UM/UWC Linkage Program

2016 Linkage Reports

Submitted by Dr. Marshall Keyster

Department of Biotechnology

University of the Western Cape

Hosted by Prof. Antje Heese (Department of Biochemistry) and Prof. David Mendoza-Cozatl (Division of Plant Sciences)

Visit period: 06 September 2016 to 22 November 2016

Original Project Abstract

Increased heavy metal concentrations in soils are a major concern worldwide, including in South Africa, as it negatively affects crop yield and nutritional quality. In addition, crop plants grown on heavy metal laden soil accumulate undesirable toxic elements. This is a food security issue that compromises human health. Heavy metal contaminants enter the soil through water waste, combustion of fossil fuels, withering of parental rock, use of fertilizers, over use of pesticides and end products from the mining industry. Plants with increased tolerance to heavy metals could provide new mechanisms to genetically engineer tolerance in heavy metal susceptible plants. Heavy metal tolerant plants could also be potential candidates for use in phytoremediation to remove heavy metals from contaminated soils. The proposed collaborative work will investigate the role of vesicular trafficking in regulating the abundance of transporters in *Arabidopsis thaliana*. We hypothesize that by altering the abundance of specific transporters we may be able to modify the uptake of heavy metals at the root level (without affecting the uptake of essential nutrients). This interdisciplinary project is based on the complimentary expertise of the labs of Dr Keyster, Prof Heese and Prof Mendoza, thus bridging research interests between MU and UWC.

Original Project Objectives or Outcomes

Heavy metals (HMs) are well-known environmental pollutants with distinctly toxic effects on plants. Toxic HM are taken up from the soil by plant metal transporters localized at the plasma membrane of plant root cells. Once within a plant cell, these toxic metals can easily displace certain essential metals like iron from a wealth of metalloproteins, and through this process disturb many normal physiological processes and cause severe developmental problems. The harmful effects of HM stress include, but are not limited to: reactive oxygen species (ROS) overproduction, higher lipid hydroperoxide contents, and chloroplast structure changes, which may lead to plant cell death.

Importantly, uptake of heavy metals into the plant cell is dependent on the correct localization and abundance of metal transporters in the plasma membrane (PM).

Recent studies show that a functional vesicular trafficking network that is required in trafficking of proteins to and from the PM plays a crucial role in regulating the correct abundance at their site of function, namely the PM. The main vesicle trafficking pathways that contribute to modulating the composition of the PM are secretion and endocytosis. In secretion, newly synthesized proteins are trafficked from the Endoplasmic Reticulum (ER) to the PM. Endocytosis is the process by which the plant cells remove and internalize proteins from the plasma membrane. We hypothesize that vesicular trafficking regulates HM transport through modulating the abundance of specific metal transporters in the PM. It remains, however, unknown which plant transporters must be present in the PM to allow uptake of HM from its environment into a plant cell

In this project, we propose to test whether mutations in genes encoding vesicular trafficking components result in less sensitivity of these Arabidopsis mutant plants. Initially, we will focus on mutants in vesicular trafficking genes available in the Heese lab and previously shown to modulate the PM abundance of a subset of proteins involved in biotic stresses. First, we will compare growth of mutant and wild-type (Col-0) plants on media containing different concentrations of essential heavy metals (e.g. 20-200 µM Zn) and toxic non-essential heavy metals (e.g. 5-100 µM Cd). In a complementary approach, plants will be grown hydroponically where the media composition can be modified accordingly. Importantly, we will focus on heavy metals that are abundant in South African soils due to mining such as cadmium, vanadium, zirconium and antimony. We hypothesize that if transporters mobilizing heavy metals cannot reach the PM, then accumulation of toxic heavy metals from surrounding soil will be reduced and therefore these plants will grow better on contaminated soils. It is possible that nutrient uptake may also be affected, therefore we will also conduct elemental profiling (ionomics) to assess which elements are being specifically affected (see below).

Next, we will investigate the ion composition of wild-type and vesicular trafficking mutants using the ionomic profiling protocol established in the Mendoza lab. Identifying whether a specific metal accumulates in wild-type (sensitive plants) but to reduced levels in a vesicular trafficking mutant (insensitive plants) will provide important information on which transporter may be important for uptake of a specific heavy metal. As a complementary approach, we will generate crosses using a As/Pi

transporter tagged with YFP and vesicle trafficking mutants available in the Heese lab. These crosses will allow us to investigate, using live-cell imaging, whether the localization of this As/Pi transporter has been altered.

The visit to the University of Missouri will be used to engage in collaborative research between the laboratories of Dr Keyster (the applicant, University of the Western Cape), Associate Prof Antje Heese (MU-Division of Biochemistry) and Assistant Prof David Mendoza-Cozatl at the University of Missouri (MU-Division of Plant Sciences). Both Prof Heese and Prof Mendoza are faculty in the College of Agriculture, Food and Natural Resources (CAFNR) and very active members of the Interdisciplinary Plant Group (IPG) at MU. The collaboration will elucidate the role of vesicle trafficking disruption in HM stress in Arabidopsis and eventually lead to engineer crop varieties with enhanced tolerance to non-essential toxic metals. Furthermore, Dr Keyster will receive training in high-throughput Agrobacterium-mediated transformation of Arabidopsis, ionomic screening, live-cell imaging and quantitative mass spec analyses. This training is critical for research at UWC and will facilitate skills transfer from the University of Missouri to South Africa (expertise in genetic transformation of plants in South Africa is extremely lacking). *Arabidopsis thaliana* will be used as the model plant, which is related to *Brassica napus*, the crop species studied in Dr Keyster's lab for heavy metal stress. In addition, *Brassica napus* is a crop of economical value and health benefit grown in South Africa.

We anticipate that results from this project will provide crucial preliminary data for submitting a collaborative grant proposal between Dr Keyster, Prof Heese and Prof Mendoza to federal and international funding agencies.

Original proposed workflow and methods

1. Arabidopsis vesicular trafficking (*ves*) mutants and wild-type from Prof Heese will be grown on MS-agar media containing increasing levels of cadmium, vanadium, zirconium and antimony. The same experiment will be initiated in hydroponic experiments.

2. Compare seedling weight, root length, aerial tissue size and chlorosis in trafficking mutant and wild-type plants exposed to heavy metals.

3. Ionomic profiling (Prof Mendoza-Cozatl) of trafficking mutants vs wild-type plants exposed heavy metal identified in 2.

4. Results from 1 and 2 would give us an idea of which transporter/ transport of ions may be affected in each of the mutants. This information will be used for a targeted approach to start transforming the mutants with specific transporters-tagged with fluorescent proteins to look at potential changes at the subcellular levels (using live-cell imaging, Prof Heese's lab) as well as within the entire organism (using Prof Mendoza-Cozatl's optimized lab approaches).

