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INSPIRATION bulletin

CL:AIRE's INSPIRATION bulletins describe practical aspects of research which have direct application to the management of contaminated soil or groundwater in an agricultural context. This bulletin describes the development of biosensors to monitor the bioavailability of heavy metals in response to biochar application as a soil amendment.

Developing biosensors to measure the bioavailability of heavy metals in soil remediation

1. Introduction

Heavy metal pollution of soils is a serious environmental problem due to potential hazards to humans and toxic effects on soil organisms. Soil microorganisms play a significant role in nutrient cycles and as decomposers of organic matter to maintain soil fertility and function (Hoorman, 2011). However, the presence of bioavailable heavy metals may influence soil microbial growth and cellular processes, such as damaging cellular membrane or disruption of enzymatic process that can cause cell death (Bruins *et al.*, 2000). These impacts may lead to the changes in soil microbial populations and diversity hence reducing soil function and quality.

Soil amendment with biochar is a low cost remediation method that can reduce the bioavailability of heavy metals through adsorption processes (Bandara *et al.*, 2017). This mechanism can potentially decrease the toxicity of metals to soil microorganisms (Beesley *et al.*, 2014). Chemical analysis of different soil fractions is often used to infer heavy metal bioavailability (Filgueiras *et al.*, 2002) but it is an indirect and imprecise measurement, as it does not account for the biologically relevant metal fraction. Instead, bacteria which respond directly to the presence of heavy metals can be used to assess the bioavailable fraction in a soil. As the site of action of heavy metals is inside the bacterial cell, bacteria-based biosensors that report cytoplasmic metal concentration directly can be developed as a more appropriate indicator. This biosensor is an alternative approach to measure the toxic effect of metals on cell physiology. The objectives of this study are to develop and apply biosensors to monitor the bioavailability of heavy metals in response to biochar application as a soil amendment. This bulletin will discuss the concept and methodology required to construct the biosensors, preliminary results, and give a forward outlook for their application in soil remediation.

2. Concept of Biosensors

Soil microorganisms can respond to heavy metal contamination in various ways. Some may survive in the presence of toxic metals due to specific cellular processes such as sequestration by exopolysaccharide (EPS), detoxification via efflux proteins on the cell membrane, enzymatic reduction (Bruins *et al.*, 2000), intracellular sequestration by metal-binding proteins (e.g. metallothioneins), precipitation as a metal salt, etc (Wasi, Tabrez and Ahmad, 2013). These mechanisms are genetically encoded in their chromosomes or plasmids and have been well studied. The properties of these genes can be utilised to develop bacteria-based biosensors to report cellular responses to the presence of heavy metals in the soil.

Biosensors exploit the performance of the Förster Resonance Energy Transfer (FRET) sensor by fusion of a metal binding protein, metallothionein (MT) to fluorescent proteins (FPs) – in this case, enhanced Cyan FP (eCFP) and the yellow Venus FP (Rajamani *et al.*, 2014). Binding of heavy metals by MT changes the molecular distance between eCFP and Venus, bringing them closer to each other (Fig. 1). This proximity enables energy transfer from eCFP (donor) to Venus (acceptor) changing the fluorescent properties of the system (Tsien, 1998; Carter *et al.*, 2014). The occurrence of FRET, and hence binding of metals to MT, can be measured by changes in the ratio of fluorescence emission of eCFP and Venus – metal binding enhances Venus fluorescence at the expense of eCFP. The expression of a FRET sensor inside a host bacterial cell allows direct, rapid measurement of intracellular metal concentrations (Fig. 1). Utilisation of soil bacteria as a host cell can potentially

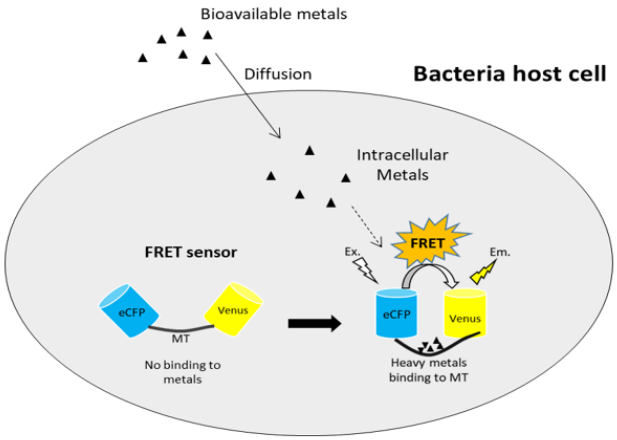

Figure 1: Concept of biosensor: FRET sensor as a fusion of eCFP-MT-Venus protein is expressed inside the bacteria host cell. Binding of intracellular metals to MT changes the distance between eCFP and Venus. This enables FRET that can be measured by ratiometric changes in fluorescence and corresponds with metal concentrations.

