DETOXIFICATION OF MARTIAN SOIL VIA PLANTS: A PRELIMINARY INVESTIGATION TO PRODUCE PLANTS CAPABLE OF PERCHLORATE BREAKDOWN

Peter Forstner[1], Alex Harris[1], Bastiaan Bargman[1], Guillaume Pilot[1], Eva Collakova[1]

1: School of Plant and Environmental Science, Virginia Tech, 24061, USA

ABSTRACT

Despite the recent focus on establishing a colony on Mars, the development of agriculture on Mars remains a significant challenge, with the presence of perchlorate in Martian regolith being a major obstacle. Some bacterial species can reduce perchlorate to the non-toxic chloride anion, but they have specific growth condition requirements, which makes them unsuitable for removing perchlorate from Martian soils. Plants can grow on Martian soils, but not efficiently as they lack the ability to metabolize perchlorate. This study lays the groundwork for genetically engineering plants capable of breaking down perchlorate within soils. Relevant bacterial perchlorate-reducing genes were synthesized and assembled into transcriptional units controlled by the plastocyanin promoter using the GoldenBraid technique and the resulting constructs were used to transform *E. coli*. However, these perchlorate-reducing genes proved toxic to *E. coli* as we failed to obtain constructs with the correct sequences. Therefore, we have explored different *E. coli* strains and lower temperatures to overcome this toxicity problem in cloning.

INTRODUCTION

With the emphasis placed recently on Martian colonization, several problems will have to be addressed and ultimately overcome before a self-sustaining colony can be established. One major obstacle that must be overcome is the ability to produce food. A challenging aspect of establishing agriculture on Mars is the presence of the perchlorate anion (ClO_4^-) in Martian regolith. Perchlorate is present at concentrations up to or even exceeding 1% weight by volume in Martian soil[1]. At this concentration, it causes a wide range of stress responses in plants, ranging from dwarfed growth habits, reduced or inhibited seed germination, disruption in photosynthesis, chlorosis, necrosis, and mortality[1]. The broad range of adverse effects caused by perchlorate at levels found in Martian soil renders it unsuitable for many uses and presents a significant challenge to future colonization efforts[1]. To utilize Martian soil a substrate for plant growth, significant remediation is required to reduce or eliminate perchlorate contamination[1]. Although perchlorate is toxic to many microorganisms, some bacteria can use perchlorate as an electron acceptor. *Azospira suillum* strain PS has perchlorate-reducing capabilities and was identified in animal waste lagoons[2]. Perchlorate reduction is achieved through a multi-step enzymatic reaction (Figure 1). In the first step, a heterodimeric enzyme *perchlorate reductase A/B* (PcrAB) reduces perchlorate to chlorite (ClO_2^-). Electrons for this process are supplied by *Perchlorate reductase C* (PcrC), a tetraheme cytochrome-type protein which transfers electrons from the respiratory electron transport chain, specifically from ubiquinone to PcrAB[3]. Electrons are then transported to PcrC via an intermediate electron transport protein. *Perchlorate reductase D* (PcrD) is a chaperone protein responsible for transporting PcrA, B, and C to the periplasmic space through the twin arginine translocation pathway (TAT)[4,5]. These four proteins combined form the perchlorate reductase complex, PcrABCD. In the second step of this reaction, chlorite produced by PcrAB is further broken down by the heme-containing enzyme *chlorite dismutase* (Cld) to chloride and oxygen[6]. Importantly, Cld does not appear to need to be coupled directly to an electron transport chain to function. Coupling PcrAB to an electron transport chain dramatically increases its efficiency[5]. However, it can still function at very low efficiency when not directly coupled to an electron transport chain[5].

Plants do not have a dedicated perchlorate reduction mechanism like bacterial perchlorate reductases and instead rely on translocation of perchlorate from the roots to the leaves, followed by a slow phytodegradation process[6,7]. It would be

beneficial to engineer plants with the capacity to enzymatically break down perchlorate for future Mars colonization efforts.