5. As a long-term goal, we also expect to pursue collaborative research with Prof Walter Gassmann (MU-IPG) to test the transporter of interest by expressing it in oocytes. These experiments will give us insight into the specific transport capabilities of selected heavy metal transporters.

6. After growing vesicle mutants, with or without heavy metals of interest, we will isolate enriched plasma membranes with-or without heavy metal compared to wild-type and then in collaboration with Prof Scott Peck (MU-IPG), perform comparative quantitative mass spec analyses to compare the mutants with the wild-type at different growth conditions.

Original project timeline

Timeframe	Activity
07-09-2016 to 09-09-2016	Departure from Cape Town and arrival at U. Missouri (Columbia)
12-09-2016 to 13-09-2016	Project planning discussions with Prof Heese and Prof Mendoza-Cozatl
14-09-2016 to 14-10-2016	Growth of Arabidopsis mutants and wild-type plants, heavy metal treatments and scoring of growth parameters (1 – 2)
17-10-2016 to 02-12-2016	Hydroponic experiments and ionomic profiling of trafficking mutants (3 - 4)
05-12-2016 to 07-12-2016	Discussion of project results, conceptualization of research papers that will be prepared for submission for publication in peer-reviewed journals, seminar presentation by Dr Keyster at U. Missouri on the work done at U. Missouri
09-12-2016 to 10-12-2016	Departure from U. Missouri and arrival in Cape Town

Deviations from the original timeline

Due to personal commitments in Dr. Keyster's schedule, we had to reschedule his trip to Columbia, Missouri. Therefore, he left Cape Town on the 5th of September 2016 and arrived in Columbia (Mo) on the 6th of September 2016. He then left Missouri on the 22nd of November 2016 and arrived back in Cape Town on the 23rd of November 2016. Due to the shorter schedule, we decided to start the growth and screening of the mutants on the same day as the hydroponic experiments.

Preliminary Results obtained

Screening the vesicular trafficking (*ves*) mutants for Cd tolerance

The control (Col-0) and mutants (*ves5*, *ves6*, *ves4* and *ves6ves4*) were sponsored by Prof. Heese where the *opt3-2* (control for iron deficiency and cadmium sensitivity) was sponsored by Prof. Mendoza-Cozatl. Seeds were sterilized and plated on MS (only) plates, iron deficient plates (containing Ferrozine) and cadmium containing plates (containing 20 µM Cd). Seeds were allowed to germinate and to grow in controlled environmental chambers. After 10 days, the plates were processed by digital photography (Fig. 1). We observed green leaves (all plants) on the MS plates as well as proper root formation. Furthermore, all plants did not perform well on the iron deficient plates. From our observations, *ves6ves4* performed the best in iron deficient conditions. When the plants were exposed to cadmium, *ves5* and *ves4* were observably more tolerant to cadmium then the rest of the mutants. As expected, *opt3-2* was sensitive to both iron deficiency and cadmium stress.

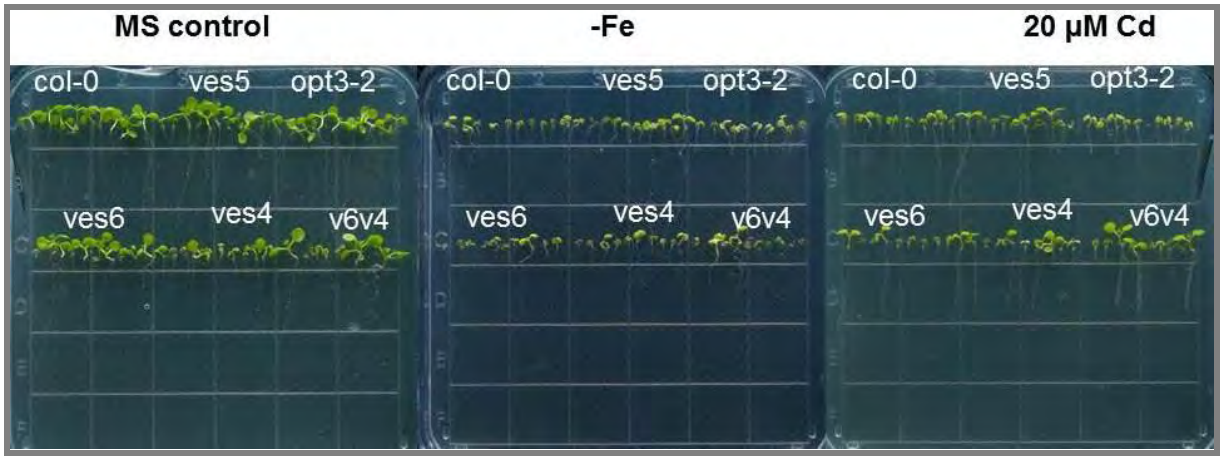

Fig. 1. Representative Murashige and Skoog (MS) agar plates used for phenotyping of *ves* mutants. MS control plate contains Murashige and Skoog medium with agar only. The Iron deficient plate (-Fe) contains Murashige and Skoog medium with agar and supplemented with Ferrozine. The Cd plate contains Murashige and Skoog medium with agar and supplemented with Cd. The *opt3-2* mutant was included as a phenotype control. Sterilized seeds were plated and grown for 10 days at 22°C in a 8-h light/16-h dark cycle in controlled environment chambers.

Microscopic observation of screened vesicular trafficking (*ves*) mutants

After digital photography, plants were subjected to microscopic analysis. Similar to the photography observations, plants were green which indicated proper chlorophyll synthesis (Fig. 2). However, when plants were exposed to iron deficient conditions, leaf yellowing was observed (Fig. 3). We also observed yellowing when plants were exposed to cadmium (Fig. 4) but not as severe as in the iron deficiency conditions.

