much stronger plant for phytoremediation purposes (Venegas-Rioseco et al., 2021). While the plant altered by a single gene had a higher phytoremediation potential than the wild, unaltered plants, it was shown that gene stacking produced an even better result with a higher accumulation potential (Zhao et al., 2014, as cited in Venegas-Rioseco et al., 2021). As with gene overexpression and CRISPR-Cas9 technologies, gene stacking faces many of the same legal and normative challenges that will hinder its exploration and implementation for phytoremediation purposes.

Conclusion

The enhancement of plants for phytoremediation purposes is one of the most promising areas of bioremediation thanks to its cost-effective, low-energy, and aesthetic nature. Many plants are already effective phytoremediators, with many of them having an affinity and tolerance for absorbing, storing, metabolizing, and/or releasing heavy metals which have come to contaminate many soil sites due to anthropogenic activities. However, it has been found that the accumulating properties of these plants can be enhanced, as well as passed on to other plants lacking these capabilities, to produce an even greater abundance of plants capable of phytoremediation.

Through gene overexpression, it can be seen that heavy metal absorption in hyperaccumulators can be enhanced by targeting and amplifying the activity of metal-binding and metal-transporting proteins. This action allows plants to experience reduced symptoms of heavy metal toxicity while allowing them to absorb and retain more heavy metals than their wild, unaltered counterparts (Venegas-Rioseco et al., 2021). By amplifying the traits these plants already have, bioengineering is turning them into phytoremediation super soldiers without having to extensively alter their genetic code.

The relatively new technology of CRISPR has allowed for the insertion of genes into plants relevant to increasing the synthesis of metal ligands, metal transport proteins, plant growth hormones, and root exudates to help plants absorb and remove heavy metals, even if they don't have an affinity for those metals to start (Basharat et al., 2018).

Finally, gene stacking brings together the techniques of both gene overexpression and CRISPR to modify and express alternative genes in plants that will impart traits relating to tolerance and accumulation potential. Gene stacking involves inserting two or more genes into a plant and then perhaps overexpressing them to gain the advantages of both genes, resulting in plants that have high affinities for heavy metals, as well as the ability to tolerate and accumulate them (Sheryar et al., 2019).

Areas for further research include studying whether heavy metal-accumulating genes can remain in the host plant without spreading to those in the surroundings, ensuring that plants are capable of taking on higher concentrations of heavy metals without reaching points of hypersensitivity, and determining if there are genes that can impart the ability to absorb heavy metals outside of the typical affinity.

With all the technologies discussed, technical, legal, and normative issues remain in the way before genetically modified plants become the norm when it comes to remediating heavy metal-contaminated soil sites. However, with increased public awareness about the benefits of phytoremediation, increased funding for scientists seeking the best ways to alter these plants, and increased legislation towards supporting more natural ways of cleaning up pollutants, phytoremediation could soon see a golden age of heavy metal cleanup.

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