PROJECT DESCRIPTION

OBJECTIVES

- Develop a modular platform for high-throughput phenotyping of multiple ion uptake kinetics to enhance understanding of root function and plant nutrient uptake.
- Characterize nutrient uptake kinetics within selected maize genetic resources to i) facilitate mapping of genetic regions underlying nutrient uptake characteristics, ii) identify entries that can be used to assess the influence of contrasting uptake kinetics in the field, and iii) provide information for breeding of more resource use efficient crops.

INTRODUCTION

Food production must double by 2050 to meet the needs of the estimated 9.7B global population (World Bank, 2014) and address one of this century's greatest challenges: global food insecurity (Funk and Brown, 2009). Optimization of crop nutrient acquisition efficiency is necessary to produce food more effectively (Lynch, 1998). In the developing world, soil nutrient availability limits plant growth (FAO, 2008), while in developed nations, intensive fertilization pollutes water and the atmosphere (Jenkinson, 2001). Global maize yield is greater than any other grain crop and maize is grown on 177 million Ha (FAO, 2012), with importance for both subsistence and commercial agriculture. Greater nutrient acquisition efficiency in maize would improve worldwide agricultural production and mitigate environmental risks.

Roots anchor the plant in the soil and forage for soil resources. Root system architecture determines both the overall extent of soil exploration and the density of roots in a particular soil volume (York *et al.*, 2013). With the development of image-based root crown phenotyping, significant advances in characterizing maize root system architecture have been made in the past few years (Bucksch *et al.*, 2014; Columbi *et al.* 2015; York and Lynch, 2015). However, while the importance of ***where roots are located*** as determined by root system architecture is well-known, questions regarding ***what roots are doing and how*** have received much less attention. Root uptake of mineral nutrients from the soil solution is of major importance for plant growth. Epstein and Hagen (1952) first used Michaelis-Menten (MM) kinetics to model uptake of nutrients by roots. The uptake rate saturates as the nutrient concentration increases in solution. In a MM kinetic model, the relationship between uptake rate and external nutrient concentration is summarized with MM parameters called I_{max}, K_m, and C_{min} (Fig. 1). I_{max} is the maximum influx rate of nutrient, K_m denotes the external nutrient concentration at which half of I_{max} is obtained, and C_{min} is the minimum external nutrient concentration at which uptake may occur. The underlying mechanistic assumption is that enzymes are actively involved. The affinity of a transporter for its substrate is represented by K_m and determines how well the transporter operates at low substrate concentrations.

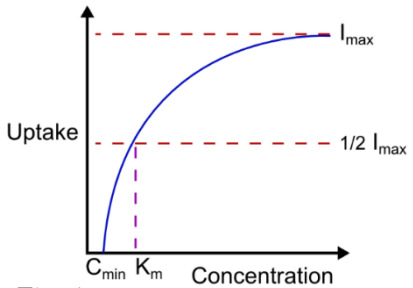

Fig. 1

Plants produce many types of transport proteins encoded by their genomes. These transporters are generally specific to particular nutrients in their most common chemical forms, and individual nutrients are known to have sub-types of transporters responsible for their uptake. For instance, a high affinity (low K_m) transport system (HATS) and a low affinity (high K_m) transport system (LATS) for nitrate have been discovered, with transporter proteins encoded by the *NRT1* and *NRT2* gene groups, respectively, in *Arabidopsis* (Tsay *et al.*, 2007). In maize, the analogous *ZmNrt1* and *ZmNrt2* genes correspond to differences in uptake relating to expression levels (Quaggiotti *et al.*, 2003, 2004; Trevisan *et al.*, 2008). Recent research has supported proton-coupled transport of nitrate by *NRT1.1* and an alternating access mechanism where a central binding site reorients to alternatively expose the bound nitrate from the external to internal solution (Parker and Newstead, 2014). Furthermore, *NRT1.1* may be post-translationally modified by phosphorylation in order to change to a high-affinity state (Parker and Newstead, 2014; Sun *et al.*, 2014). While nitrate is generally regarded as the nutrient most commonly limiting maize growth, the other macronutrients are also of interest, especially in poor soils. Transporters have also been identified for ammonium (Howitt and Udvardi, 2000; Sohlenkamp *et al.*,

2000), phosphate (Raghothama, 2000), potassium (Coskun *et al.*, 2013), and sulfate (Takahashi *et al.*, 2012). While most commonly discovered and studied in *Arabidopsis*, maize analogs are known (Hopkins *et al.*, 2004).