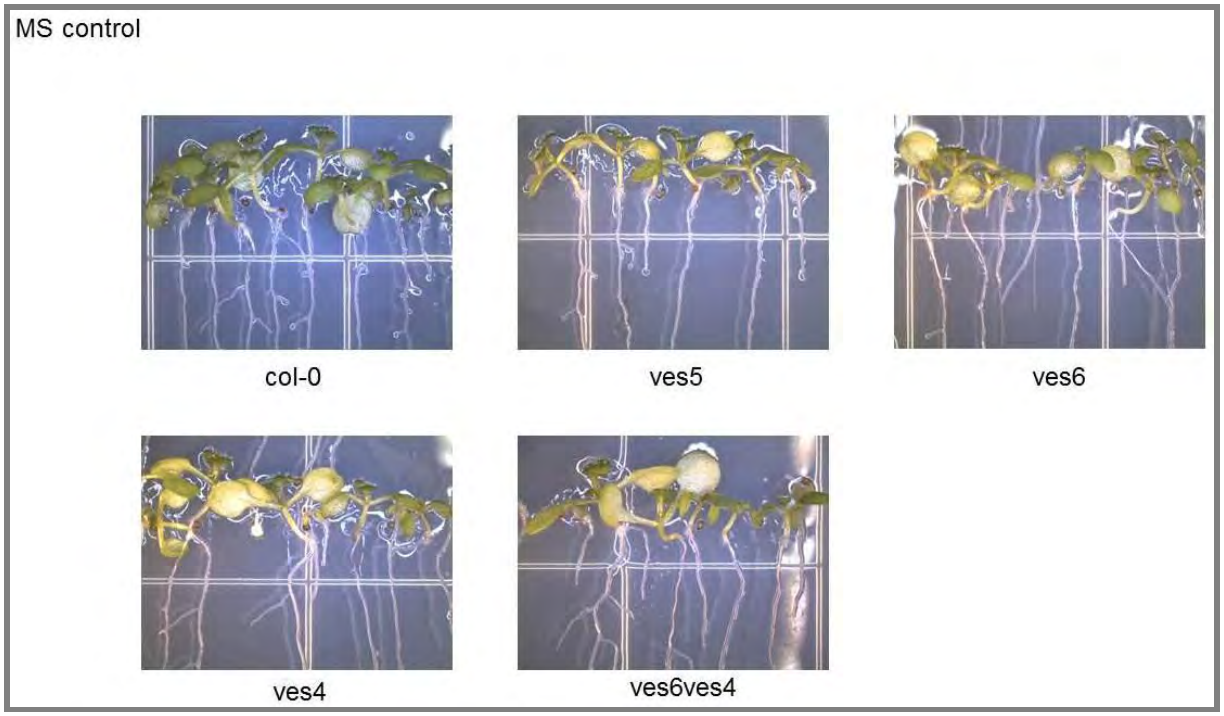

Fig. 2. Representative microscopic images of Murashige and Skoog (MS) agar plates used for phenotyping of *ves* mutants.

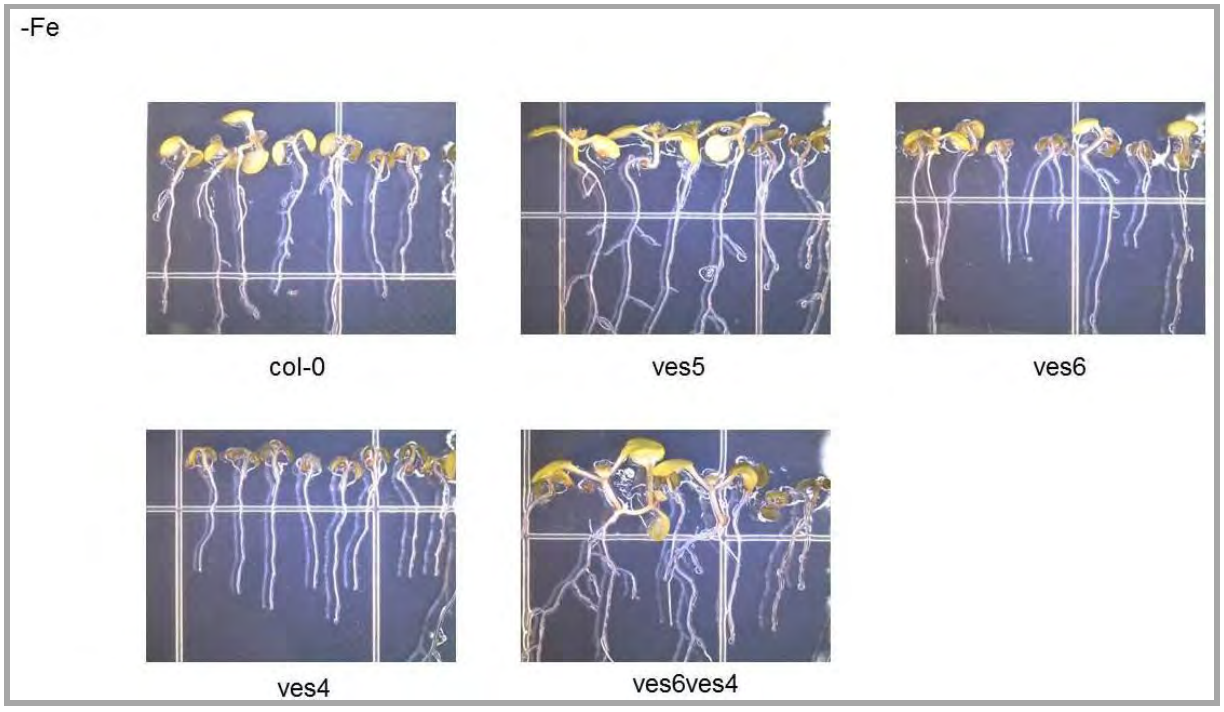

Fig. 3. Representative microscopic images of Murashige and Skoog medium (with agar) supplemented with Ferrozine used for phenotyping of *ves* mutants.

