EXECUTIVE SUMMARY REPORT

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Executive Summary Report

Introduction

The establishment of vegetation habitats, for on-site production of biomaterials and sustainable food production at ground space stations, is one of the biggest challenges for space exploration. High level of radiation, low temperature, low light intensity, low atmospheric pressure, and low oxygen in the atmosphere as well as high level of toxic compounds in soil are some of the most difficult hurdles to overcome in order to establish vegetation in space. Martian regolith contains high level of toxic compounds that are dangerous for both plants and human, including perchlorates and heavy metals. In order to address these environmental challenges the genetic mechanisms for stress resistance was introduced from environment resilient organisms into plants.

The main objective of the project was to generate plants engineered with the perchlorate degradation pathway from the bacteria *Dechloromonas aromatica* and the melanin biosynthesis pathway from the fungus *Alternaria alternata*, with the aim to produce plant lines that are able to withstand high concentrations of both perchlorate and heavy metal at levels similar to what is found in Martian regolith. In addition, expression of fungal melanin in these plants should also provide higher tolerance to ionizing radiation as it is a major component of fungal protection against extreme stress including high energy radiation.

Generating plants with high tolerance to perchlorate, ionizing radiation and heavy metals would be of great importance for Mars missions. Such plants would be also valuable for bioremediation of soils and water contaminated with perchlorate and heavy metals on Earth.

Engineering of tolerance to perchlorate in plants

Many plants can accumulate perchlorate in their tissue, indicating a native tolerance to low concentration of this pollutant. Reduction products of perchlorate have also been detected in plant tissue upon initial uptake, suggesting an enzymatic stepwise reduction of perchlorate in plant cells.

Some plants can naturally tolerate high amount of perchlorate and are used for bioremediation of contaminated soil and water on Earth. The current process for bioremediation is to grow tolerant plants on contaminated areas with subsequent removal of plant biomass that accumulated perchlorate to a specific processing and detoxification site. This way perchlorate is slowly removed from soil and other agricultural crops can be planted after bioremediation.

We believe that engineering of plants with an efficient bacterial pathway for perchlorate degradation can both increase the amount of perchlorate absorbed by plants from soil and degrade perchlorate in plants, thus accelerating time for bioremediation of contaminated sites.

In order to generate efficient perchlorate tolerance, we introduced the perchlorate degradation pathway from the bacteria *Dechloromonas aromatica* into tobacco plants using advanced synthetic biology tools developed in our laboratory.

We observed that introduction of bacterial perchlorate degradation pathway in plants provided significant tolerance to up to 0.5% of perchlorate in soil, which is similar to the levels of perchlorate found in Martian regolith. This level of perchlorate is considered toxic to both humans and plants, and engineering of tools for regolith detoxification is of paramount importance for future Mars missions.

Our study showed that expression of bacterial perchlorate degradation enzymes significantly increased plant tolerance to toxic levels of perchlorate (Figure 1).