However, although transporter genes are largely known, and their protein structures are being determined (Parker and Newstead, 2014), the linkages with uptake kinetics at the root system level as well as genetic variation for ion uptake kinetics remain underexplored. Nutrients can have competitive interactions in terms of plant uptake (Cox and Reisenauer, 1973), but, although critical under natural conditions, genotypic variation relative to these interactions has not been investigated. Multiple ion uptake kinetics (MIUK) are necessarily aggregate phenes (units of phenotype, *sensu* York *et al.*, 2013) that result from numbers and types of transporters and other processes, and consequently are complex to study. We are not aware of any platforms that can efficiently handle the number of nutrient solutions (combinations of different nutrients x different concentrations of individual nutrients) that are needed to advance fundamental understanding of MIUK. The development of a platform for high-throughput phenotyping of root functional phenes will open new doors to investigate MIUK in a large number of genotypes and, consequently, enable the identification of the genetics underlying MIUK. This, in turn, will facilitate germplasm development and breeding for improved nutrient acquisition.

We propose to develop a high-throughput platform for characterizing MIUK of roots that allows examination of large numbers of genotypes, consequently opening the door for genetic studies of root function and the identification of genotypes that can be employed to breed for improved crop nutrient uptake. System development and initial efforts will focus on maize and macronutrients that can limit crop productivity: nitrate, ammonium, phosphate, potassium, and sulfate (NAPPS), but the platform will be adaptable for other plant species and any ion of interest. As opposed to the wealth of data being collected by phenotyping root system architecture, high-throughput approaches to characterize root function are virtually non-existent. To our knowledge, neither simultaneously measuring the uptake kinetics of multiple ions nor phenotyping uptake kinetics for the numbers of plants required for genomic associations have been attempted.

BACKGROUND

Our team has substantial experience in root physiology, phenotyping, genetic analysis, and big data analytics. All members are familiar with interdisciplinary studies scaling from molecular to field levels, and thus are well positioned to successfully implement the objectives of this ambitious, potentially transformative research.

Kinetics physiology is ready to transform to kinetics phenomics

Phenotyping of uptake kinetics is in its infancy because uptake kinetics in physiological studies have generally been measured only for individual genotypes. Uptake kinetics have primarily been measured using whole root systems (Pace and McClure, 1986; Hasegawa and Ichii, 1994), or for excised roots (Rao *et al.*, 1997). In maize, differential ^{15}N accumulation was demonstrated for the primary root tip, other zones of the primary root, and the primary root laterals, but neither I_{max} nor K_m were reported (Lazof *et al.*, 1992). In another case, I_{max} and K_m were determined along intact maize primary roots using a compartmented chamber, but no other root classes were included (Sorgonà *et al.*, 2011). Ammonium and nitrate kinetics were determined for intact crop and tree root tips in the field by carefully removing soil and placing the tips in solutions containing varying concentrations (Bassirirad *et al.*, 1999). Despite advances in measurement of small segments of roots, the method is difficult and throughput is low. York *et al.* (2016) demonstrated that while nitrate uptake kinetics varied among maize root classes, lateral roots dominated root system uptake. Therefore, measurement of an entire root system is a reasonable approximation of a genotype's inherent maximum uptake potential. Determination of uptake can be based on depletion of nitrate from an external solution, or more directly based on accumulation of a radiotracer such as ^{13}N inside the roots (Kronzucker *et al.*, 1995). In general, the depletion-based method is convenient because no radioactive material is used, only standard analytical chemistry instruments are needed, and the uptake time is short enough that plasticity does not confound results.

Nutrient uptake kinetic parameters rarely have been phenotyped across multiple maize (or other crop) genotypes. Nutrient uptake kinetics for several maize genotypes were determined for nitrate (Pace and McClure, 1986), phosphate (Nielsen and Barber, 1978), sulfate (Cacco *et al.*, 1978), and multiple ions (measured separately: K, Ca, Mg, P; Baligar and Barber, 1979) more than 30 years ago. These pioneering studies confirmed that MM kinetics generally model root uptake of nutrients well, and also identified substantial variation among the few genotypes studied. However, the complexities and labour requirements associated with such experiments, intensive data analysis, and the rise of molecular biology likely led to few researchers attempting this approach over the past couple of decades. Increasing the extent of measurements to hundreds of individual genotypes and simultaneous determination of uptake kinetics for several ions will transform the study of plant nutrient uptake.