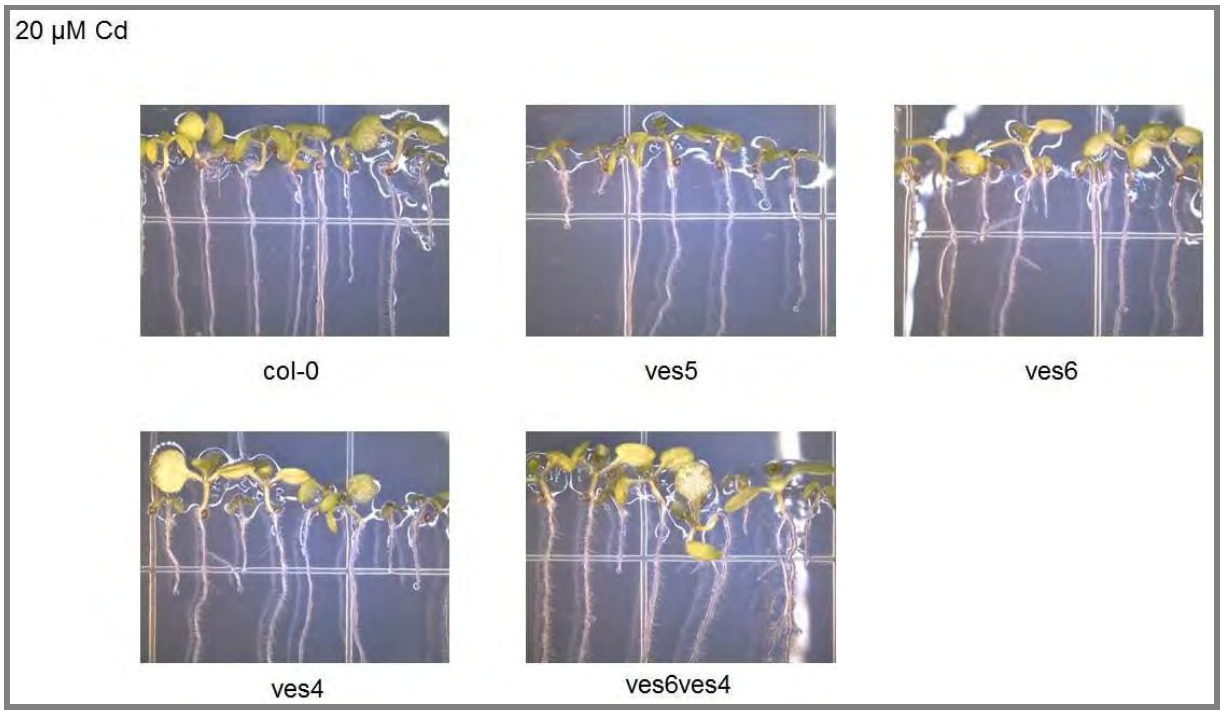

Fig. 4. Representative microscopic images of Murashige and Skoog medium (with agar) supplemented with Cd used for phenotyping of *ves* mutants.

Fresh weight measurement of *ves* mutants

We investigated the effect of iron deficiency and cadmium stress on seedling biomass obtained after digital photography (Fig. 1) and microscopy (Fig. 2, 3, and 4). We used three seedlings (of similar size) to measure accurate seedling fresh weight. We calculated the average fresh weight of the seedlings after weighing atleast four sets of seedlings (n=4). We observed that *ves5* and *ves6ves4* were heavier than Col-0 and that *opt3-2* and *ves4* weighed the least of all the seedlings (Fig. 5). Furthermore, as expected, *opt3-2* was the most sensitive to iron deficient conditions (followed by *ves6*). The *ves4* mutant was the most tolerant to cadmium followed by the *ves5* mutant.

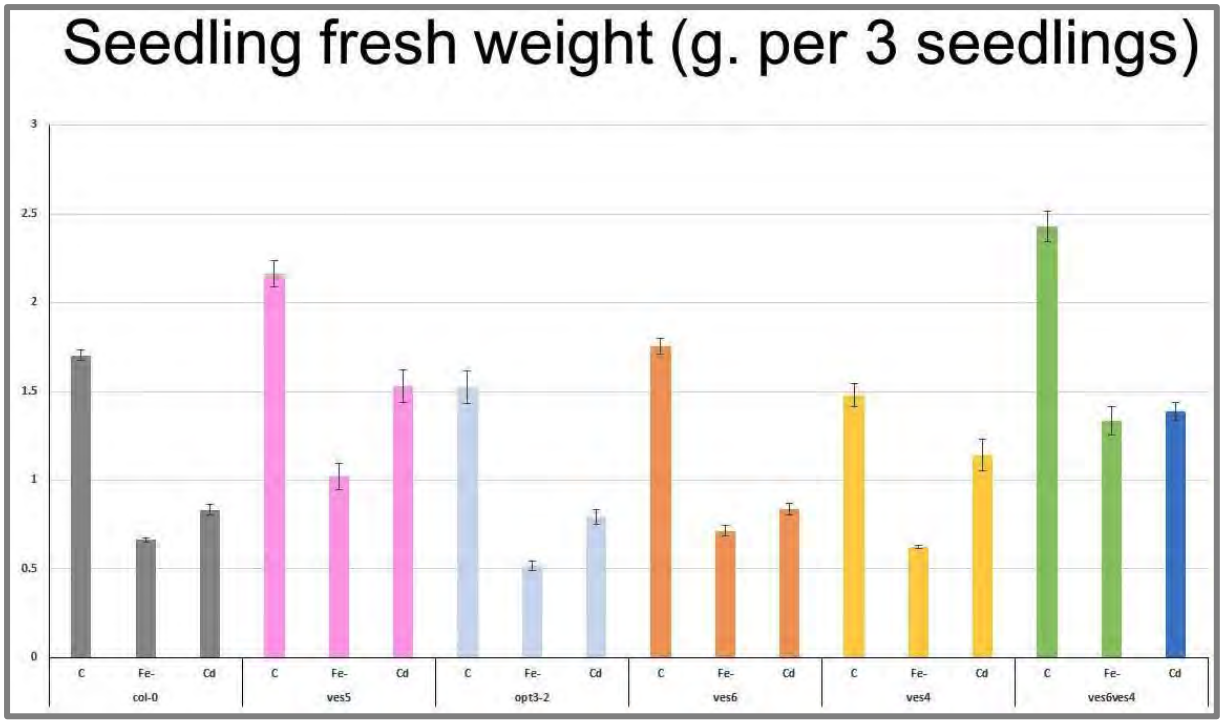

Fig. 5. Seedling fresh weight of the *ves* mutants from control, Ferrozine and Cd MS plates. Seedlings were weighed as sets of 3, and at least 4 replicates were measured per screen (n=4).

Screening of a second set of *ves* mutants for Cd tolerance

Seeds were sterilized and plated on MS (only) plates, iron deficient plates (containing Ferrozine) and cadmium containing plates (containing 20 µM Cd). Furthermore, seeds were allowed to germinate and to grow in controlled environmental chambers (8-h light/16-h dark cycle). After 10 days, the plates were processed by digital photography (Fig. 6). We observed green leaves in all plants plated on the MS plates. We observed proper root formation in all the mutants except for the *ves3* mutant. This mutant showed a “small root” phenotype on MS plates. Furthermore, Col-0, *opt3-2* and *ves1* did not perform well on the iron deficient plates where *ves3* and *ves2* performed observably better under iron deficiency. When the plants were exposed to cadmium, *ves3* and *ves2* (which were more tolerant to iron deficiency) were observably more tolerant to cadmium then the rest of the mutants. Again, as expected, *opt3-2* was sensitive to both iron deficiency and cadmium stress.

