

*Molecular and biochemical  
characterisation of transgenic banana  
lines containing iron uptake and storage  
enhancing genes*

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Submitted in fulfilment of the requirements for the degree of  
Doctor of Philosophy

Centre for Tropical Crops and Biocommodities  
Faculty of Science and Engineering  
Queensland University of  
Technology  
2016

## Keywords

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banana, biofortification, bunchy top virus 4 (BT4), FRO2, IRT1, soy-ferritin, OsNAS1, OsNAS2, OsYSL2, maize polyubiquitin (Ubi), nicotianamine (NAS), cauliflower mosaic virus 35 promoter (CaMV35S), iron uptake, storage enhancement, recommended dietary intake (RDI), micronutrient deficiency, iron, zinc, malnutrition, micronutrient

# Abstract

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Over three billion people in developing countries face iron deficiency anaemia (IDA) (WHO, 2000). Further, nutritional deficiencies like iron ( $\text{Fe}^{2+}$ ), zinc ( $\text{Zn}^{2+}$ ) and vitamin A account for almost two-thirds of the child mortality worldwide (Bouis et al., 2011; Hossain and Mohiuddin, 2013; Saltzman et al., 2013). These deficiencies are typically caused either by diets with high intake of staple foods such as, banana that are low in micronutrients, or by low intake of rich micronutrient sources like vegetables, fruits and fish products.

In Uganda, IDA has been identified as a critical public health problem, where 73% of six to 59 month old Ugandan children exhibit mild to severe anaemia (MOH, 2002; UDHS, 2011). IDA causes a range of health problems in the human population, including pregnancy-related complications, brain damage in infants, and reduced productivity (Frossard et al., 2000). Traditional strategies to alleviate mineral deficiency in susceptible populations have relied on supplementation, food fortification and dietary diversification programs (Bouis, 1999; Bouis and Welch, 2010; Graham et al., 2001). These have seen limited success in alleviating IDA in developing countries like Uganda as they require reliable transport infrastructure and consistent policy support, which are currently unavailable. Biofortification has been reported to be a suitable option to complement other approaches (Bouis, 2003; Bouis et al., 2011). Biofortification is the development of micronutrient-dense staple crops using traditional biotechnology or genetic modification (Nestel et al., 2006).

Banana (*Musa* spp.) is one of the major staple foods in sub-Saharan Africa, and global production is approximately 97 million tons annually (FAOSTAT, 2010). Compared to other starchy staples, it is rich in many minerals but is deficient in nutrients like Fe, Zn and pro-vitamin A. East African Highland bananas are an important group of genetically similar cultivars (Tushemereirwe et al., 2001) that have not changed over several generations as they are sterile and parthenocarpic triploids. These biological factors make their genetic improvement using classical breeding methods difficult. Therefore, the application of biotechnological tools has the potential to add desired traits without changing highly valued characteristics of the target crop. Such an approach requires well characterised genes and promoters. A number of genes involved in plant Fe assimilation have been isolated from *Arabidopsis thaliana* and other plants. Strategies to enhance Fe accumulation in edible plant parts include improving uptake (Connolly et al., 2002; Vasconcelos et al.,

2006), Fe storage (Ravet et al., 2009b) and enhancing Fe transportation (Lee et al., 2009; Masuda et al., 2009).

However, there are no published studies on known banana cultivars with naturally elevated Fe accumulation in fruit pulp, and Fe metabolism in bananas is not well understood. Thus, in this study, cooking and dessert banana cultivars from different agro-ecological zones were investigated for the effect of genotype by environment interactions in addition to other environmental factors such as soil Fe, pH and texture on Fe and Zn accumulation in fruit pulp during development. The interaction of these factors was determined using principal components analysis and agglomerative hierarchical clustering. The effect of leaf position on Fe accumulation at vegetative, flowering and fruiting stages of development were also investigated. Biofortification of plants to enhance mineral accumulation in the edible plant parts has shown potential in plants like rice and cassava (Ihemere et al., 2012; Narayanan, 2011; Masuda et al., 2012; Johnson et al., 2011). Recently transgenic bananas, cultivars '*Sukali Ndiizi*', '*Nakinyika*' and '*Cavendish*', transformed with Fe enhancement genes were developed in Uganda and at Queensland University of Technology. Transgenic plants evaluated in the field harbour *IRT1*, *FRO2*, *Sfer*, *FEA1*, and *OsNAS1*, whereas plants transformed with *OsNAS2+OsYSL2* were only evaluated under glasshouse conditions. Detailed molecular and biochemical analyses were done on selected lines that produced fruits. The effect of gene expression on mineral accumulation was determined at different maturity stages. ICP-OES was used to determine mineral accumulation in the different plant tissues. The data shows that transgenic lines transformed with *IRT1*, *FRO2* and *FEA1* did not show significant amount of Fe in the fruit pulp while those transformed with ferritins, and *OsNAS1* showed elevated amount ranging from 1.12 - 1.8 fold as compared to wild type. All transgenic banana leaves accumulated more Fe compared to the wild type indicating that constitutive expression using 35S and Ubi promoters was more effective in the vegetative parts of banana plants compared to the fruit pulp. *OsNAS1* enhanced Fe and Zn accumulation in both fruit pulp and leaf tissue although reduced Zn concentration was observed in the latter. In contrast, lines containing *Sfer*, *FRO2*, *IRT1* and *OsNAS2-OsYSL2* showed enhanced Fe content in both fruit and leaf tissue with reduced Zn content in these tissues. The results indicate that *OsNAS* genes are a desirable strategy particularly where both Fe and Zn are required in the diet.

This research is part of a broader project currently being undertaken by researchers at Queensland University of Technology (QUT, Australia) and at the National Agricultural Research Organization (NARO, Uganda), to address Fe and pro-vitamin A micronutrient

deficiency in consumer acceptable banana varieties *via* genetic engineering. Therefore, this study provides valuable information on how different genes affect Fe metabolism in fruit and leaf tissues and pinpoints specific insights for future development of Fe or Zn rich bananas.

# Table of Contents

---

|                                                                                                      |      |
|------------------------------------------------------------------------------------------------------|------|
| KEYWORDS .....                                                                                       | II   |
| ABSTRACT .....                                                                                       | III  |
| TABLE OF CONTENTS .....                                                                              | VI   |
| LIST OF FIGURES .....                                                                                | XI   |
| LIST OF TABLES .....                                                                                 | XIII |
| LIST OF ABBREVIATIONS .....                                                                          | XIV  |
| STATEMENT OF ORIGINAL AUTHORSHIP .....                                                               | XVI  |
| ACKNOWLEDGEMENTS .....                                                                               | XVII |
| CHAPTER 1: LITERATURE REVIEW .....                                                                   | 1    |
| 1.1 Micronutrient malnutrition .....                                                                 | 1    |
| 1.1.1 Dietary Fe: sources and functions in the human body .....                                      | 1    |
| 1.1.2 Iron deficiency and associated health implications .....                                       | 2    |
| 1.1.3 Traditional strategies to combat micronutrient deficiencies .....                              | 4    |
| 1.1.4 Soil Fe, amendments as a strategy to meet the challenge of micronutrient<br>malnutrition ..... | 5    |
| 1.1.5 Biofortification as a strategy to meet the challenge of micronutrient<br>malnutrition .....    | 6    |
| 1.2 Iron metabolism in plants .....                                                                  | 7    |
| 1.2.1 Iron uptake in plants .....                                                                    | 7    |
| 1.2.2 Strategy I plants .....                                                                        | 8    |
| 1.2.3 Strategy II plants .....                                                                       | 12   |
| 1.2.4 Fe redistribution with plants .....                                                            | 14   |
| 1.2.5 Iron storage mechanisms in plants .....                                                        | 15   |
| 1.2.6 The intercellular and intracellular transport of Fe .....                                      | 16   |
| 1.2.7 Transgenic approaches to improve Fe content in edible plant parts .....                        | 19   |
| 1.3 Bananas and their origin .....                                                                   | 20   |
| 1.3.1 Importance of bananas to Uganda's economy .....                                                | 21   |