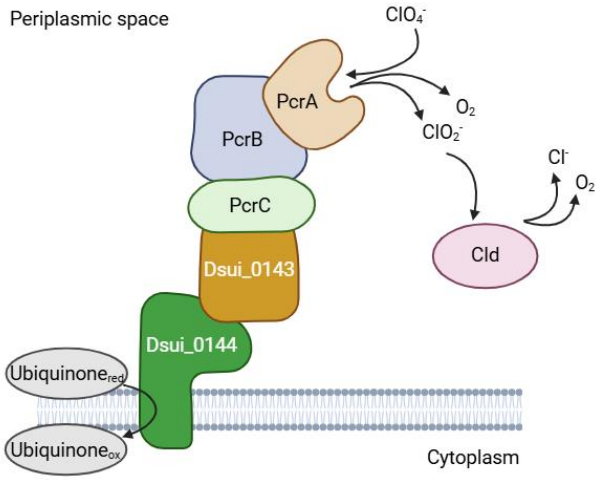

Figure 1. Simplified perchlorate reduction pathway in *Azospira suillum* strain PS. Dsui_0143 is an electron transport protein, and Dsui_0144 oxidizes ubiquinone. PcrABC forms a complex with Dsui_0143 to reduce perchlorate to chlorite. Cld further reduces chlorite to chloride. Made with BioRender.

Introducing the bacterial perchlorate-degrading pathway into plants could give plants the ability to remediate perchlorate contaminated soils. However, the corresponding enzymes use electrons from respiration in bacteria. In plants, perchlorate is localized to leaves, and possibly chloroplasts, using the nitrate assimilation pathway[6]. Therefore, our metabolic engineering strategy includes linking these enzymes to the photosynthetic electron transport chain via protein fusion with one of the Arabidopsis ferredoxin reductases. Here, we present the progress related to the cloning of these genes encoding bacterial perchlorate-reducing enzymes and the development of a perchlorate-induced degradation assay for screening Arabidopsis transformants.

MATERIALS AND METHODOLOGY

Rapid Leaf Disc Chlorophyll Degradation Assay

To determine whether it is possible to rapidly detect the effects of perchlorate in leaf tissue samples for screening purposes, a plate reader assay was developed to estimate chlorophyll breakdown in leaf discs exposed to perchlorate. *Nicotiana benthamiana* (*N. benthi*) was grown under standard conditions for 4 weeks[9]. Leaf discs were submerged either in deionized water (control) or in a 1% (w/v) magnesium perchlorate solution (treatment) for 2, 24, or 72 hours. These concentrations of perchlorate are found in Martian regolith[1]. After soaking, the reflectance of leaf disc was read at 666, 653, and 470 nm to estimate chlorophyll a, chlorophyll b, and carotenoid content, respectively.

Cloning of PcrABCD and Cld

Genetic coding sequences for the bacterial PcrA, PcrB, PcrC, PcrD, Cld, the *Arabidopsis* Feredoxin Reductase 2 enzyme (FNR2), RUBISCO small subunit chloroplast localization tag (Rbs-NT), heat shock protein terminator (t-HSP), and the plastocyanin promoter (petE; shown to induce gene expression in leaves[10]) were all obtained from the GenBank. The sequence for Rbs-NT was attached to the 5' end of the sequences of PcrA, B, D, and Cld to localize these proteins to the chloroplast. These sequences are herein referred to as Rbs-NT::gene. The coding sequence of FNR2 including its chloroplast localization tag was attached to the 5' end of the PcrC coding sequence using the GS flexible linker protein sequence[11]. Alphafold2 was used to simulate the folding of the FNR2::PcrC fusion protein to verify that no unintended tertiary or quaternary structures would result. Using the GoldenBraid Domesticator tool[12], all sequences were domesticated for compatibility with the GoldenBraid cloning system[13]. Bacterial sequences were also domesticated for use in plants. These modified sequences are included in supplementary material 1.