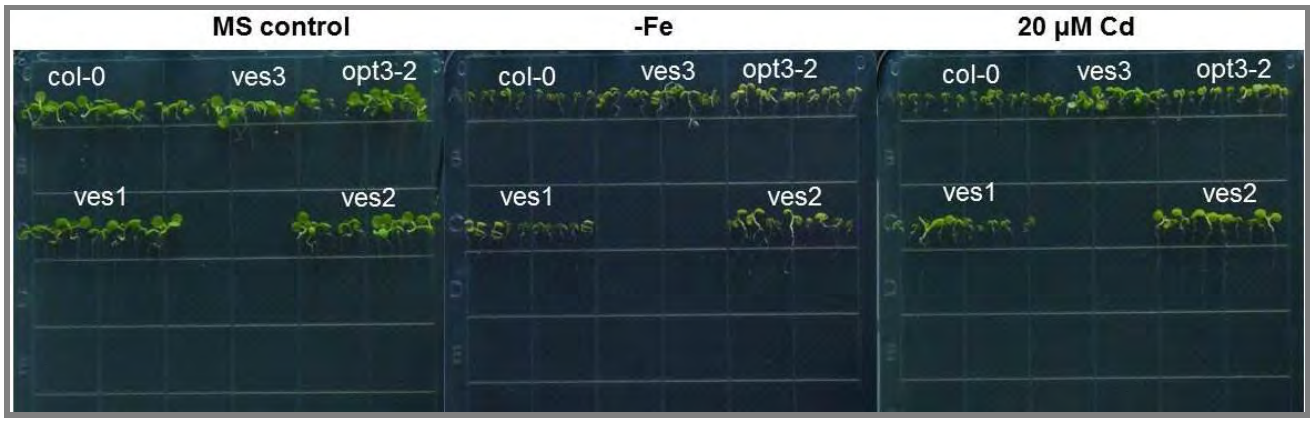

Fig. 6. Representative Murashige and Skoog (MS) agar plates used for phenotyping of *ves1, ves2* and *ves3* mutants. MS control plate contains Murashige and Skoog medium with agar only. The Iron deficient plate (-Fe) contains Murashige and Skoog medium with agar and supplemented with Ferrozine. The Cd plate contains Murashige and Skoog medium with agar and supplemented with Cd. The *opt3-2* mutant was included as a phenotype control. Sterilized seeds were plated and grown for 10 days at 22°C in a 8-h light/16-h dark cycle in controlled environment chambers.

Microscopic observation of screened *ves1, ves2* and *ves3* mutants

After digital photography, plants were subjected to microscopic analysis. Similar to the photography observations, plants were green which indicated proper chlorophyll synthesis (Fig. 7). Furthermore, the “small root” phenotype was clearly visible on the MS plates. Nevertheless, when plants were exposed to iron deficient conditions, leaf yellowing was observed (Fig. 8). However, we observed much greener leaves in the *ves3* mutant (on iron deficient plates). We also observed yellowing when plants were exposed to cadmium (Fig. 9) but the yellowing was not as pronounced in the *ves3* mutant even though cadmium stress clearly affected the rooting of *ves3*.

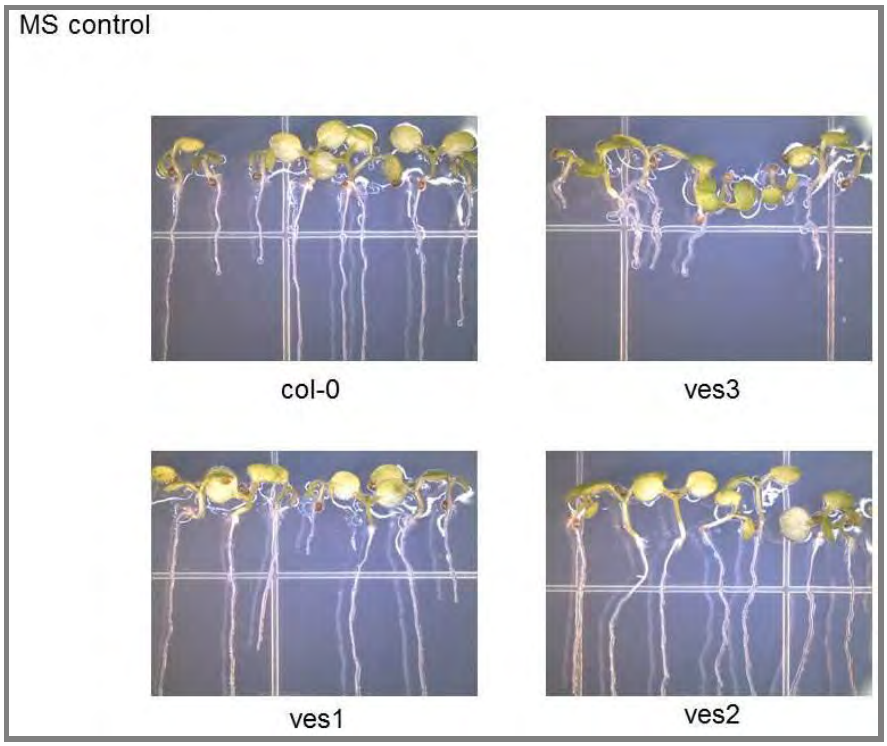

Fig. 7. Representative microscopic images of Murashige and Skoog (MS) agar plates used for phenotyping of set 2 mutants (Mutants *ves1* and *ves2*).

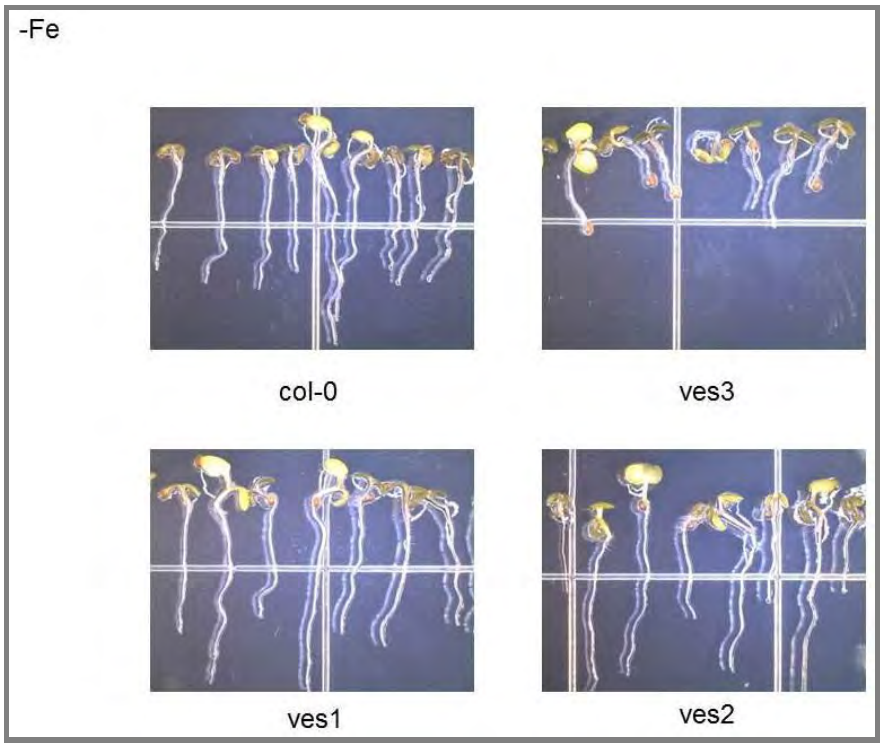

Fig. 8. Representative microscopic images of Murashige and Skoog medium (with agar) supplemented with Ferrozine used for phenotyping of *ves1*, *ves2* and *ves 3* mutants.