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Inspiration

enable the measurement of bioavailable metals in contaminated soil samples. The choice of host cell is important as it must be viable in soil, but also its physiology (e.g. presence of metal efflux proteins) will influence the intracellular concentrations of metals to be sensed.

3. Methodology of Biosensor Development

The properties of biosensors - the metal binding protein, reporter protein and host cell - were characterised to measure bioavailable Cd, Zn and Pb in contaminated soil. A gene encoding a metal binding protein (MT) from chicken (Rajamani *et al.*, 2014) was synthesised and optimised for bacterial expression. This gene was inserted between two reporter protein genes encoding eCFP and Venus, in a plasmid using the Gateway Recombination method (Hartley *et al.*, 2000). This plasmid allows for an inducible protein expression in bacterial host cells (Fig. 2). The plasmid (pGWF1-CMT) construct and orientation were verified by gel electrophoresis and DNA Sequencing.

The pGWF1-CMT was transformed into *Escherichia coli* BL21 Rosetta 2(DE3) pLysS for initial expression and characterisation of the FRET sensor (*in vitro* and *in vivo*). The transformant cells were grown in liquid Luria Bertani (LB) medium supplemented with ampicillin. Protein expression was induced by the addition of 0.4 mM isopropyl thiogalactoside (IPTG) under the control of T7 promoter. The proteins were extracted from host cells using sonication and purified by using Sephadex size-exclusion column to purify the FRET protein prior to characterisation of heavy metal binding.

In vitro characterisation was carried out by mixing different concentrations of $CdCl_2$, $ZnSO_4$, and $Pb(NO_3)_2$ solutions with the biosensor protein. Fluorescence intensities were measured before and after heavy metal addition using a spectrofluorometer with the following parameters: eCFP: λexcitation 435 nm, λemission 478 nm and Venus λemission 527 nm (Kaper *et al.*, 2008). Initial *in vivo* characterisation was performed by harvesting the *E.coli* host cells expressing the FRET sensor from the growth medium and resuspending the cells in M9 minimal media pH7. The cell suspension was dispensed into microplate 96 wells and heavy metal solutions were added. Fluorescence intensities of the host cells were monitored before and after heavy metal addition using a microplate reader. The emission peaks from eCFP (478 nm) and Venus (527 nm) were identified and the ratio was calculated for each metal concentration.

4. Preliminary Results

In vitro characterisation was used to demonstrate the concept of the heavy metal FRET biosensor. The results showed that the emission intensity of eCFP and Venus changed due to the addition of Cd and Zn. The ratios of Venus and eCFP emission (FRET ratio) were then plotted against the heavy metal concentrations (Fig. 3). In the absence of metals, the FRET ratio was ~1.8 and increased with increasing metal concentrations. The FRET ratio in response to Cd increased from 2.01 to 2.38 over a concentration range 1 μM to 5 mM. The FRET ratio in response to Zn increased from 2.14 to 2.4 over a concentration range 1 μM to 1 mM. These results indicate that binding of Cd and Zn to the biosensor led to quantitative changes in the ratio of fluorescence emission due to metal-induced FRET.

Measuring the fluorescence ratio changes also allowed the quantification of heavy metals inside the host bacterial cell. Preliminary *in vivo* tests using *E.coli* as a host cell showed that the FRET ratio changed 90 minutes after Cd or Zn addition. In the case of Pb, precipitation of Pb in the medium prevented testing of the biosensor.