The Rbs-NT::gene, petE, and t-HSP parts were synthesized by Twist Biosciences (San Francisco, CA) for use in GoldenBraid assembly. Domestication of the parts into pUPD2 vectors was performed according to a standard GoldenBraid domestication[13]. *E. coli* strain 'Mach1' cells were transformed with the assembled constructs[14] and transformants selected on chloramphenicol, as well as X-gal IPTG selection[15]. Plasmid DNA was extracted and purified using the GenCatch™Plus Plasmid DNA Miniprep Kit (Epoch Life Sciences,

Missouri City, TX) and sequenced (Plasmidsaurus, Louisville, KY).

The cloned parts were then assembled into level 1 expression vectors to place them under the control of the petE promoter and t-HSP terminator. To assemble these expression vectors into a single vector for Arabidopsis transformation, each part was ligated into a different expression vector following standard GoldenBraid protocols[13]. These constructs were used to transform Mach1 E. coli cells. Colony PCR[16] was also used to quickly verify transformation success in the resulting colonies and all constructs were sequenced (Plasmidsaurus).

RESULTS AND INTERPRETATION

Chlorophylls and Carotenoids Degrade in the Presence of Perchlorate

To leverage chlorophyll and carotenoid breakdown due to perchlorate as means of rapid selection of transformants in Arabidopsis, we measured reflectance from control and perchlorate-treated leaf discs. No statistically significant differences were observed at 2 and 24 hours (data not shown). Leaf discs soaked for 72 hours in perchlorate showed statistically significant reductions in reflectance at all wavelengths compared to the control leaf discs (Figure 2). These results indicate that leaf discs can take up perchlorate, leading to chlorophyll and carotenoid degradation as a sign of oxidative stress. This assay will be used to screen for transformants that have active perchlorate degradation system.

Simulated GoldenBraid assembly of final vector

Benchling was used to simulate the final assembly of the vector containing PcrABCD and Cld to facilitate the cloning process. The final vector will be 22.7kb in length (Figure 3).

Metabolic Engineering Strategy for linking perchlorate-reducing enzymes with photosynthetic electron transport chain

The bacterial perchlorate-reducing enzyme complex draws electrons from the respiratory electron transport chain rather than from a reductant such as FADH2 or NAD(P)H[5]. Due to its similarity to nitrate, we hypothesize perchlorate likely uses nitrate transporters to accumulate in chloroplasts, where it could be slowly degraded by nitrate reduction[6].

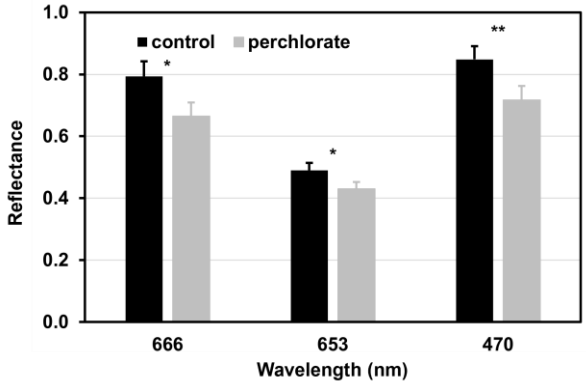

Figure 2. Leaf disc reflectance. Leaf discs were soaked in water (control) or 1% perchlorate for 72 hours and reflectance was measured at 470, 653, and 666 nm as described in MATERIAL AND METHODOLOGY. Results are presented as an average ± SD of reflectance values for 6 biological replicates. Significant at *p*-values < 0.005* and 0.001**.

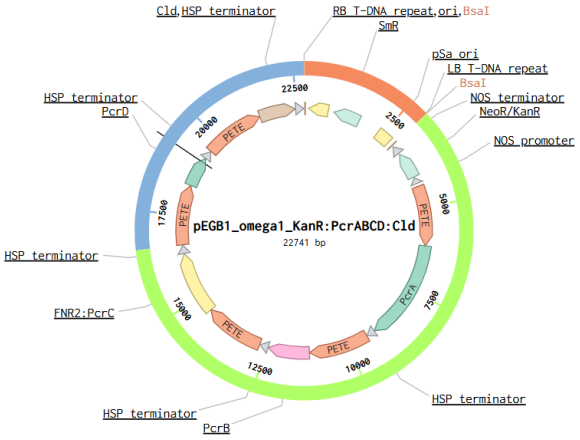

Figure 3. Simulated final assembly of the pEGB1_omega1 vector containing perchlorate reduction genes. The overall construct structure is shown by the outside circle, where different colors correspond to component vectors which will be assembled together. The individual genes and regulatory components and their orientation and order is shown in the inner circle.