|                                         |                                                                 |           |
|-----------------------------------------|-----------------------------------------------------------------|-----------|
| 1.3.2                                   | Banana nutritional characteristics .....                        | 22        |
| 1.3.3                                   | Rationale for banana biofortification .....                     | 22        |
| 1.3.4                                   | Strategies for improving bananas for increased Fe content ..... | 23        |
| 1.3.5                                   | Banana transformation and regeneration .....                    | 23        |
| 1.4                                     | Project aims .....                                              | 25        |
| <b>CHAPTER 2: GENERAL METHODS .....</b> |                                                                 | <b>26</b> |
| 2.1                                     | General Methods for sampling.....                               | 26        |
| 2.1.1                                   | Soil sampling.....                                              | 26        |
| 2.1.2                                   | Banana fruit sampling.....                                      | 26        |
| 2.1.3                                   | Banana leaf sampling.....                                       | 26        |
| 2.2                                     | Soil sample analysis .....                                      | 27        |
| 2.2.1                                   | pH determination.....                                           | 27        |
| 2.2.2                                   | Soil texture determination .....                                | 27        |
| 2.2.3                                   | Soil iron determination .....                                   | 27        |
| 2.3                                     | Banana sample processing.....                                   | 28        |
| 2.3.1                                   | Banana leaf processing.....                                     | 28        |
| 2.3.2                                   | Banana fruit processing.....                                    | 28        |
| 2.3.3                                   | Banana leaf and fruit elemental analysis .....                  | 28        |
| 2.4                                     | General methods for nucleic acid purification from plants ..... | 31        |
| 2.4.1                                   | Overview .....                                                  | 31        |
| 2.4.2                                   | DNA extraction .....                                            | 31        |
| 2.4.3                                   | Purification of total RNA from banana leaf tissue.....          | 32        |
| 2.4.4                                   | cDNA synthesis .....                                            | 33        |
| 2.5                                     | General methods in nucleic acid amplification .....             | 33        |
| 2.5.1                                   | PCR amplification .....                                         | 33        |
| 2.5.2                                   | Agarose gel electrophoresis .....                               | 34        |
| 2.5.3                                   | Purification of DNA from agarose gels .....                     | 34        |
| 2.5.4                                   | Restriction enzyme digestion of DNA .....                       | 34        |
| 2.5.5                                   | Southern hybridisation analysis.....                            | 34        |

|                                                                                                                                        |           |
|----------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <b>CHAPTER 3: DETERMINING THE MINERAL CONTENT OF BANANA AND SOIL SAMPLES COLLECTED FROM DIFFERENT REGIONS OF UGANDA .....</b>          | <b>37</b> |
| 3.1 Introduction.....                                                                                                                  | 37        |
| 3.2 Materials and methods .....                                                                                                        | 39        |
| 3.2.1 Sample collection sites and sampling.....                                                                                        | 39        |
| 3.2.2 Soil characterisation.....                                                                                                       | 40        |
| 3.2.3 Banana leaf and fruit analysis.....                                                                                              | 40        |
| 3.3 Results .....                                                                                                                      | 42        |
| 3.3.1 Analysis of soil properties .....                                                                                                | 42        |
| 3.3.2 Mineral content of cooking bananas.....                                                                                          | 43        |
| 3.3.3 Mineral content of dessert bananas .....                                                                                         | 44        |
| 3.3.4 Investigating the relationships between fruit mineral content and environmental properties .....                                 | 47        |
| 3.3.5 Biochemical analysis of banana leaf tissue .....                                                                                 | 50        |
| 3.4 Discussion .....                                                                                                                   | 63        |
| 3.4.1 Introduction.....                                                                                                                | 63        |
| 3.4.2 Status of fruit mineral content .....                                                                                            | 63        |
| 3.4.3 Contribution of environmental factors (external factors) to fruit mineral content .....                                          | 65        |
| 3.4.4 Contribution of banana leaves mineral (internal factors) to fruit mineral content.....                                           | 66        |
| 3.4.5 Conclusion: Future directions and summary statement .....                                                                        | 68        |
| <b>CHAPTER 4: MOLECULAR AND BIOCHEMICAL CHARACTERISATION OF TRANSGENIC BANANAS CONTAINING IRON UPTAKE AND STORAGE TRANSGENES .....</b> | <b>70</b> |
| 4.1 Introduction.....                                                                                                                  | 70        |
| 4.2 Materials andMethods .....                                                                                                         | 72        |
| 4.2.1 Production of transgenic plants .....                                                                                            | 72        |
| 4.2.2 Molecular analysis of transgenic plants.....                                                                                     | 73        |
| 4.2.3 RT-PCR analysis.....                                                                                                             | 74        |
| 4.2.4 Southern hybridisation .....                                                                                                     | 75        |
| 4.2.5 Banana leaf and fruit analysis.....                                                                                              | 75        |
| 4.3 Results .....                                                                                                                      | 75        |

|                                                                                                                                    |                                                                                                                                      |           |
|------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|-----------|
| 4.3.1                                                                                                                              | PCR screening of transgenic lines.....                                                                                               | 75        |
| 4.3.2                                                                                                                              | RT-PCR analysis.....                                                                                                                 | 77        |
| 4.3.3                                                                                                                              | Southern analysis of transgenic lines transformed with Exp-Sfer, BT4-Sfer, 35S-FRO2 and 35S-IRT1 transgenes.....                     | 78        |
| 4.3.4                                                                                                                              | Biochemical analysis of transgenic banana lines.....                                                                                 | 78        |
| 4.3.5                                                                                                                              | Phenotypic characteristics of transgenic banana plants under field conditions .....                                                  | 85        |
| 4.4                                                                                                                                | Discussion.....                                                                                                                      | 88        |
| 4.4.1                                                                                                                              | Introduction .....                                                                                                                   | 88        |
| 4.4.2                                                                                                                              | Effect of transgenes on mineral accumulation in banana fruit transformed with the banana ferritin gene .....                         | 88        |
| 4.4.3                                                                                                                              | Effect of transgenes on mineral accumulation in banana fruits transformed with IRT1 and FRO2 transgenes using the 35S promoter ..... | 90        |
| 4.4.4                                                                                                                              | Effect of transgenes BT4-Sfer and Exp1-Sfer on mineral accumulation in banana leaf tissues .....                                     | 90        |
| 4.4.5                                                                                                                              | Effect of transgenes 35S-IRT1 and 35S-FRO2 on mineral accumulation in banana leaf tissues .....                                      | 91        |
| 4.4.6                                                                                                                              | Conclusion .....                                                                                                                     | 93        |
| <b>CHAPTER 5: MOLECULAR AND BIOCHEMICAL CHARACTERISATION OF TRANSGENIC BANANAS CONTAINING IRON REDISTRIBUTION TRANSGENES .....</b> |                                                                                                                                      | <b>95</b> |
| 5.1                                                                                                                                | Introduction.....                                                                                                                    | 95        |
| 5.2                                                                                                                                | Materials and Methods .....                                                                                                          | 96        |
| 5.2.1                                                                                                                              | Production of transgenic plants.....                                                                                                 | 96        |
| 5.2.2                                                                                                                              | Glass house acclimatization .....                                                                                                    | 97        |
| 5.2.3                                                                                                                              | Fe uptake screening assay.....                                                                                                       | 97        |
| 5.2.4                                                                                                                              | Molecular analysis of transgenic plants .....                                                                                        | 98        |
| 5.2.4.1                                                                                                                            | Genomic DNA isolation and PCR analysis .....                                                                                         | 98        |
| 5.2.5                                                                                                                              | RT-PCR analysis.....                                                                                                                 | 99        |
| 5.2.6                                                                                                                              | Southern hybridisation.....                                                                                                          | 99        |
| 5.2.7                                                                                                                              | Banana leaf and fruit analysis .....                                                                                                 | 99        |
| 5.3                                                                                                                                | Results.....                                                                                                                         | 100       |
| 5.3.1                                                                                                                              | PCR screening of transgenic lines.....                                                                                               | 100       |
| 5.3.2                                                                                                                              | RT-PCR analysis.....                                                                                                                 | 101       |