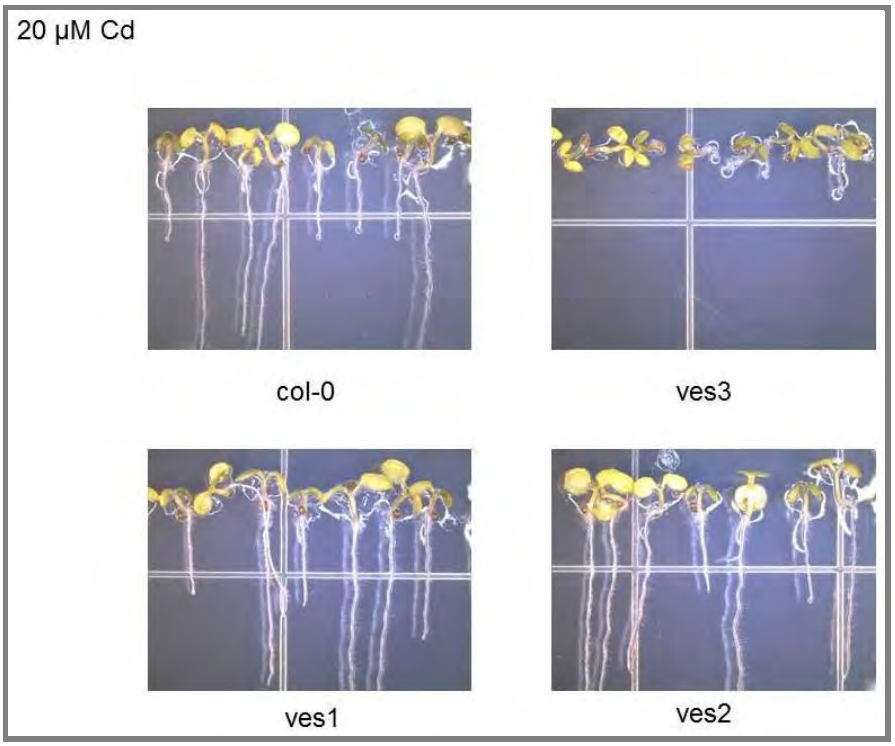

Fig. 9. Representative microscopic images of Murashige and Skoog medium (with agar) supplemented with Cd used for phenotyping of *ves1, ves2* and *ves 3* mutants.

Fresh weight measurement of *ves1, ves2* and *ves3* mutants

We investigated the effect of iron deficiency and cadmium stress on seedling biomass obtained after digital photography (Fig. 6) and microscopy (Fig. 7, 8, and 9). Again, we used three seedlings (of similar size) to measure accurate seedling fresh weight. We calculated the average fresh weight of the seedlings after weighing four sets of seedlings (n=4). We observed that *ves3* and *ves1* were similar in weight as Col-0 and that *opt3-2* and *ves2* weighed the least of all the seedlings (Fig. 10). Furthermore, again as expected, *opt3-2* was the most sensitive to iron deficient conditions (followed by *ves1*). The *ves3* mutant was the most tolerant to cadmium followed by the *ves2* mutant. This observation correlated positively to the phenotypic screening (Fig. 6) and the microscopic observations (Fig. 9).

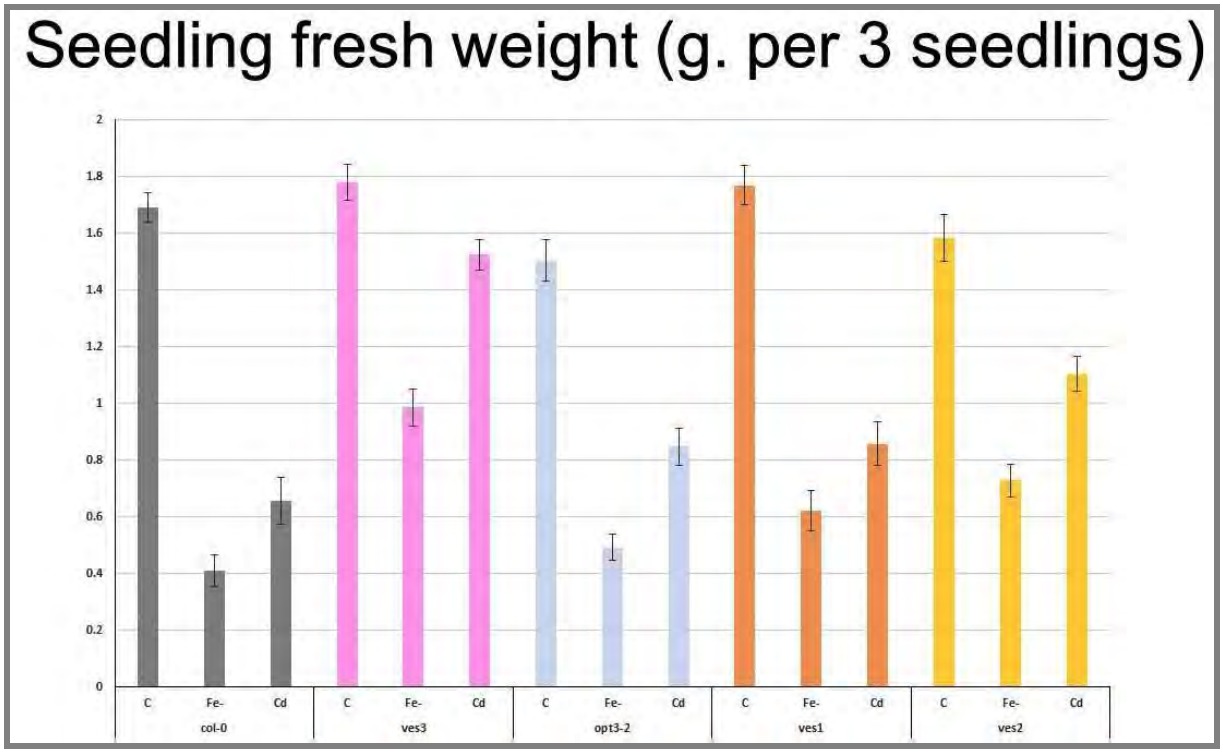

Fig. 10. Seedling fresh weight of the set 2 mutants from control, Ferrozine and Cd MS plates. Seedlings were weighed as sets of 3, and at least 4 replicates were measured per screen (n=4).