Therefore, targeting these bacterial enzymes to the chloroplasts and linking them to the photosynthetic

electron transport chains offers a chance to improve their efficiency in transformed plants. Rbs-NT was used for targeting of genes to chloroplasts.

PcrC is the enzyme that accepts electrons from ubiquinone in *Azospira Suillum*. The idea was to link PcrC with a photosynthetic electron donor such as ferredoxin reductase (Figure 4). In Arabidopsis, there are two genes for this enzyme, one of which, FNR2, is loosely associated with the electron transport chain. We chose FNR2 to prevent any major negative impacts on photosynthesis due to competition for electrons with NADP. PcrC was coupled to FNR2 using a flexible "GS" linker protein[11] after confirming by AlphaFold2 that this FNR2::PcrC fusion will not form any unwanted tertiary or quaternary structures, while preserving the 3D structures of both proteins (Figure 5). The flexible linker protein holds FNR2 in close proximity with PcrC. We hypothesize that this close proximity will be sufficient for increased electron transfer between FNR2 and PcrC.

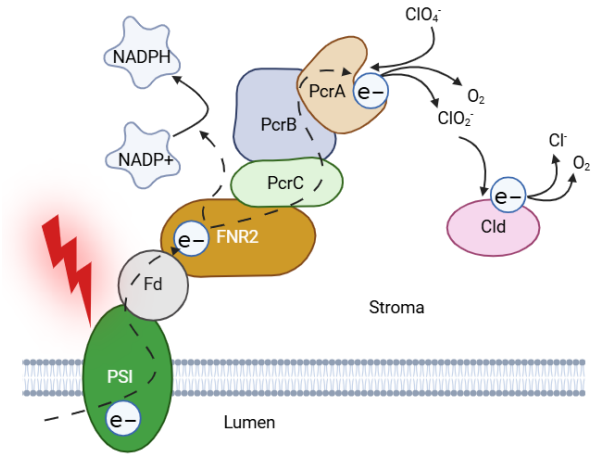

Figure 4. Engineered perchlorate reduction system in plants. Electrons from photosystem I (PSI) are transferred to FNR2 through ferredoxin (Fd). PcrC accepts electrons from FNR2, competing with NADP reduction. The PcrABC complex reduces perchlorate to chlorite which is then reduced by Cld to chloride. Made with BioRender.

Cloning of Perchlorate Reductase Genes

Sequencing revealed that all Rbs-NT::gene components ligated in a domestication vector had no mutations (Supplemental Material 2). Note that FNR2 has a native chloroplast targeting sequence, so Rbs-NT was not used for the FNR2::PcrC fusion shown in Figure 4. Overall, the synthesized genes of interest were all successfully domesticated into the GoldenBraid system. When assembled into GoldenBraid expression vectors however, the transformation efficiency for these constructs was extremely low when compared to domestication vectors. Very few colonies resulted (Figure 6). Sequencing of expression vectors purified from these colonies showed the same type of gene excision for all constructs. Each perchlorate reduction gene was excised from the plasmid along with one or both GoldenBraid restriction enzyme recognition sites (Figure 7) and parts of the left and right border T-DNA repeats present within the expression vector backbone.

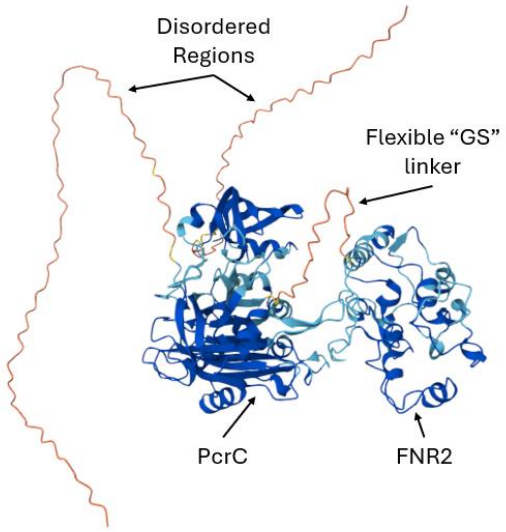

Figure 5. Structure analysis of the FNR2::PcrC protein fusion. The protein structures of the fusion protein and the individual proteins (not shown) were simulated in AlphaFold2.