Instruments that allow accurate and high-throughput analysis of ion concentrations are available

Depletion-based methods for uptake kinetics commonly use ion chromatography (IC), which is readily available, reliable, sensitive, precise, and accurate. Ion chromatography can be used to measure several ions from one sample, but generally of one charge. However, by switching columns IC can measure several anions in one stream, and several cations in another stream. IC instruments with an autosampler allow unsupervised high-throughput measurements of more than 100 samples a day, and can reach 1000 samples a week. Promising alternatives exist that are increasingly used for ionomics, such as ICP-MS and ICP-AES (Salt *et al.*, 2008); however, generally the methods have to be combined for coverage of this project's focal nutrients, elements cannot always be distinguished in different chemical forms, and the instruments are much more expensive. Thus, IC with autosampling forms the backbone of the proposed high-throughput phenotyping of multiple ion uptake kinetics.

Recent advances in maize genetics allow for efficient phenotyping of target phenes

The maize nested association mapping (NAM) population was created by crossing 25 diverse maize inbreds to a common reference inbred line to generate 25 families each consisting of 200 recombinant inbred lines (McMullen *et al.*, 2009). The reference inbred line, B73, was used for the public physical map and the Maize Sequencing Project, thus representing an invaluable resource for maize researchers. The NAM population and associated analysis tools make use of substantial genotypic and phenotypic variation to allow phene-to-gene mapping with great resolution. However, phenotyping all 5000 NAM lines with necessary replication is a daunting task. Fortunately, empirical work with 19 phenes across 11 environments suggests that phenotyping the NAM parents followed by selection of a family based on maximum phenotypic distance of the parents may be an efficient method for minimizing the number of plants phenotyped while maximizing the probability of locating important genetic loci (Hung *et al.*, 2012). This approach will be employed in the current project to maximize the chances of success in confirming known genetic associations with nutrient uptake, and, more importantly, in discovering novel genetic associations with MIUK.

Statistical programming now allows big data approaches to fitting kinetics curves

Originally, MM parameters were inferred graphically from experimental data using techniques such as the Hanes-Woolf plot (Haldane, 1957). The advent of non-linear regression using numeric minimization algorithms allowed parameters to be derived by using initial parameters to fit a curve, computing the sum-of-squares between the differences of the raw data and fitted curve, then iteratively changing the parameters to minimize the sum-of-squares (a measurement of the error, Marquardt, 1963). These numerical solutions have benefitted greatly from the advances in computer speed. Nonetheless, fitting many curves for multiple genotypes using the most common statistical programs can be time consuming because the user must initiate each fitted curve. The *R* statistical programming language allows automation of this process with user-defined programs that call upon the *nls* (nonlinear least squares) function to sequentially fit MM curves to subsets of large datasets based upon factors such as genotype using the *aggregate* function. Co-PI York has already developed such programs and can automate the entire process including data visualization such that hundreds of curves can be fitted with relative ease.

SPECIFIC AIMS

Developing high-throughput phenotyping of MIUK and applying the results to generate new knowledge will require several integrated activities. Specifically, our aims include:

Aim 1: Develop an automated modular hydroponic system with a 24-channel peristaltic pump and a fraction collector

To determine the genetic potential of a maize genotype for multiple ion uptake kinetics, measurements need to be conducted when the plants are operating at their maximum uptake capacities. Generally, this potential is unlocked by depriving the plants of the focal nutrient for a period of time followed by exposure to high nutrient concentration for a period of hours to induce the transport systems. After induction, uptake kinetics and MM parameters can be assessed by two depletion-based methods. The first method will be referred to as 'depletion-over-ranges', and relies on characterizing nutrient uptake of separate plants placed in different concentrations of nutrient that represent the range of relevant concentrations (originally in Epstein and Hagen, 1952). The second method will be referred to as 'depletion-over-time', and relies on measuring nutrient uptake by a single plant starting at a high nutrient concentration and measuring depletion over time (originally in Claassen and Barber, 1974). Depletion-over-ranges has the advantage of requiring less time for the uptake study, but entails the use of multiple plants and vessels and, therefore, introduces interplant variability. On the other hand, depletion-over-time requires only a single plant and vessel, which simplifies the experimental setup. However, sampling multiple times is more complicated, the solution may not be depleted to low enough concentrations to accurately determine K_m, and plants may exhibit plasticity in response to the changing nutrient concentrations. Following optimization, the proposed system will facilitate both approaches and will allow us to compare the uptake kinetics and potential biological implications (e.g. plasticity) associated with the two approaches.