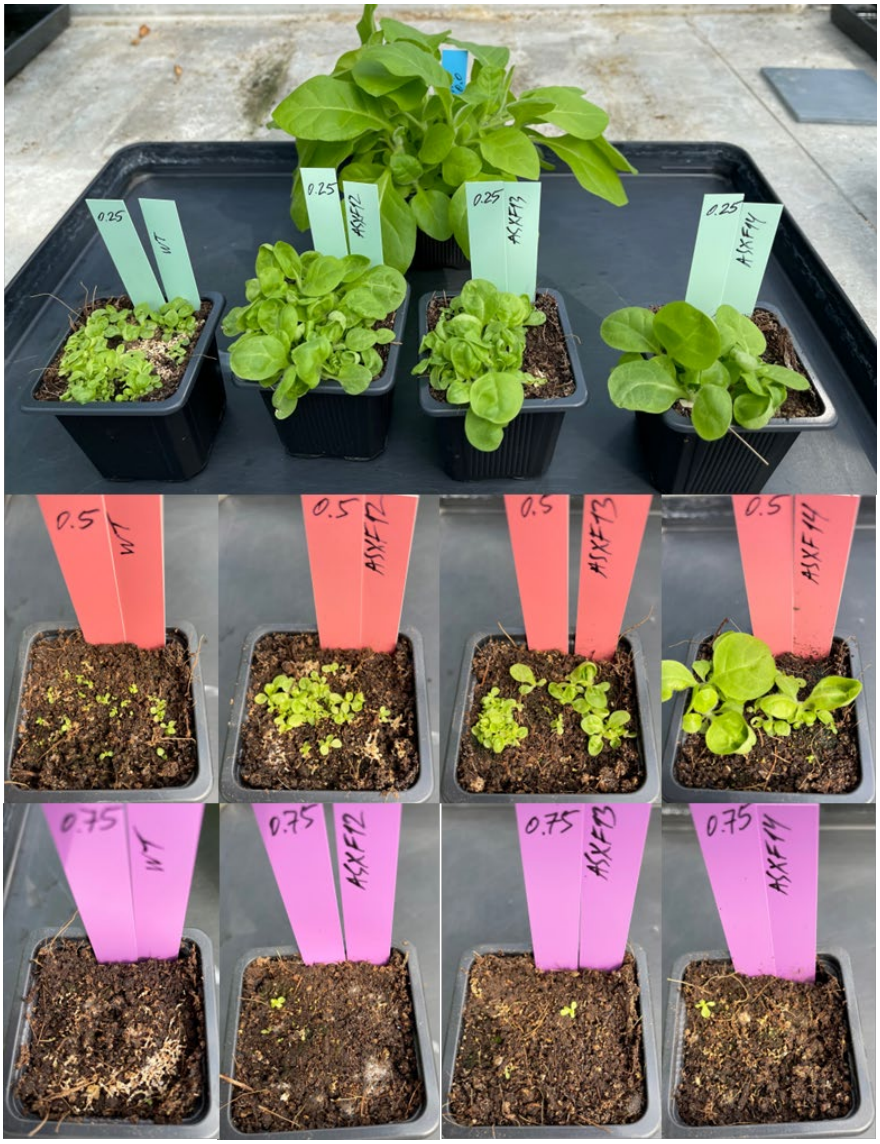

Figure 1. Germination of tobacco seeds and seedlings tolerance to perchlorate contaminated soil. Top panel- 0.25%, middle panel- 0.5%, lower panel- 0.75% of perchlorate. WT- non-transgenic plants, ASXF12, ASXF13, ASXF14- engineered lines with perchlorate degradation pathway.

The engineered plants were developing well on 0.25% perchlorate concentration and although we observed a slight suppression of plant growth initially, they generated normal biomass and seeds. Wild type plants was significantly suppressed on this concentration and no seeds could be obtained (Figure 2). Seeds of wild type tobacco can germinate on 0.5% of perchlorate, but seedlings died within one month while engineered plants could tolerate 0.5% of perchlorate

(Figure 2). Although their growth was significantly suppressed, they produced higher biomass on 0.5% perchlorate compared to wild type plants grown on 0.25%. Thus, expression of bacterial perchlorate degradation pathway in plants provide significant increase in tolerance to Martian level of perchlorate contamination.

Previous research has shown that activity of bacterial perchlorate degradation enzymes is much higher under low oxygen concentration, therefore it may be possible to further enhance activities of these enzymes in plants by reducing oxygen level in plant cells. Enzymes called "oxygen scavengers" can be used for this purpose and we will further pursue this approach to engineer plants with better tolerance to higher perchlorate concentrations.

Figure 2. Development of wild type and engineered ASXF plants on soil contaminated with perchlorate. WT: wild type plants were grown in soil supplemented with 0.25% of perchlorate, as they did not survive on 0.5% concentration. Transgenic ASXF lines were grown on 0.5% perchlorate concentration in soil.

Engineering of tolerance to heavy metals and ionising radiation in plants

Another challenge for space exploration as well as on Earth, is soil contamination with heavy metals.

Heavy metals are toxic for plants and for human health and there is a strong demand for bioremediation technologies. In order to address this problem, we have investigated fungal melanin, which has very strong binding and neutralisation capacity for heavy metals, and has strong protective capabilities to many types of stress. The biosynthetic pathway for melanin production is well described in melanin producing fungi and we explored the possibility to produce fungal melanin in plants with a purpose to increase heavy metal tolerance. Tobacco plants were engineered for production of fungal melanin and tested on soil contaminated with

different concentration of copper sulphate salt, which provide a source of heavy metal. From the initial set of constructs we did not observe strong accumulation of melanin in plant tissue. However, low accumulation of fungal melanin in plants could still provide sufficient protection against heavy metal contaminated soils. Indeed, engineered plants demonstrated strong tolerance to 1% of copper sulphate in soil, while wild type plants already died on 0.5% of copper sulphate (Figure 3).