An initial hypothesis from this observed result was that the GoldenBraid ligation reaction failed in some way. To test this, multiple repetitions of the GoldenBraid reaction were conducted, with all yielding the same result. To increase screening throughput of the few colonies resulting from transformation with expression vectors, colony PCR was performed. Colony PCR confirmed the results from sequencing: all surviving colonies exhibited mutations excising the genes of interest from the plasmid. Additionally, sequencing showed

the excisions happened in slightly different locations within the T-DNA repeats of the vector backbone in all sequenced plasmids, further supporting the hypothesis that this result was not caused by a failed GoldenBraid ligation. Had a failed GoldenBraid ligation been responsible for the observed lack of any genes of interest, the missing areas within the T-DNA repeats would have been consistent, exhibiting consistent cuts made by a restriction enzyme or showing that no restriction enzyme cuts had been made.

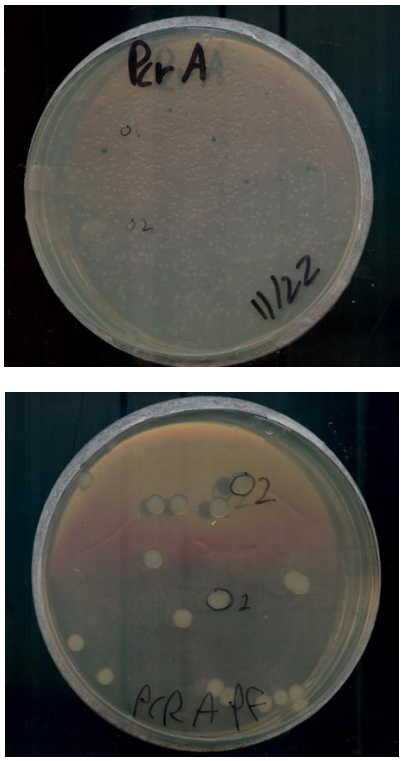

Figure 6. Selection plates for representative domestication and expression vectors. The plate on the top exhibiting ~200 colonies contains the domestication vector of PcrA while the plate on the bottom exhibiting <20 colonies contains PcrA in the expression vector controlled by petE.

The most obvious explanation is that, while the individual genes themselves are not toxic (the initial individual domestication of the Rbs-NT::gene components was successful), when placed under the control of a promoter, these proteins were expressed and proved to be cytotoxic to *E. coli*. For this to be the case, leaky expression of the perchlorate reduction genes must have been occurring within the Mach1 cells. This is surprising (but not unheard of) because they were under the control of a plant promoter, petE, that should not be active in bacteria. Further examination of the sequence of the petE promoter revealed that it contained nine TATA box motifs within the sequence. Bacteria are known to initiate translation at these simple TATA box motifs[17], indicating the possibility of a leaky expression of the perchlorate reduction genes. In addition, the Mach1 *E. coli* strain does not contain the T7 lysozyme[18], which inhibits RNA polymerase and reduces leaky expression from plasmids.