To facilitate adaptation to different needs and objectives of different investigators, we will design and develop a modular automated system that can be easily scaled up for high-throughput phenotyping (Fig. 2). The module will be set up to simultaneously grow and assay 24 plants in individual vessels. These vessels will be interconnected such that commonly-used hydroponics principles will allow filling of the vessels to the same volume levels. Large storage tanks will accommodate pure water, growth solution, deprivation solution, induction solution, and experimental solutions for depletion-over-ranges as well as depletion-over-time methods as appropriate. Using a manifold system connecting the components, pumps, and valves the solutions can be pumped in and out of the vessels in a controlled, user-defined method. Pure water will be used to rinse the vessels, tubing, and apparatus between runs. During growth, nutrient solutions will be replenished daily from large reservoirs. The manifold system will be designed such that individual vessels can be filled with solutions of different concentrations. Automated sampling of the assay solution for IC will be accomplished using a scientific-grade 24-channel peristaltic pump capable of taking small samples (mL). While the growth conditions setup for the depletion-over-time method is simpler due to only one experimental solution being used, the multiple sampling times require extra consideration. However, the same 24-channel peristaltic pump can be programmed to sample through time, and the output lines can be fed to a fraction collector that can move the lines to new tubes. The 24-channel pump and fraction collector identified for this project are laboratory-grade equipment that are programmable and designed to work together. For analysis, samples will be transferred to an IC with an autosampler.

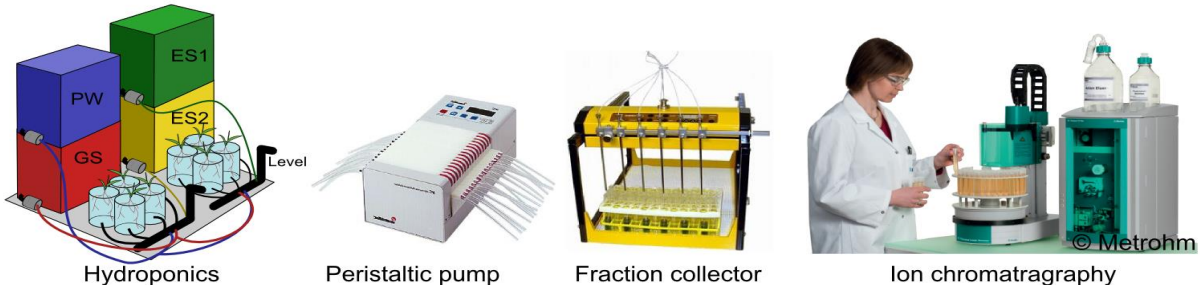

Fig. 2. Illustration of components of the high-throughput phenotyping platform. PW = pure water, GS = growth solution, ES1 and ES2 = experimental solutions 1 and 2.

In parallel to the development of a system based on laboratory-grade components, the development of a system based on low-cost "hobbyist" components will be pursued. This activity lends to the transformative aspect of this project because the same 24-channel design could potentially be achieved at an approximately 20-fold lower cost. Inexpensive single-line peristaltic pumps can be controlled from a circuit board that is connected to a Raspberry Pi computer. These hobbyist computers are increasingly popular due to their low cost and ease of programmability. For this aspect of the project we will recruit vocational students through the Mechatronics program at the Southern Oklahoma Technology Center, with the aim to identify interested minority and female students. Student-led teams have a history of successfully completing such projects at the University of Missouri, including a project in Fritschi's lab. One student will be selected for part-time work in the lab as a dedicated developer. The performance of the resulting modular system achieved with substantially decreased cost will be compared with the original system and, if successful, will allow the rapid propagation of the method to other research groups.

Aim 2: Optimize experimental conditions for MIUK phenotyping

Seeds will be germinated in an incubator using germination paper and 0.2 mM $CaSO_4$ solution. After 3 days, seedlings will be transferred to containers with 0.25x Hoagland's growth solution (Hoagland and Arnon, 1950; York *et al.*, 2016) and grown until transfer to the deprivation solution and initiation of the protocol for the kinetic assays. In all growth systems, the hydroponic solution will be monitored and adjusted for pH as needed. The growth solution will be changed on a regular schedule to ensure nutrient availability, and will be aerated vigorously to avoid hypoxic conditions. Assays will be conducted in growth chambers controlled at 28/20 °C day/night, 65% RH, 700 µmol m^{-2} s^{-1} light intensity, and a 14-hour photoperiod.