Figure 3. Tolerance of engineered ASWF lines to 1% of copper sulfate in soil. Left pot- wild type (WT) tobacco plants, three pots on the right- ASWF9, ASWF14, ASWF25- engineered lines.

Further improvement for expression of a crucial gene from melanin biosynthetic pathway generated further improvement of copper tolerance, as plants accumulated considerable higher biomass on this non-permissive copper concentration (Figure 4).

Figure 4. Tolerance of melanin engineered plants to 1% of copper sulfate in soil. Wild type tobacco plants could not withstand this copper concentration and died, while engineered plants grew normally and produced seeds. These plants with optimised gene expression performed much better on copper contaminated soil with higher biomass accumulation as compared with engineered ASWF lines (see Figure 3).

Thus, low accumulation of fungal melanin in engineered plant provides protection to plants against high concentration of heavy metals in soil.

Harsh ionizing and ultraviolet (UV) radiation is another critical challenge for space exploration. Although plants have adjusted to handle low levels of UV and ionizing radiation on Earth, chronic subjection of plants to such environmental factors is damaging for plant growth. High level of UV at high altitude prevents growth of many plant species, in particular trees. Few plant species can survive in mountain area because of harsh UV radiation. Mars does not have a dense atmosphere, providing insufficient protection to high levels of UV and ionizing radiation. Engineering of plants with strong tolerance to these factors would be a highly desirable trait for manned Mars missions.

We attempted to engineer plants with strong tolerance to UV and ionizing radiation. For this purpose we came back to the production of fungal melanin. Fungal melanin provides fungi with strong shield against ionizing radiation, some fungal species can even thrive on the wall of Chernobyl nuclear reactor, where radiation level is deadly for many leaving organisms. Fungi, which did not produced melanin due to a mutation in melanin biosynthesis genes, rapidly die after their exposure to UV. This pointed out that this compound could serve as a reliable shield for protection against radiation. In our initial experiments for engineering heavy metal tolerance in plants we did not succeed in producing high level of melanin in plants.

The melanin production system was optimised by expressing genes responsible for efficient synthesis of melanin precursor, which dramatically increased the level of melanin in plant tissue, making leaves completely black (Figure 5).

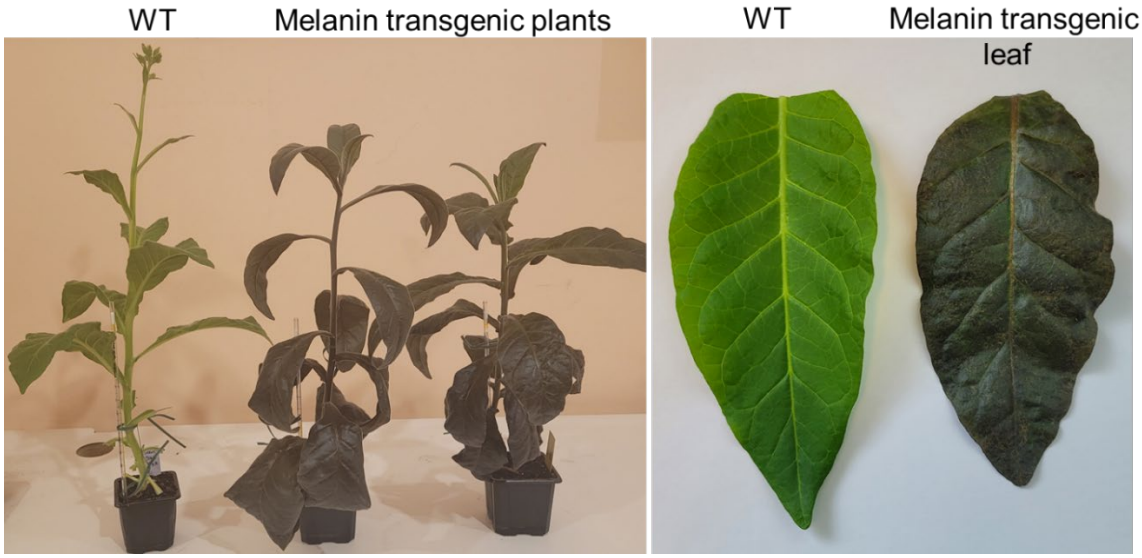

Figure 5. Engineering of plants with high level of melanin accumulating. WT- wild type tobacco plant and leaf of melanin engineered plant.