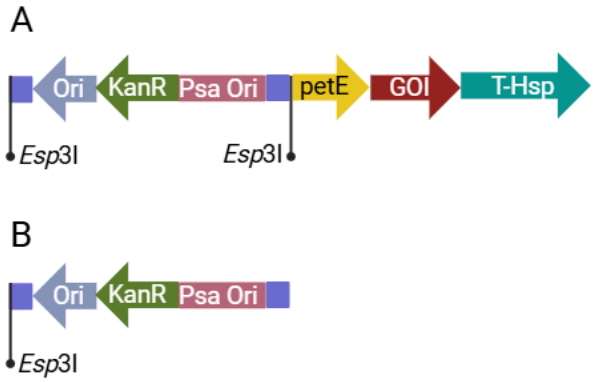

Figure 7. Schematic diagram of excision mutations. A. Correct assembly and **B.** Example of observed assembly with excisions. In all cases, the gene of interest (GOI), promoter, and terminator were excised from the expression vector. Left and right border T-DNA repeats (blue boxes) were randomly cut within. The right-most Esp3I recognition site was always excised and the leftmost recognition site was sometimes also excised. Ori – *E. coli* origin of replication, KanR – kanamycin resistance gene, Psa Ori – *Agrobacterium* origin of replication, petE – plastocyanin promoter, T-Hsp – heat shock protein terminator, *Esp*3I - *Erwinia* species RFL3 restriction enzyme.

FUTURE STEPS

While this potential gene toxicity represents a significant setback that has slowed down the progress of the proposed work, it provided indirect evidence that the perchlorate-reducing complex may be active. This setback is also possible to overcome. Growing *E. coli* at lower temperatures has been shown to reduce leaky expression of toxic proteins by allowing proteins to fold into more stable configurations[19]. Using a different strain of competent *E. coli* cells with reduced leaky

expression, such as BL-21-AI, could also reduce the production of any cytotoxic proteins[20]. This would allow for the plasmid containing a perchlorate reduction gene and its associated plant promoter to be replicated successfully. BL-21-AI is a well-established strain commonly used in molecular biology and has well-established transformation and growth protocols in literature[21]. Work is currently in progress exploring the viability of using the BL-21-AI and Top-10 strains of *E. coli* rather than Mach1. Work is also in progress investigating whether lowering the incubation temperature of the *E. coli* cultures from 37ºC to either 28ºC or 20ºC will reduce cell death caused by the toxic proteins.

Additionally, investigating different promoters could help to reduce the leaky expression. While petE was chosen to allow for leaf-specific expression in transformed plants, many other leaf-specific promotors are available. Screening these promoters for TATA box motifs and selecting one with no or low numbers of TATA boxes could prevent the transformed *E. coli* from producing toxic proteins. Placing the perchlorate reduction genes under the control of the 35S promoter could also serve as a proof of concept, demonstrating that in the absence of leaky expression of the genes, cell death and mutation rate would be drastically reduced. Preliminary work on placing the PcrA, PcrB, and Cld genes under control of the 35S promoter rather than the petE promoter is currently underway. The Cld construct will be also used to transform *Arabidopsis* which should be capable of breaking down toxic chlorite to chloride. Additionally, transforming *Arabidopsis* with this assembly should allow a test of whether this gene would be toxic to *Arabidopsis*. If Cld, as the end stage of the perchlorate reduction pathway, is shown to effectively break down chlorite in plants, it will provide a promising first step towards generating plants fully capable of breaking down absorbed perchlorate.

Modifying the GoldenBraid reaction to perform a one-pot assembly of all parts without the need to grow, extract, and purify individual plasmids for further assembly into level 2, 3, and 4 expression vectors may also represent an alternative method to generate the final required expression vector containing PcrABCD and Cld. Examples in literature have demonstrated the viability of GoldenBraid assemblies of up to 52 parts simultaneously without the need to run multiple assembly reactions[22]. This may represent a third strategy to create the final required expression vector needed for *Arabidopsis* transformation.

AKNOWLEDGEMENTS

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CITATIONS

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SUPPLIMENTAL MATERIAL 1: GoldenBraid Optimized Gene Sequences

Rbs-NT::PcrA

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ccaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttc

SUPPLIMENTAL MATERIAL 3: Example expression vector sequences with perchlorate reduction genes excised

Ex. 1: petE::PcrA::T-Hsp in pEGB1

ttgagatccttttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactcttct
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Ex. 2: petE::PcrA::T-Hsp in pEGB1

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Ex. 3: petE::PcrB::T-Hsp in pEGB1

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Ex. 4: petE::PcrB::T-Hsp in pEGB1

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