|                                                                                                                                                 |                                                                                                                                        |            |
|-------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------|------------|
| 5.3.3                                                                                                                                           | Southern analysis of transgenic lines transformed with <i>Ubi-FEA1</i> , <i>Ubi-OsNAS2+YSL2</i> and <i>Ubi-OsNAS1</i> transgenes ..... | 102        |
| 5.3.4                                                                                                                                           | Biochemical analysis of transgenic banana lines .....                                                                                  | 103        |
| 5.3.5                                                                                                                                           | Effects of transgenes on phenotypic characteristics of bananas .....                                                                   | 108        |
| 5.4                                                                                                                                             | Discussion .....                                                                                                                       | 109        |
| 5.4.1                                                                                                                                           | Effect of <i>FEA1</i> and <i>OsNAS1</i> transgenes on Fe and Zn accumulation in banana fruit .....                                     | 109        |
| 5.4.2                                                                                                                                           | Effect of transgenes on mineral accumulation in banana leaf under glasshouse conditions.....                                           | 110        |
| 5.4.3                                                                                                                                           | Conclusion.....                                                                                                                        | 112        |
| <b>CHAPTER 6: GENERAL DISCUSSION .....</b>                                                                                                      |                                                                                                                                        | <b>114</b> |
| <b>BIBLIOGRAPHY .....</b>                                                                                                                       |                                                                                                                                        | <b>121</b> |
| <b>APPENDICES .....</b>                                                                                                                         |                                                                                                                                        | <b>140</b> |
| Appendix I: Analysis of potential heavy metal contamination in banana fruit samples analysed by ICP-OES in Chapter 3.....                       |                                                                                                                                        | 140        |
| Appendix I: Continued .....                                                                                                                     |                                                                                                                                        | 141        |
| Appendix II: Analysis of potential heavy metal contamination in banana fruit samples analysed by ICP-OES in Chapter 4 (Figure 4.5 and 4.6)..... |                                                                                                                                        | 142        |
| Appendix III: Analysis of potential heavy metal contamination in banana fruits samples analysed by ICP-OES in Chapter 5.....                    |                                                                                                                                        | 143        |

# List of Figures

---

|                                                                                                                                      |    |
|--------------------------------------------------------------------------------------------------------------------------------------|----|
| Figure 1.1: Reduction-based strategy for iron uptake in the model plant <i>Arabidopsis thaliana</i> .....                            | 10 |
| Figure 1.2: Chelation-based strategy for iron uptake in graminaceous monocots.....                                                   | 14 |
| Figure 3.1: Map of Uganda showing sample collection.....                                                                             | 39 |
| Figure 3.2: Banana leaves at different stages of development .....                                                                   | 41 |
| Figure 3.3: Banana fruit processing procedure prior to mineral analysis.....                                                         | 42 |
| Figure 3.4: Mineral content of fruit from cooking banana cultivars from selected Ugandan districts.....                              | 45 |
| Figure 3.5: Mineral content of fruit from dessert banana cultivars from selected Ugandan districts.....                              | 46 |
| Figure 3.6: PCA plot of different cooking banana cultivars from different districts of Uganda.....                                   | 48 |
| Figure 3.7: Clustering analysis of cooking bananas from different districts of Uganda. ....                                          | 49 |
| Figure 3.8: Mineral content of banana leaves from Nakitembe cultivar during plant development .....                                  | 52 |
| Figure 3.9: Mineral content of banana leaves from <i>Mpologoma</i> cultivar during plant development. ....                           | 53 |
| Figure 3.10: Mineral content of banana leaves from M9 cultivar during plant development. ....                                        | 55 |
| Figure 3.11: Mineral content of banana leaves from Dwarf Cavendish cultivar during plant development .....                           | 58 |
| Figure 3.12: Mineral content of banana leaves from Kivuvu (Bluggoe) cultivar during plant development .....                          | 61 |
| Figure 4.1: Schematic representation of binary expression vectors used for transformation of banana.....                             | 73 |
| Figure 4.2: PCR analysis of genomic DNA from different transgenic and non-transgenic banana plants using gene specific primers. .... | 76 |
| Figure 4.3: RT-PCR analysis of transgenic banana lines to determine gene expression. ....                                            | 77 |
| Figure 4.4: Southern blot analysis on selected transgenic banana lines.....                                                          | 78 |
| Figure 4.5: Mineral content of transgenic Nakinyika lines. ....                                                                      | 79 |
| Figure 4.6: Mineral content of transgenic Sukali Ndiizi lines.....                                                                   | 80 |
| Figure 4.7: Mineral content of banana leaves cultivar Nakinyika transformed with BT4-Sfer during development. ....                   | 82 |
| Figure 4.8: Mineral content of transgenic banana leaves cultivar Sukali Ndiizi during plant development. ....                        | 84 |
| Figure 4.9: Phenotype analysis of selected transgenic banana lines.....                                                              | 85 |

|                                                                                                                                                 |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Figure 5.1:</b> Schematic representation of binary expression vectors used for transformation of banana. ....                                | 98  |
| <b>Figure 5.2:</b> PCR screening of representative transgenic banana lines for the presence of each respective gene of interest. ....           | 101 |
| <b>Figure 5.3:</b> RT-PCR analysis of representative transgenic banana lines to determine whether the integrated transgenes are expressed. .... | 102 |
| <b>Figure 5.4:</b> Southern blot analysis on selected banana lines transformed with different transgenes. ....                                  | 103 |
| <b>Figure 5.5:</b> Analysis of fruit mineral content in transgenic Cavendish banana lines. ....                                                 | 104 |
| <b>Figure 5.6:</b> Mineral content of transgenic banana leaves transformed with Ubi-FEA1 and Ubi-OsNAS1 transgenes. ....                        | 106 |
| <b>Figure 5.7:</b> Mineral content of transgenic banana leaves transformed with Ubi-OsNAS2+Ubi-YSL2 transgenes. ....                            | 107 |
| <b>Figure 5.8:</b> Effect of transgenes on the banana phenotype. ....                                                                           | 108 |