The nutrient solutions for initial experiments will contain an optimal level of all nutrients using the standard Hoagland's solution. Over the course of the first experiments, vessel volume (0.5 – 3 L), nutrient concentration ranges (1 – 5000 µM), plant age (2 – 3 wks), deprivation (6 – 48 h) and induction times (1 – 10 h) will be optimized, in that order. Container volume determines the total amount of nutrient available, which affects depletion time. The ranges of concentrations will be determined for each nutrient separately before being combined for multiple ion uptake analysis; literature cited above provides relevant ranges to test. Ideally, proportions of Hoagland's solution will be identified that can cover needed ranges for all nutrients (eg. 1%, 10%, 20%, 50%, 100%). The optimization results will also influence decisions in designing the automated pumping and fraction collecting system. These optimization experiments will not only benefit future high-throughput phenotyping, but will provide novel biological information for B73, the maize NAM parent that will be used for these experiments.

Following transporter induction, the depletion-over-ranges method requires each plant to be placed in an individual vessel of varying NAPPS concentrations ranging (for example) from 1 – 100 µM. After a defined time period (e.g. 1 h), a known volume (e.g. 5 mL) of the solution is removed, and uptake is determined as the difference between the initial concentration and final concentration using IC. The depletion-over-time method requires multiple samplings from the same vessel over a period of several hours, and necessitates careful tracking of all time points and nutrient solution volume removed from the vessel. An IC with an autosampler will be used for determining ion concentrations in all aims of this project.

Following uptake measurements, plants will be immediately removed from the nutrient solutions, roots severed from the shoots, and stored frozen until scanning on a flatbed scanner. Analysis of root length and volume will be carried out using WinRhizo software. Determination of lengths within diameter classes allows useful approximation of the proportions of seminal, lateral, and nodal root length to inform subsequent analysis of uptake standardized to root architectural measurements across plants.

In parallel with the optimization experiments, the R statistical programming language (R Core Team, 2014) will be used to develop a script to automate combining of data output files from ion chromatography and WinRhizo into a single file with the necessary information and processing of the data. This will include calculations of calibration curves for determining ion concentrations as needed, and will lead to the creation of a standard file format that can be used by any researcher attempting to do similar work. From this file format, non-linear regression and aggregation features of R will allow

the high-throughput computation of MM parameters from the raw data. The output of this tool will also be a standard format that, importantly, will include phene ontology metadata (Oellrich *et al.*, 2015), including the species used and experimental conditions, to allow future metaanalyis. This R data analysis script will be uploaded to CyVerse (Antin, 2016) as a publicly available tool, and an app will be created that CyVerse users can include in their dashboard for custom phenotyping pipelines.

Aim 3: Quantifying the importance of competitive and facilitative interactions of ion uptake by measuring focal ion kinetics in factorial combinations of varying levels of other ions

Early work on uptake kinetics demonstrated competition effects between tracer analogs and the respective nutrient, such as rubidium and calcium (Epstein and Hagen, 1952). However, in general the study of nutrient uptake has focused on a single nutrient at a time, often from solution containing only that nutrient. While useful for fundamental understanding of the uptake kinetics for a specific nutrient, this approach complicates the translation to real-world conditions where soil solutions generally contain a complex complement of nutrients in varying concentrations. Since electrochemical gradients influence plant nutrient uptake, the uptake of a nutrient is influenced by other nutrients with the same as well as opposite charges (Cox and Reisenauer, 1973). Although most relevant under field conditions, this area of nutrient uptake kinetics is critically understudied. In order to better understand nutrient interactions with regard to uptake kinetics, we will phenotype B73 for MIUK using factorial combinations of nutrient concentrations to determine the competitive / facilitative interactions. We will employ the automated modular system developed and optimized as part of Aims 1 and 2 for these experiments, which will allow testing of a large number of combinations in a short period of time.

Aim 4: Phenotype and map a maize NAM population using the optimized procedure for MIUK

With an automated system and optimized protocols in hand, all 26 parents of the maize NAM populations will be phenotyped for multiple ion uptake kinetics. The population of the parent most divergent from B73 for MIUK will be selected and all 200 recombinant inbred lines will be phenotyped. This will represent the first time a mapping population is phenotyped not only for the uptake kinetics of a single nutrient, but for the uptake kinetics of a set of the most important agricultural nutrients. The number of replicates will be at least three, but will be based on data from Aim 1. Only a subset of these lines will fit in the growth chamber for each sampling day, thus sampling day will be treated as a statistical nesting factor. The MM parameters determined from this study will be used for QTL analysis to link MIUK to genetic loci. The MM parameters from each replicate will be modelled using linear mixed effects correcting for nesting of lines within sampling day as a fixed term in order to calculate best linear predictors (BLUPs) for both parameters. The publically available map of the respective population and the BLUPs will be used for fitting a single QTL model using the extended Haley-Knott method on imputed genotypes using the R *qtl* package. QTL candidates will be identified using logarithm of odd scores above 2.5.