Hydroponic experiment for ionomic profiling of *ves3* mutant

We selected the mutant which was the most tolerant to cadmium (*ves3*) for the hydroponic experiment. The hydroponic setup and experiment was optimized in Prof. Mendoza-Cozatl's laboratory. The *ves3* mutant and Col-0 (control) plants were grown in nutrient solution containing cadmium or no cadmium. Afterwards, the plants were harvested and separated into leaves and roots. The leaves and roots were used for subsequent ICP-OES ionomic (micro-nutrient) screening. The material (root and leaf) was dried and subjected to acid digestion. After complete digestion, the samples were processed on an ICP-OES machine using the appropriate standards. For cadmium content, we observed significantly more cadmium in *ves3* roots than in Col-0 roots (Fig. 11). However, we observed much less cadmium in the *ves3* leaves than in the Col-0 leaves. For iron content, we observed more iron in the Col-0 control roots than in the ves3 control roots. However, we observed a decrease in iron content when Col-0 was exposed to cadmium stress. In contrast, we observed an increase in iron content when *ves3* was exposed to cadmium (when comparing the control to the cd).

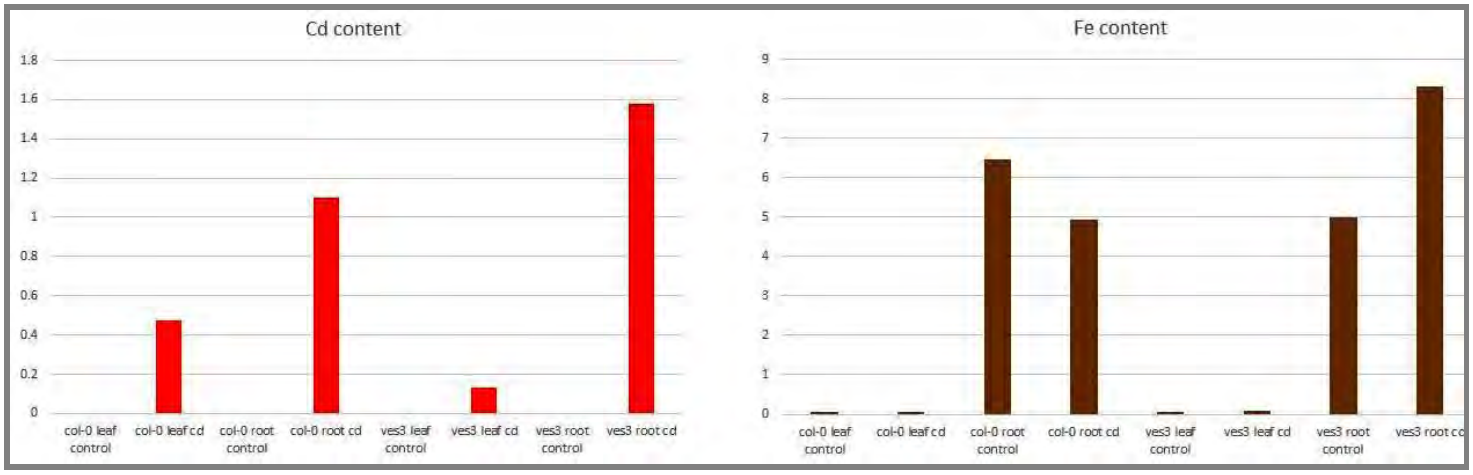

Fig. 11. ICP-OES quantification of Cd and Fe after *ves3* mutant was propagated in hydroponics (control and cd solutions).

For copper content, we observed that Col-0 accumulated more copper in control leaves than the *ves3* mutant (Fig. 12). However, we observed more copper in the *ves3* roots in comparison with the Col-0 roots in control media. However, when both plants were subjected to cadmium stress, the *ves3* mutant accumulated significantly more copper in the roots than Col-0. For manganese content, we observed approximately similar manganese content in Col-0 and *ves3* leaves. However, we observed a more pronounced increase in manganese content in *ves3* leaves under cadmium content compare to Col-0. Furthermore, we observed much more manganese in *ves3* roots than Col-0 roots under control conditions. However, when both plants were exposed to cadmium, Col-0 plants increased root manganese content where *ves3* plants significantly decreased root manganese content.

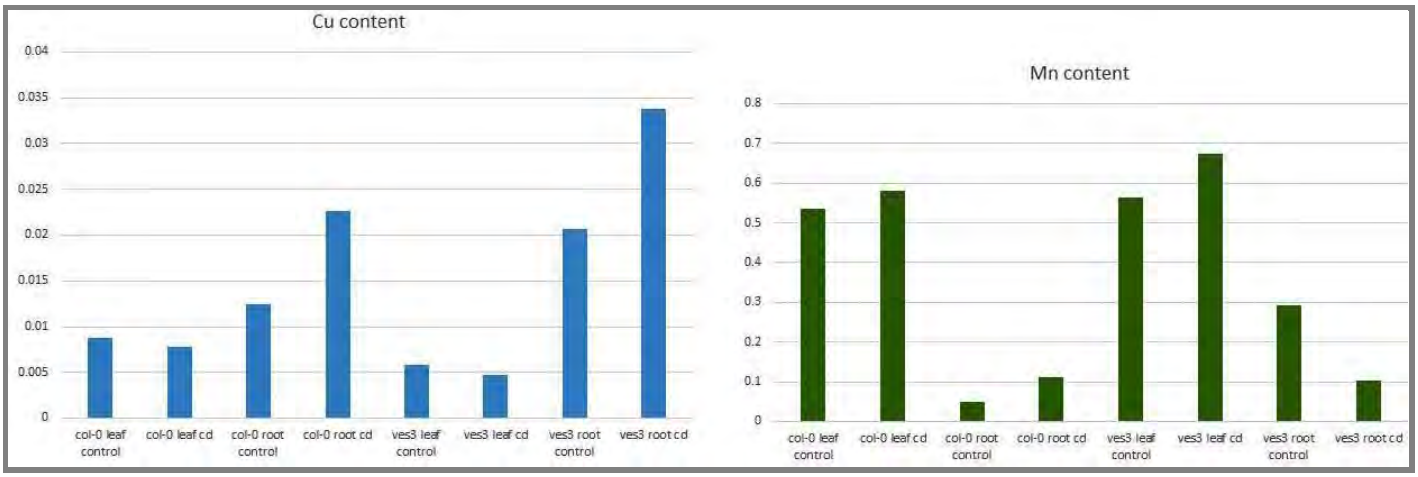

Fig. 12. ICP-OES quantification of Cu and Mn after set 2 mutant *ves3* was propagated in hydroponics (control and cd solutions).