# List of Tables

---

|                                                                                         |     |
|-----------------------------------------------------------------------------------------|-----|
| Table 2.1: Instrumental conditions of the ICP OES. ....                                 | 30  |
| Table 2.2: Optical emission lines used to analyse elements of interest.....             | 30  |
| Table 3.1: Selected districts for banana sample collection and analysis in Uganda ..... | 40  |
| Table 3.2: Soil properties of different districts .....                                 | 43  |
| Table 4.1: Description of plant expression vectors used in the study .....              | 73  |
| Table 4.2: List of primers used for PCR analysis of transgenic banana plants .....      | 74  |
| Table 4.3: List of primers used for RT-PCR analysis of transgenic banana plants .....   | 74  |
| Table 4.4: PCR screening results summary.....                                           | 75  |
| Table 4.5: Field phenotypic expression of transgenic banana lines.....                  | 87  |
| Table 4.6: Post flowering phenotypic expression observed among transgenic banana .....  | 87  |
| Table 5.1: Description of plant expression vectors used in this study .....             | 97  |
| Table 5.2: List of primers used for PCR analysis of transgenic banana plants .....      | 98  |
| Table 5.3: List of primers used for RT-PCR analysis of transgenic banana plants .....   | 99  |
| Table 5.4: PCR screening results summary.....                                           | 100 |

# List of Abbreviations

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| Abbreviation      | Description                                                                |
|-------------------|----------------------------------------------------------------------------|
| Al <sup>3+</sup>  | Aluminium                                                                  |
| ANOVA             | Analysis of variance                                                       |
| AP                | Anti-dioxygen polyamide                                                    |
| <i>A.thaliana</i> | <i>Arabidopsis thaliana</i>                                                |
| bHLH              | Basic helix loop helix                                                     |
| bp                | Base pair                                                                  |
| <i>AtFRO</i>      | <i>Arabidopsis Ferric chelate reductase oxidase</i>                        |
| Cd <sup>2+</sup>  | Cadmium                                                                    |
| cDNA              | Complementary deoxyribonucleic acid                                        |
| CTAB              | Cetyltriethylammonium bromide                                              |
| CTCB              | Center for Tropical Crop Biocommodities                                    |
| Cu <sup>2+</sup>  | Copper                                                                     |
| DEPC              | Diethylpyrocarbonate                                                       |
| DMA               | Deoxymugineic acid                                                         |
| DMAS              | Deoxymugineic acid synthase                                                |
| DMSO              | Dimethyl sulfoxide                                                         |
| DNA               | Deoxyribonucleic acid                                                      |
| DTT               | Dithiothreitol                                                             |
| EAHB              | East African Highland Bananas                                              |
| EDTA              | Ethylene-diamine tetraacetic acid                                          |
| Fe <sup>2+</sup>  | Iron                                                                       |
| FEA1              | Algae iron assimilatory gene                                               |
| FeSO <sub>4</sub> | Ferrous sulphate                                                           |
| Fe-EDDHA          | Ethylene diamine-N,N bis(2 hydroxyphenylacetic acid) Ferric sodium complex |
| Fe-EDTA           | Iron ethylene-diamine tetraacetic acid                                     |
| FIT               | Fe deficiency induced transcription factor                                 |
| FRO2              | Ferric chelate reductase oxidase 2                                         |
| FRD3              | Ferric Reductase Deficient 3 proteins                                      |
| gDNA              | Genomic deoxyribonucleic acid                                              |
| g                 | Gram/s                                                                     |
| HIV               | Human immunodeficiency virus                                               |
| ICP-OES           | Inductively coupled plasma optical emission spectroscopy                   |
| IDA               | Iron deficiency anaemia                                                    |
| IRT1              | Iron regulated transporter 1                                               |
| kbp               | Kilo base pair                                                             |
| LN <sub>2</sub>   | Liquid nitrogen                                                            |
| MA                | Mugineic acid                                                              |
| Ubi               | Maize polyubiquitin                                                        |

|                    |                                                    |
|--------------------|----------------------------------------------------|
| Min                | Minutes                                            |
| Mn <sup>2+</sup>   | Manganese                                          |
| MOH                | Ministry of Health                                 |
| mqH <sub>2</sub> O | Milli-Q water (purified to 18.2 MΩ·cm resistivity) |
| NA                 | Nicotianamine                                      |
| NAS                | Nicotianamine synthase                             |
| NARO               | National Agricultural Research Organisation        |
| Ni <sup>2+</sup>   | Nickel                                             |
| <i>nptII</i>       | Neomycin phosphotransferase                        |
| <i>NRAMP</i>       | Natural Resistance Associated Macrophage Protein   |
| <i>OsNAS</i>       | <i>Oryza sativa</i> Nicotianamine synthase         |
| <i>OsVIT</i>       | <i>Oryza sativa</i> Vacuolar Iron Transporter      |
| pCambia            | binary plasmid vector-pCambia                      |
| PCR                | Polymerase chain reaction                          |
| pDNA               | Plasmid deoxyribonucleic acid                      |
| PS                 | Phytosiderophores                                  |
| <i>Ps</i>          | <i>Pisum sativum</i>                               |
| <i>PsFRO</i>       | <i>Pea</i> Ferric chelate reductase oxidase        |
| PVP                | Polyvinylpyrrolidone                               |
| QUT                | Queensland University of Technology                |
| RDA                | Recommended daily allowance                        |
| RNA                | Ribonucleic acid                                   |
| RT                 | Reverse transcriptase                              |
| RT-PCR             | Reverse transcription polymerase chain reaction    |
| TAE                | Tris-acetate EDTA                                  |
| <i>Taq</i>         | <i>Thermus aquaticus</i>                           |
| TE                 | Tris-ethylene-diamine tetraacetic acid             |
| UBOS               | Uganda bureau of statistics                        |
| UDHS               | Uganda demographic health survey                   |
| VIT                | <i>Vacuolar Iron Transporter</i>                   |
| WHO                | World Health Organisation                          |
| YSL                | <i>Yellow stripe-like</i>                          |
| Zn <sup>2+</sup>   | Zinc                                               |

## Statement of Original Authorship

---

I, Moses Matovu, do hereby declare that "Molecular and biochemical analysis of transgenic bananas containing Fe uptake and storage enhancement genes" is original and has not been submitted to any other University or Institution for degree award. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: \_\_\_\_\_



Date: \_\_\_\_\_

17/03/12

# Acknowledgements

---

I wish to thank my supervisor Distinguished Professor James Dale for guidance, encouragement and selecting me during the project and editorial advice on this thesis. James you have been patient with me during the tough project times of my Candidature. I am very grateful for all the things you have done.

My special thank you goes to Prof Wilberforce Tushemereirwe, Dr Harjeet Khanna, Dr Geoffrey Arinaitwe, Dr Jerome Kubiriba, and Dr Priver Namanya for the excellent support to enable me to be a PhD student at NARO and CTCB, QUT and to complete this project.

I would like to express my sincere thank you to my mentor and principal supervisor Dr Bulukani Mlalazi for your willingness to help and offer excellent assistance during my PhD project.

Special thanks must go to Dani, JY, Betty, Steven, Anthony James, Phong from the CTCB; Shane, Aarshi and Dr Sunny Hu from CARF for technical support; Joseph, Betty, Ruth, Wilson, Florence, Richard and Abdullah from NARO.

My sincere thanks go to my student colleagues Isaac, Mary, Steven, Betty, Grace, Brinin, Bernard, Jimmy, and many others at CTCB labs whose names are not here, Daniela, Maiko, Di and Banana research groups for their support and good friendship.

I thank the Director General NARO Dr Ambrose Agona for allowing me time off to undertake my PhD studies.

I would like to thank the Bill and Melinda Gates Foundation for their generosity in funding the Banana 21 project which has supported my PhD candidature.

Many thanks to Lyndon Palmer from WAITE analytical labs (Adelaide) for your generous support and for sharing your knowledge on micronutrient analysis.