Schedule of activities over 24 months

Aim	Activity	3 m	6 m	9 m	12 m	15 m	18 m	21 m	24 m
1	Hydroponics								
1	Lab autosample								
1	Pi autosample								
2	Optimize								
2	R script to CyVerse								
3	Interactions								
4	NAM parents								
4	NAM population								

Intellectual merit and fit for EAGER

Development of the proposed high-throughput platform for MIUK has the potential to transform studies on nutrient acquisition by roots. The direct deliverables of this proposal will demonstrate variation in nutrient kinetics within the *Mays* genus, demonstrate possible interactions among the uptake of different ions, and map genetic loci of MIUK. Although phenotyping the uptake kinetics of several ions simultaneously by roots has never been attempted and is not guaranteed to work, this project and extensions thereof are expected to address substantial knowledge gaps that exist as to how variation in MIUK emerges from a complex system with multiple transporter types, ATPase proton pumps, and ion assimilation processes. In the future, omics data, such as transcriptomic and plasma membrane-enriched proteomic data (Zhang *et al.*, 2013; Voothuluru *et al.*, 2016), could be collected to link variation in MIUK, genetics, and plasma membrane proteins in genotypes contrasting for MIUK. Data obtained will also enhance mathematical models of ion uptake by roots already under development by Co-PI York and colleagues.

This research will be conducted in hydroponics and may not directly translate to nutrient uptake under field conditions. However, Nielsen and Barber (1978) found that hydroponics measurements of phosphate uptake kinetics of different maize genotypes did correspond to phosphorus uptake in the field. Given that nutrients are ultimately acquired from soil solution, using MIUK as determined in hydroponics as a proxy for field uptake capacity is reasonable. In any case, this and future work will allow the selection of genotypes contrasting in MIUK for testing under field conditions where uptake can be determined both by shoot concentrations and by depletion from soil solution using a promising technique called microdialysis (Shaw *et al.*, 2014).

This project encompasses a large method-development aspect associated with the coupling of different components including fluid handling for plant growth systems as well as sampling of hydroponic solutions, measurements of several ions, upscaling to hundreds of plants, and data integration and analysis. Successful coupling of all of these components is associated with considerable risk and necessitates delivery of proof of concept. Thus, this project is not a good match for traditional NSF funding but is an appropriate fit for EAGER. Particularly, the technologies involved in the fluid handling have not previously been used for this purpose, and their reliability and accuracy are unknown in this context. Nutrient uptake kinetics are known to be plastic (van Vuuren *et al.*, 1996), therefore a large source of variability potentially exists through replication of plants and through time in the growth chamber. Last, uptake kinetics in hydroponics may not translate to field uptake because of the many differences in substrate. However, if successful this research will result in a transformative technology that enables high-throughput examination of root function, a highly understudied area of plant biology. This technology extends the types of phenotypic data collected, can potentially integrate with other phenotyping platforms such as for root architecture or anatomy, will use appropriate ontology based on root class, and will allow verification of modelling activities. Therefore, this project is consistent with the EAGER requirement of extending plant phenomics.

Broader impacts

Increasing nutrient uptake efficiency by crops will benefit global food security and decrease environmental pollution; however, the ion uptake kinetics of plant roots are not well-understood and have never been the target of a breeding program in any crop. Identifying maize genotypes that contrast for MIUK will allow future work to characterize field performance, and genetic mapping will allow the development of molecular markers that can be used in breeding programs. While maize is the most widely grown crop globally, knowledge gained about its physiology is applicable to other cereal crops including rice, wheat, sorghum, and barley, and the phenotyping platform is scalable and can easily be adapted for other plant species. Involving students from underrepresented groups in STEM in development of the scalable automated sampling system will foster diversity in the US education system. The development of a lower-cost platform and addition of the analysis package to CyVerse will enhance dissemination of the technology around the world and increase adoption of the approach by different research groups, including those in developing countries. Movies explaining the platform and uptake kinetics will be generated in PowerPoint, narrated, and shared on YouTube.