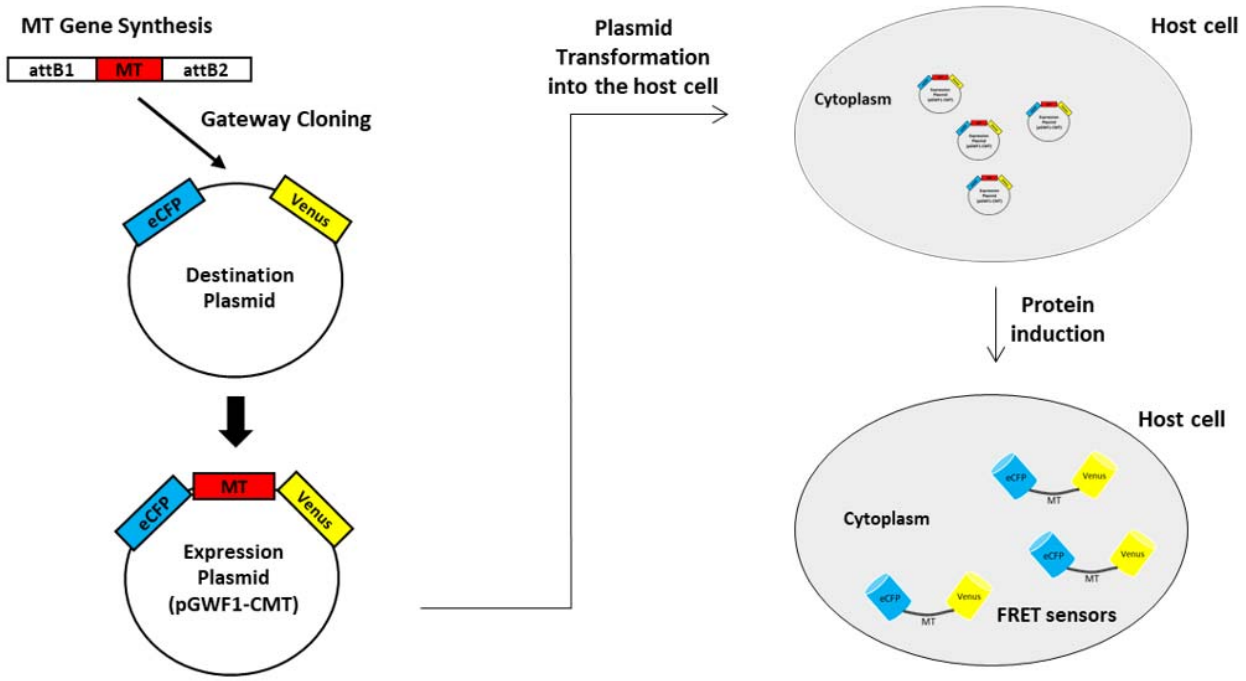

Figure 2: The stages of FRET biosensor development. A gene encoding MT was synthesised and cloned into a plasmid encoding eCFP and Venus to generate the expression plasmid (pGWF1-CMT). The pGWF1-CMT was transformed into a bacteria host cell and the FRET sensor protein production was induced by the addition of IPTG.

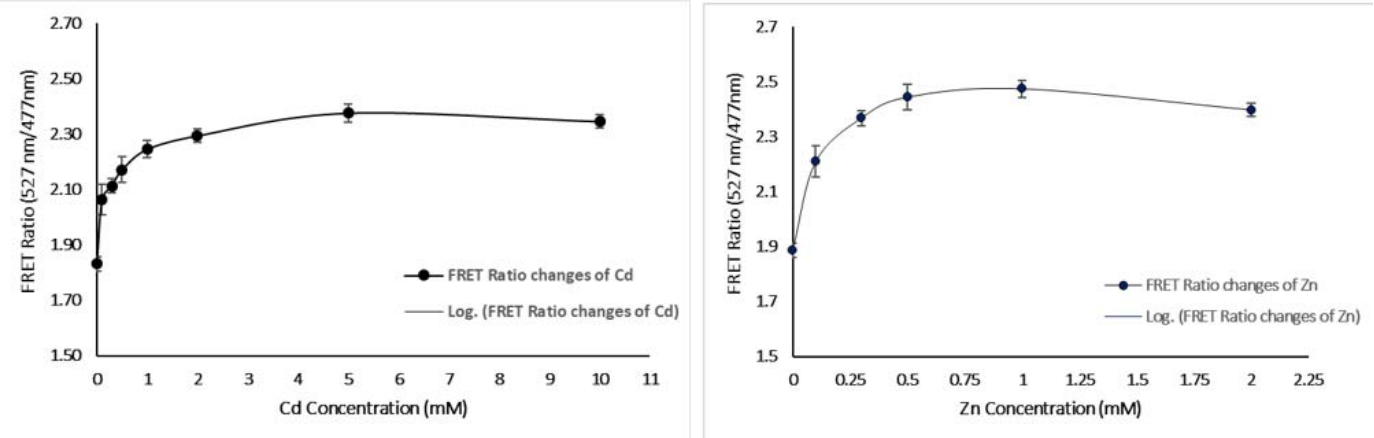

Figure 3: FRET Ratio changes (In vitro) at various concentration of (a) Cd and (b) Zn. The experiment was conducted in 3 replications (n=3).

5. Future Work

Further developments will express the biosensor inside a soil bacterium, e.g. *Pseudomonas putida* KT2440. This strain is considered to be a robust host cell typically found in contaminated soils.

The biosensors will be used as monitoring tools to measure the change of bioavailable heavy metals due to biochar amendment in contaminated soil. This can be conducted by extracting the soil pore water from the contaminated soil samples and adding them to the biosensor assay and monitoring results using a microplate reader. Alternatively, microbes can be mixed directly with the soil and metal concentrations monitored using fluorescence microscopy. The measurement of metal concentrations from the biosensors will be integrated with an analysis of plant-bioavailable metal concentrations and changes in soil microbial community to assess the remediation performance. The outcomes of these analyses are expected to provide information about the conditions that can improve the soil quality to support plant growth and microbial activity for the restoration of contaminated land.

Acknowledgments

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