I am very grateful to my family and friends for their long term support and love. To my wife Rayan, children Menisha, Rahma and Areeb, my brother Idris and sisters and parents for your unconditional support without you this PhD would not have been possible. Allahamed Lillah.

# Chapter 1: Literature review

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## 1.1 MICRONUTRIENT MALNUTRITION

Globally, malnutrition and other nutrition-related deficiencies are responsible for more deaths than any other cause and accounting for over 20 million mortalities annually (Bhullar and Gruissem, 2013). Micronutrient malnutrition alone affects more than two billion people, the majority of whom are from resource-poor populations in developing countries, with iron (Fe), iodine (I), zinc (Zn), and vitamin A deficiencies being most prevalent (Kennedy et al., 2003). Humans require at least 49 nutrients to meet their daily metabolic needs (Welch and Graham, 2004). Some are required in large amounts whilst others, such as Fe, Zn, copper (Cu), iodine (I) and selenium (Se), are required in trace amounts given their cytotoxic effects at higher concentrations (White and Broadley, 2005; Kennedy et al., 2003). Micronutrient deficiencies have several socio-economic consequences such as elevated mortality and morbidity; impaired growth, development and learning ability in infants and children; and loss of work capacity in adults (Zhao and Shewry, 2011). Furthermore, even mild levels of micronutrient malnutrition may damage cognitive development, decrease disease resistance in children and reduce the likelihood that mothers survive childbirth (Bhullar and Gruissem, 2013).

### 1.1.1 Dietary Fe: sources and functions in the human body

Fe is not only essential for plant growth and development, but it also plays an integral role in human and animal health. Its ability to readily gain and lose electrons has made it a cofactor for enzymes involved in a wide variety of oxidation-reduction reactions critical for basic metabolic functions in humans (Hentze et al., 2004; Curie and Briat, 2003). It is required for cellular functions such as respiration and photosynthesis. For example, Fe is a vital component of blood in the form of haemoglobin which accounts for approximately 2 g of Fe in an average adult while a small amount is stored in circulating storage proteins such as transferrin (Gómez-Galera et al., 2010).

Broadly, dietary Fe is obtained as either haem or non-haem Fe. Haem Fe is usually found in meat, milk, eggs and fish products, while non-haem Fe is found in cereals, pulses, fruits and vegetables and is a major contributor to the diet in the developing countries. (López and Martos, 2004). Although Fe is an essential nutrient, consumption in excess amounts could be a potential health risk. Moreover, the damage caused by excess Fe is thought to be

modulated *via* free radical production since *in vitro* free Fe is a potent generator via the Fenton reaction (Frossard et al., 2000). Free Fe can react with oxygen and generate reactive oxygen species such as superoxides which are destructive to cells (Curie and Briat, 2003). In order to supply sufficient Fe for cell metabolic functions while avoiding toxicity, multicellular organisms have evolved highly regulated mechanisms for Fe homeostasis during its uptake, partitioning to various organs and storage (White and Broadley, 2009). Assuming a normal balanced diet, the recommended dietary allowance (RDA) for Fe is 10 mg/day for children and lactating mothers, 8 mg/day for adult males, 18 mg/day for adult females and 27 mg/day for pregnant women (Trumbo et al., 2001).

### 1.1.2 Iron deficiency and associated health implications

Dietary Fe has been identified as an essential nutrient that is often lacking in human diets, either due to insufficient intake or to poor absorption. Haem Fe is primarily derived from the haemoglobin and myoglobin found in meat while non-haem Fe is derived from both plant foods and animal tissues (Theil and Briat, 2004). Haem Fe represents 30 to 70% of lean meat Fe content and is usually well absorbed (Frossard et al., 2000; López and Martos, 2004) while non-haem Fe absorption is highly dependent on the presence of enhancing or inhibitory substances in an individual's meal (Frossard et al., 2000; Valderrama-Cháirez et al., 2002). The main Fe and Zn absorption enhancing substances include ascorbic acid and  $\beta$ -carotene from fruits and vegetables (White and Broadley, 2009; Hurrell and Egli, 2010) whilst cysteine-rich polypeptides from plant and animal sources promote the absorption of Zn and Cu in addition to Fe (White and Broadley, 2009; Frossard et al., 2000). In plant-based diets, phytates and polyphenols play a major role in Fe absorption inhibition (Hurrell and Egli, 2010). Phytates accumulate in the aleurone and embryo cells of cereals and legume seeds making non-haem Fe absorption from plants difficult (Zhao and Shewry, 2011). Prebiotics, indigestible carbohydrates used as food ingredients, have been reported to promote mineral absorption in animal models and in humans (Bouis and Welch, 2010; Hurrell and Egli, 2010). Fructans, including inulin, improve systemic absorption and utilization of micronutrients through promotion of beneficial microbiota in the hind gut of humans (Abrams et al., 2005; Abrams et al., 2007), with the potential added benefit that these bacteria metabolise nutrient absorption inhibitors such as phytates and polyphenols.

Dietary Fe absorption occurs in two steps: absorption from the intestinal lumen into mucosal cells, and transfer from mucosal cells to the plasma where the Fe is bound to storage proteins such as ferritin and hemosiderin (Hurrell and Egli, 2010). This process is regulated by a complex enzyme-mediated homeostatic mechanism and is strongly

influenced by dietary components that bind Fe in the intestinal lumen (McKie et al., 2001). Generally, the amount of dietary Fe absorbed depends on the type of meal an individual consumes. Overall, haem Fe represents 10 to 15% of dietary Fe intake in high meat consuming individuals while non-haem Fe absorption is in the region of 1 to 20% (Frossard et al., 2000).

Anaemia, the lack of sufficient Fe in blood, is characterised by a low level of haemoglobin in the blood, and decreases the amount of oxygen reaching the tissues and organs of the body, thus reducing their capacity to function (Goto and Yoshihara, 2001). Although there are many causes of anaemia, inadequate intake of Fe, folate, vitamin B12, or other nutrients usually accounts for the majority of cases in many populations. This may be caused by low consumption of Fe-rich foods such as meat, fish, or poultry, as is the case in poor communities (Bhargava et al., 2001). In Uganda, malaria also accounts for a significant proportion of anaemia in children under five in malaria endemic areas (UDHS, 2011). In addition, other causes of anaemia include thalassemia, sickle cell disease, and intestinal worm infestation (UDHS, 2006). Severe anaemia increases both morbidity and mortality of mothers and children at child birth and decreases resistance to infection (White and Broadley, 2005; Frossard et al., 2000; Poletti et al., 2004).

Iron deficiency anaemia (IDA) is a leading nutritional disorder that affects both developed and developing countries (WHO 2011). However, comparatively, the prevalence of IDA in developing countries has been reported at four times higher (WHO, 2001). Nutritional Fe deficiency arises when physiological requirements cannot be met by dietary Fe absorption (Zimmermann and Hurrell, 2007) either due to an absolute low Fe dietary intake or in diets with low proportions of haem. The prevalence of Fe deficiency and IDA is influenced by age, gender, diet, physiological and pathological factors (Zhao and Shewry, 2011). Worldwide, IDA has been associated with serious socioeconomic consequences such as increased morbidity and mortality, particularly among women and children; impaired growth, cognitive and psychomotor development in infants and children; and loss of work capacity in adults (Bhullar and Gruissem, 2013; Kennedy et al., 2003; Zhao and Shewry, 2011).