Despite good tolerance of leaves to high melanin accumulation, the development of the root system was drastically affected. Engineered plants developed nicely at early stage, but collapsed later due to shortage in water supply to the whole plant from the roots (Figure 6).

Figure 6. Engineering of melanin producing plants. Left top panel shows process from regeneration to transferring plants to soil. Plant leaves have good tolerance to high level of melanin accumulation, however, high level of melanin accumulation suppressed development of roots. As a result, engineered plants died at the stage of flowering, as root system cannot provide sufficient water supply to support plant biomass development. Low panel shows roots of wild type plant on the left and melanin affected roots of engineered plants on the right.

The rooting issue could be easily addressed by regulation of gene expression, namely, providing high level of expression of melanin biosynthesis genes in leaves and low level of expression in roots. As indicated in Figure 4, low expression of melanin biosynthesis genes in engineered plants provided high tolerance to heavy metals. We did not observe any suppression of root development in those plants. Thus, reduction of gene expression in roots would still provide efficient protection of plants against heavy metals, while high expression in leaves would drive high level of melanin accumulation and strong protection against UV and ionizing radiation.

Finally, upon recovery of melanin accumulating plants with low expression in roots, we would like to combine both perchlorate and heavy metal tolerance, as well as enhanced protection of plants against UV and ionizing radiation provided by a high level of melanin accumulation in leaves. These plants will be evaluated in the Marsimulator chamber in Toulouse by combining soil contamination with perchlorate and heavy metals, with irradiation with UV and gamma rays.

We believe that our engineered plants could open new research avenues for bioremediation of contaminated soil and subsequent crop production for Earth applicationsand manned Space exploration.

Conclusions

The major purpose of the project was to obtain a proof of concept for generation of environment resilient plants which could tolerate perchlorate and heavy metals in contaminated soil, as well as withstand severe UV and ionizing radiation. For this purpose expression of bacterial perchlorate degradation pathway and fungal melanin biosynthesis in plants was evaluated.

Perchlorate tolerance

- Engineering of plants with tolerance to high perchlorate contamination in soil is feasible by expression of a bacterial pathway for perchlorate degradation
- Engineered plants could withstand up to 0.5% of perchlorate in soil
- Cytoplasmic localization of the oxygen sensitive perchlorate degradation enzymes is preferable, as oxygen level in the cytoplasm is lower than in the chloroplasts.
- Our attempt to reduce oxygen content in plant cells using our oxygen scavenging system was not successful possibly due to the generation of high level of hydrogen peroxide which is causing growth defects and plant sterility.

Melanin production and heavy metal tolerance

- Production of fungal melanin in plants was successfully achieved by introduction of a fungal melanin biosynthesis pathway and critical precursor gene for 1.8-dihydroxynaphtalen biosynthesis in plants
- High accumulation of melanin in plant tissue was observed by targeting melanin biosynthesis enzymes both to the cytoplasm and chloroplast, **this is a very significant breakthrough as high yielding fungal melanin accumulation in plants has not been reported previously.**
- We observed that a low accumulation of fungal melanin in plant tissue is sufficient to protect the engineered plants against high concentration of heavy metals in soil
- The leaves of engineered plants have good tolerance to high accumulation of melanin, however roots are strongly affected and cannot provide sufficient water for plant survival, plants that can survive are not producing viable seeds.

Combining properties for perchlorate tolerance, heavy metal resistance and protection against radiation

- We initially planned to cross plants transformed with the perchlorate degradation pathway together with melanin producing plants to combine all properties into one plant and test for a combination of stress parameters (perchlorate, heavy metal and ionizing radiation), this has not been possible during the timeline of the project due to issues linked to fertility and strong effect of melanin accumulation in roots.