For zinc content, we observed more zinc in the Col-0 leaves than in the *ves3* leaves under control conditions (Fig. 13). This zinc content did not statistically change when both plants were exposed to cadmium. Nonetheless, we observed significantly more zinc in *ves3* roots than in Col-0 roots under control conditions. However, when both plants were exposed to cadmium, we observed a decrease in root zinc content.

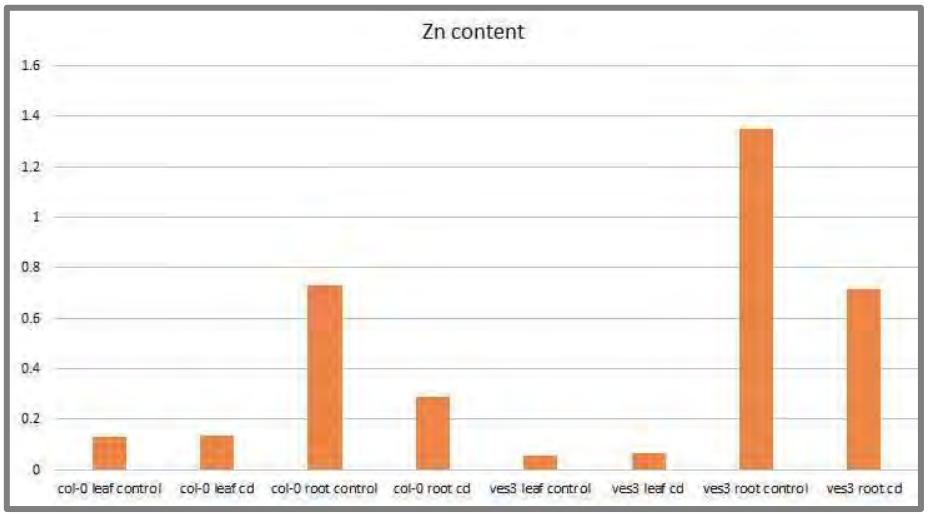

Fig. 13. ICP-OES quantification of Zn after set 2 mutant *ves3* was propagated in hydroponics (control and cd solutions).

Other outputs

During Dr. Keyster's stay in Columbia (MO), he participated in the 30-year anniversary celebration of the partnership between Missouri University-system and the University of the Western Cape. Dr. Keyster presented a 10 minute talk entitled "The role of vesicle trafficking in regulating the abundance of transporters in *Arabidopsis thaliana*" on the 27th of September 2016. On the same day, a web article was published on the website of the Interdisciplinary Plant Group (IPG) which explains our collaboration. Furthermore, as proposed in the original project timeline, Dr. Keyster presented a seminar presentation (~1 hour) in the IPG fall seminar series entitled "Towards Multidisciplinary Research in the Environmental Biotechnology Laboratory" (on the 7th of November 2016).

Conclusion

In conclusion, we have screened *Arabidopsis thaliana* vesicle trafficking mutants for tolerance and sensitivity to cadmium stress. We identified four cadmium tolerant mutants namely *ves4*, *ves5*, *ves3* and *ves2*. These mutants are classified into set 1 (*ves4* and *ves5*) mutants and set 2 (*ves3* and *ves2*) mutants. Furthermore, we selected *ves3* (our most cadmium tolerant mutant) for hydroponics and ICP-OES ionomics. We observed that *ves3* differentially regulate micro-nutrient (iron, copper, zinc and manganese) contents under both control and cadmium stress conditions and this pattern differs in Col-0. Interestingly, *ves3* accumulates more cadmium in the roots than Col-0 but the opposite pattern was observed in the leaves. The Col-0 control plants accumulated significantly more cadmium in the leaves than the *ves3* mutant plants. Provisionally, the lack of cadmium translocation from roots to leaves could be a mechanism which *ves3* mutants employ to tolerate cadmium stress.

Acknowledgements

Firstly, I would like to thank the two partner universities (MU and UWC) for driving such a wonderful initiative. I would like to acknowledge the two steering committees from both universities who selected my application for funding. Especially, the two chairpersons Prof. Rodney Uphoff (MU) and Prof. Ramesh Bharuthram (UWC). Furthermore, I would like to thank the assistants Mrs. Brenda Dennis and Mrs. Debra Lamson for coordinating my whole trip to Columbia (Mo). I would also like to acknowledge the following funding bodies: UMSAEP for providing the majority of the funding for my trip, UWC-ECRS funding which contributed to matching funds and my NRF Thuthuka grant (Grant no. 93983) which contributed to both matching funds and subsistence funding. I would like to thank the staff of Respect Hall (residence) who made my stay a very comfortable experience. Furthermore, I would like to thank the following people who contributed to wonderful friendship and entertainment during my stay in Columbia: Mr. Sfiso Khanyile, Mr. Veli Thipe, Dr. Mark Herbet, Dr. Kene Obikeze, Prof. David Fisher, Miss. Lyn Lawrence, Mr. Landry Meleine and Mr. Sebastian Cardona Ramirez. I would also like to thank the students from the hosting laboratories (DMC lab and Heese lab) who shared knowledge and skills with me during experiments. Lastly, I would like to thank the following people who not only contributed to the scientific advancement of the project but who became close friends: Prof. David Mendoza-Cozatl (host), Prof. Antje Heese (host), Prof. Scott Peck, Prof. Walter Gassmann, Dr. Norma Castro-Guerrero, Dr. Mather Khan and Dr. Michelle Leslie.

Weblinks

1. 30-year celebration of the partnership between MU and UWC

https://www.umsystem.edu/ums/news/news_releases/um_system_marks_30_year_anniversary_of_visionary_partnership_with_uwc

2. University of the Western Cape Researcher Visits MU

https://ipg.missouri.edu/feature-stories/Universi_09272016.cfm

3. Mizzou events - Dr. Marshall Keyster seminar

http://calendar.missouri.edu/bond_life_sciences_center/calendar/day/2016/11/7

4. Hydroponic experiment

Article: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5091364/pdf/jove-113-54317.pdf

Video: https://www.jove.com/video/54317/hydroponics-versatile-system-to-study-nutrient-allocation-plant