Peak prevalence of IDA in young children occurs at approximately 18 months of age and then falls as Fe requirements decline (Black et al., 2008). Women of childbearing age are at high risk of Fe deficiency because of blood loss during menstruation and the substantial Fe demands of pregnancy (Brock et al., 1994; Shamah-Levy et al., 2003). In Uganda, anaemia has been reported to be an underlying cause of maternal mortality, spontaneous abortion,

premature birth, and low birth weight (UDHS, 2011). Further, just under half of all women are anaemic (49%), with 35% mildly anaemic, 13% moderately anaemic, and 1% severely anaemic (UDHS, 2006).

#### **1.1.3 Traditional strategies to combat micronutrient deficiencies**

Micronutrient malnutrition can be avoided in human populations through several strategies. The most established methods are: supplementation with pharmaceutical preparations, food fortification and dietary diversification (Poletti et al., 2004).

Supplementation and fortification of foods with Fe has been successful in industrialized countries (Poletti et al., 2004). Fe supplementation has also been ongoing in developing countries for rapid improvement of Fe status in deficient individuals, but this strategy is relatively expensive and often has poor compliance, particularly with Fe because of unpleasant effects on food taste and colour (Frossard et al., 2000). Food fortification with Fe is difficult because of technical problems related to the choice of Fe compounds. Unfortunately, the relatively highly bioavailable Fe compounds such as ferrous sulphate, often cause unacceptable colour and flavour changes in fortified foods (Bhullar and Gruissem, 2013; Frossard et al., 2000). In contrast, the organoleptically inert forms of Fe, such as elemental Fe, are usually poorly absorbed (Bouis, 2003; Hurrell and Egli, 2010). However, scalable flame aerosol technology for the production of nano-sized Fe and Fe/Zn compounds may be a solution. Based on rat feeding experiment, these compounds were as bioavailable as  $\text{FeSO}_4$ , but caused less colour change in reactive food matrices than conventional Fe compounds (Hilty et al., 2010).

Cost and benefit economic analysis of Fe supplementation and fortification, in terms of cost per saved life and productivity gained, has shown fortification to be more effective than supplementation (Hunt, 2002). However, although both approaches are aimed at addressing micronutrient malnutrition, they have not been successful in developing countries due to logistical limitations. Many such programs are also disadvantaged due to their short term and donor-dependent nature. As a result, their success as well as long term impact has been constrained by lack of adequate and sustainable funding to support continuity. In addition, political instability hinders implementation in some countries.

For example, Uganda is amongst countries where there are very high levels of anaemia among children, women of reproductive age (15 to 49 years) and pregnant women (UDHS 2011). Therefore, the National Anaemia Policy of Uganda mandates that, if the prevalence of IDA is at least 40%, children under five years of age should receive appropriate

supplementary Fe tablets per day depending on their age. However, only 5.5% of the children below five years are reported to receive Fe supplements (UBOS, 2006). The effective implementation of this policy is hindered by inefficiencies in the health system such as supply constraints, distribution logistics, performance of health workers and lack of awareness among pregnant women especially in rural communities (Mwadime, 2001).

Food diversification, with an emphasis on the consumption of foods crops that are already Fe-rich, like green leafy vegetables and legumes is a another option (Schuler and Bauer, 2012). This is because simple diets with low diversification are the main cause of micronutrient deficiency (Nair and Iyengar, 2009). The structure of agriculture, the green revolution and need to supply sufficient food to rapidly increase the population has made food diversification impossible in developing countries for both economic and social reasons (Schuler and Bauer, 2012). Thus, the current strategies for combating Fe deficiency have encountered limited success. Further, although dietary diversification and use of fortified foods is desirable, these are usually expensive and therefore often unavailable to communities whose primary daily dietary nutrient source is a staple food (Zhu et al., 2007), and for whom the purchase of fortified foods is considered a luxury. All these issues taken together make such approaches unreliable in the long term.

#### **1.1.4 Soil Fe, amendments as a strategy to meet the challenge of micronutrient malnutrition**

Although Fe is assumed to be abundant in soils and sediments, its phytoavailability is usually low mainly because soil Fe occurs most often as insoluble hydroxides, oxy-hydroxide and oxide polymers, which are very stable with low solubility in aerobic environments with pH between neutral and alkaline (Sperotto et al., 2012; Lemanceau et al., 2009). In rice, findings have shown that increased Fe uptake leads to increased growth and seed yield, but does not affect Fe loading to the seeds. In contrast, however, excessive Fe availability as found in waterlogged conditions, leads to Fe toxicity, resulting in yield reduction due to stress (Sahrawat, 2005).

Several soil amendment strategies are being applied to counteract the problem of Fe deficiency in soils (Frossard et al., 2000). However, the application of fertilizers containing Fe compounds, such as  $\text{FeSO}_4$ , has generally shown a very low efficiency in increasing crop yield (Frossard et al., 2000). This has been attributed to the rapid and strong binding of the added Fe to soil constituents (Frossard et al., 2000). Soil application of Fe chelates such as Fe-EDDHA for calcareous soils or Fe-EDTA for acidic soils, have shown a better efficiency than simple Fe salts. However, these compounds are expensive and are not always

successful (Curie and Briat, 2003). Application of foliar  $\text{FeSO}_4$  or Fe chelates, enables direct uptake of Fe by plants through cuticular pores located around the guard cells present on the leaf surface (Marschner, 1996). Foliar fertilization has been reported to efficiently correct Fe chlorosis in various plants such as beans, sorghum, peanuts and fruit crops. This approach has a greater impact on overall crop yield than increasing the Fe content of the grain. As such, this might be the only available fertilization practice that can increase the Fe content of grains (Rengel et al., 1999). Fe fertilization has been reported to be even more complicated, as Fe has a stronger tendency towards insolubility, unless used in large quantities (Shuman, 1998). Therefore, the use of other methods that regulate Fe homeostasis is required to ensure adequate uptake to achieve efficient growth and yield, proper translocation to sink tissues, and to prevent accumulation in organs where Fe enhancement would either be unnecessary or detrimental.

#### **1.1.5 Biofortification as a strategy to meet the challenge of micronutrient malnutrition**

In recent years, efforts using biotechnology techniques have focused on the production of staple crops with improved essential nutrient content through breeding, a process known as biofortification. Due to the magnitude of micronutrient malnutrition and the existing limitations in its control, research towards production of crops with improved nutrient content are an attractive option to explore. To this end, biofortification of staple crops through plant breeding offers a great opportunity to create a product that has comprehensive benefits for both the producers and consumers, even in underdeveloped countries (Bouis et al., 2011). This strategy offers a long term, affordable option and is expected to have positive effects on human health (Goto and Yoshihara, 2001; Bouis, 2003). Therefore, micronutrient-enriched foods could reach larger numbers of people than nutrient supplements or fortified foods, and would also be less expensive (Bouis, 2003; Poletti et al., 2004). Moreover, increasing the concentration of bioavailable elements in edible portions before harvesting could be a more efficient and cost effective solution to combat IDA (Sperotto et al., 2012). Hence, there is considerable interest in breeding mineral-efficient crops that produce high yields and accumulate essential minerals from infertile soils.

Improvement of nutrient content through conventional breeding exploits the genetic variation that exists within crops and related species to introduce new alleles through cross breeding. This has great potential as its success takes advantage of the benefits of the green revolution, where the rate of increased production of grains such as rice, wheat and

maize in developing countries overtook population growth such that the prices of rice reduced by 30 to 40% (Bouis, 2003). In order to solve the malnutrition challenge, several international organizations within the Consultative Group on International Agricultural Research (CGIAR) have embarked on programs to study the genetic variation of micronutrient content and feasibility of breeding programs for nutrient enhancement. Conventional breeding uses inherent traits of a crop and its sexually compatible relatives. This has a far reaching impact on communities and has fewer regulatory constraints compared to genetically modified varieties (Zhu et al., 2007). However, for staple crops such as bananas where cultivated varieties are vegetatively propagated and are infertile, cross breeding is a limited resource. According to the early banana breeding reports, the development of hybrid bananas by a conventional breeding approach is hindered by long cropping cycles, complex genetic constitution and the low female fertility of most banana cultivars, thereby limiting the successful application of classical breeding methods (Ortiz et al., 1995; Vuylsteke, 1996). As a result, for such crops, genetic engineering is an invaluable alternative and a complimentary solution to conventional breeding efforts. In addition, genetic engineering has the advantage that characterised traits can be directly introduced into elite varieties and the process is not limited by the existing gene pool of a specific crop (Zhu et al., 2007).

## **1.2 IRON METABOLISM IN PLANTS**

Transgenic approaches to mineral biofortification involve improving mobilisation from the soil, uptake from the rhizosphere, translocation to the shoot and accumulation of bioavailable mineral forms in edible tissues (White and Broadley, 2005). These efforts are mainly focused on Fe and Zn due to their high nutritional significance. For example, Fe uptake and tolerance of Fe-deficient soils can be improved in non-graminaceous plants by over expressing genes encoding Fe (III) reductases (Boardman and McGuire, 1990), and in graminaceous plants by increasing the synthesis and exudation of phytosiderophores (Frossard et al., 2000).

### **1.2.1 Iron uptake in plants**

Nutrient availability to a crop can be defined as the quantity of the nutrient that can it can assimilate from the soil during a growing season (Frossard et al., 2000). Plants take up most of their nutrients from the aqueous soil solution in the form of free ions (e.g.  $\text{Fe}^{2+}$ ) *via* the roots. The availability of these nutrients to the plant is a function of soil physicochemical and biological properties, which govern their release from the solid phase of the soil into solution, and of the plant properties that govern nutrient uptake (Briat and Lobréaux,

1997). The availability of a mineral to a plant depends on its speciation in the soil, where the mineral can be present in many different physicochemical forms. Minerals in soil solution can be in a free ionic form which is totally available to the plant. Alternatively, the mineral may be present: in solution as a chelate, adsorbed onto mineral or organic surfaces, as precipitates, in lattice structures, or as part of the live or dead soil biomass (Colangelo and Guerinot, 2006). These forms are either partly available or completely unavailable to plants. The most important soil properties governing mineral availability are: soil pH, redox conditions, cation exchange capacity, microorganism activity, soil structure and water content (Frossard et al., 2000). Among these, soil pH can vary in agricultural soils from 4.0 to 9.0, thus exerting a substantial control over the free ion concentration in soil solution.

Plants play a key role in the food chain by mining minerals from the soil. Minerals enter the plant cells through transport proteins located in the plasma membrane of the cell. Membranes contain different types of transport proteins such as ATPases (ATP-powered pumps), channel proteins, and co-transporters (Jeong and Connolly, 2009). In plant cells, H<sup>+</sup>-ATPases pump protons across the plasma membrane generating the proton motive force responsible for ion uptake. Channel proteins facilitate the diffusion of water and ions down energetically favourable gradients. Co-transporters can move solutes either up or down gradients (Guerinot, 2009). However, these processes alone are insufficient to meet the critical needs for plant growth processes. Therefore, Fe uptake by plants requires a preliminary step, either chelation or acidification in the rhizosphere (Briat et al., 2007). Two major strategies are used by plants to acquire Fe via their roots. These mechanisms are termed either "Strategy I" for dicotyledonous and non-graminaceous monocotyledonous plants or "Strategy II" for graminaceous species (Vansuyt et al., 2000; Jeong and Connolly, 2009).

### 1.2.2 Strategy I plants

#### 1.2.2.1 Overview

Dicotyledonous plants, such as fruit trees, pea (*Pisum sativum*) and tomato (*Solanum lycopersicum*), as well as non-graminaceous monocots, employ a reduction-based strategy (Ivanov et al., 2012). In response to Fe deficiency, Strategy I plants acidify the rhizosphere through proton extrusion, increasing Fe (III)-chelate solubility and concomitant reduction by a ferric reductase (Robinson et al., 1999; Jeong and Connolly, 2009). Under Fe-deficient conditions, these plants acidify the soil through activation of a specific plasma membrane

H<sup>+</sup>-ATPase of the root epidermal cells, potentially encoded by the *AHA2* gene in *Arabidopsis* (Curie and Briat, 2003; Ivanov et al., 2012; Grotz and Gueriot, 2006). This acidification lowers the pH of the rhizosphere, thereby increasing the amount of free soluble Fe(III), and also establishes an electrochemical gradient that provides the driving force for the transport of Fe into the root plasma membrane *via* Fe<sup>2+</sup> transporters (Grotz and Gueriot, 2002; Marschner and Römheld, 1994) (Figure 1.1). The resulting ferrous Fe is then transported inside the roots, likely by ferrous transporters of the IRT family (Eide et al., 1996; Vert et al., 2002).

Strategy I plants can also show an enhanced release of reducing and chelating compounds such as, phenolics, from the roots into the soil (Marschner and Römheld, 1994). Under Fe deficiency conditions, changes in root morphology and anatomy can also occur, such as the formation of transfer cell-like structures (Frossard et al., 2000). These transfer cells are presumably sites with enhanced net excretion of protons and reducing capacity, as well as enhanced release of phenolic compounds (Frossard et al., 2000). More than one type of reductase in the root-cell plasma membrane is capable of transferring electrons from the cytosol to Fe<sup>3+</sup>. This increases the reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> at the outer surface of the plasma membrane which in turn correlates with enhanced uptake of Fe and other cations (Welch et al., 1993). The reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> by ferric chelate reductase is thought to be an obligatory step in Fe uptake as well as the primary factor in making Fe available for absorption by all Strategy I plants (Luqing Zheng, 2010; Yi and Gueriot, 1996).

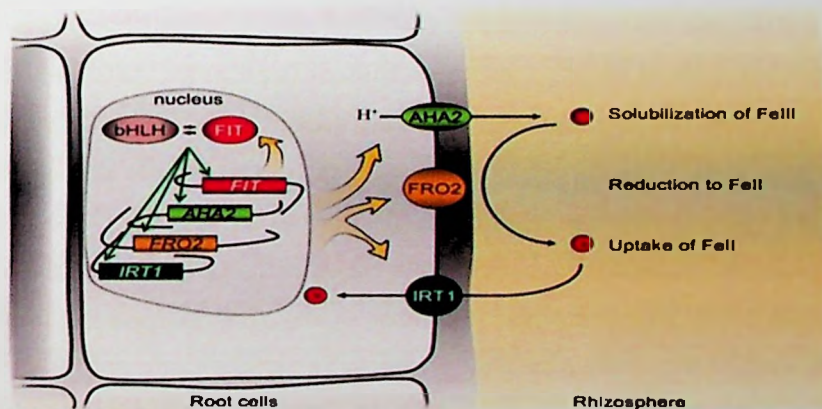
#### 1.2.2.2 *FRO*

Strategy I plants induce the expression of ferric chelate reductase (FRO) enzymes in addition to rhizosphere acidification under Fe limiting conditions (Connolly et al., 2003; Gueriot, 2007a). FRO enzymes act by reducing chelated ferric Fe (Fe<sup>3+</sup>) into soluble Fe (Fe<sup>2+</sup>) at the surface of root hairs, particularly when Fe is limiting (Colangelo and Gueriot, 2006; Gueriot, 2007b). After Fe<sup>3+</sup> reduction, Fe<sup>2+</sup> is absorbed across the plasma membrane of root cells bound to an Fe<sup>2+</sup> transport protein (Connolly et al., 2003).

The reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> by *Arabidopsis* FRO2 (*AtFRO2*) has shown to be an obligatory step in Fe absorption by Strategy I plants (Frossard et al., 2000). The *AtFRO2* gene encodes an Fe deficiency-inducible ferric chelate reductase (Robinson et al., 1999) which has intramembranous binding sites for haem and cytoplasmic binding sites for nucleotide cofactors that donate and transfer electrons. *AtFRO2* encodes a 725-amino acid protein with eight putative transmembrane domains and shares similarity with both the human phagocytic

NADPH oxidoreductase gp91phox subunit and yeast ferric chelate reductases (Curie and Briat, 2003). The function of *AtFRO2* in reducing ferric Fe has been demonstrated in *Arabidopsis* (Robinson et al., 1999). However, the function of the other seven *Arabidopsis* *FRO* gene family members has not been determined.

The pea *FRO1* (*PsFRO1*) gene encodes a protein that is 55% identical to *AtFRO2* (Schagerlöff et al., 2006). Based on a correlation established between *PsFRO1* mRNA abundance and root ferric reductase activity, *PsFRO1* is thought to represent the pea reductase involved in root Fe acquisition (Schagerlöff et al., 2006). In contrast to *AtFRO2*, *PsFRO1* gene plays an additional role in Fe distribution throughout the plant because it is expressed in both root and shoot (Curie and Briat, 2003). However, although *FRO2* is primarily expressed in the outer layer of the root cells in response to Fe deficiency in the soil (Connolly et al., 2003), other members of the *FRO* family are expressed in other plant parts. For example, *AtFRO6*, *AtFRO7* and *AtFRO8* are expressed in green tissues, while *AtFRO3* is induced by Fe limitation both in shoots and roots and is limited to the vascular cylinder (Jeong and Connolly, 2009). This therefore suggests an additional role in uptake and distribution of Fe within the plants.



**Figure 1.1:** Reduction-based strategy for iron uptake in the model plant *Arabidopsis thaliana*. Iron is first solubilized by rhizosphere acidification through the action of the H<sup>+</sup>-ATPase AHA2, and is then reduced from ferric ( $Fe^{3+}$ ) to ferrous ( $Fe^{2+}$ ) iron by the reductase *FRO2*. Bivalent iron is then imported into the root cell by the metal transporter *IRT1*. The activity of this uptake system is dependent on the action of the transcription factor *FIT*. Under iron deficiency, up-regulation of the iron-responsive genes is achieved through a complex including *FIT* and at least one of the two *bHLH* proteins. The *FIT* gene is induced by this system and thus it undergoes a feed-forward regulation, where the gene product positively regulates the source gene. Induction of *FRO2* and *IRT1* activity is co-regulated in response to iron deficiency, while that of *AHA2* seems to be regulated in an independent manner. Adopted from Ivanov et al. (2012).

### 1.2.2.3 *IRT*

Expression of an *Arabidopsis* cDNA library in the *Saccharomyces cerevisiae* *fet3fet4* double mutant strain, impaired in both low and high affinity Fe transport, enabled cloning of a cDNA encoding a putative *Arabidopsis* Fe<sup>3+</sup> transporter named Iron regulated transporter 1 (*IRT1*) (Eide et al., 1996).

*IRT1* encodes a 347 amino acid polypeptide and is a member of the ZIP family. This protein contains eight putative transmembrane domains and is related to eukaryotic metal ion transporters in rice, yeast, nematodes, and humans, (Guerinot, 2000). Biochemical studies with *IRT1* indicated that it functions as a metal transporter with a broad substrate range (Korshunova et al., 1999). However, additional findings suggested that *IRT1* gene is exclusively expressed in the root epidermis and it is a plasma membrane localized protein which shows that this transporter functions in Fe uptake from the soil (Henriques et al., 2002). In *Arabidopsis*, *IRT1* is expressed in the roots and is induced by Fe deficiency. It also exhibits altered regulation in plant lines containing mutations that affect the Fe uptake system (Frossard et al., 2000). The *Arabidopsis IRT1* takes up Fe, as well as other divalent metals, from the soil upon Fe deficiency (Vert et al., 2002).

*IRT1* plays a major role in regulation of plant Fe homeostasis, as was observed by the severe chlorosis and lethality of an *irt1-1* knockout mutant (Vert et al., 2002; Henriques et al., 2002). The *IRT1* gene is highly expressed in Fe starved root peripheral cell layers such as the epidermis and underlying cortex (Vert et al., 2002). This allows proper growth and development under Fe-limited conditions (Henriques et al., 2002). The *IRT1* protein localizes to early endosomal compartments but cycles with plasma membrane and traffics to the vacuole for constant turnover (Barberon et al., 2011). Despite the importance of *IRT1*, other transporters may also play a role in Fe uptake (Curie and Briat, 2003). For instance, *IRT2*, an *Arabidopsis* gene, belonging to the ZIP family and closely related to *IRT1*, encodes a protein for selective transport of Fe and Zn, but not Mn and Cd, when expressed in yeast (Vert et al., 2002).

### 1.2.2.4 *Regulation of strategy 1*

During Fe deficiency strong *IRT1* gene expression is induced in the root peripheral cell layers via the bHLH transcription factor Fe-Deficiency-Induced Transcription Factor (FIT), which is a positive regulator of the Fe deficiency response (Colangelo and Guerinot, 2004). Interestingly, expression of *FRO2* and *IRT1* is tightly co-regulated both in response to the Fe supply and diurnally, this suggests that they are physically controlled by the same set of

regulators (Vert et al., 2003). However, the expression pattern of *IRT1* (Colangelo and Guerinot, 2004) suggests that another factor might be able to activate this gene in absence of *FIT*. Interestingly, a similar scenario might be applicable in the case of *IRT1*, where transgenic plants expressing *IRT1* mRNA under the control of a ubiquitous viral promoter were unable under Fe-deficient conditions, to produce IRT1 protein in any plant tissue except the root (Connolly et al., 2002). Additionally, findings have shown that in the weak *fit-1* mutant, despite the relatively high *IRT1* expression under low Fe availability, IRT1 protein was not detectable. This has led to a proposal that FIT regulates a factor that is required for the stability of IRT1 (Colangelo and Guerinot, 2004). Furthermore, evidence for post-translational control of IRT1 is its rapid disappearance upon resupply of Fe (Connolly et al., 2002). IRT1 protein is targeted to the plasma membrane, suggesting a function as a metal importer while IRT2 is localised in intracellular compartments (Vert et al., 2009), suggesting a sequential role of the two genes in the transport of Fe in the cell.

### 1.2.3 Strategy II plants

#### 1.2.3.1 Overview

Graminaceous plants acquire Fe using a unique mechanism known as Strategy II, secreting Fe-chelating compounds into the rhizosphere when the plants sense Fe deficiency (Marschner and Römheld, 1994). These Fe-chelators are known as mugineic-acid (MA) family phytosiderophores (Mizuno et al., 2003), and Strategy II plants take up Fe (III)-MA complexes through specific transporters (Inoue et al., 2008). MAs dissolve Fe in the rhizosphere, followed by reabsorption of the Fe (III)-MA complexes through Yellow Stripe1 (YS1) transporters in the plasma membrane (Curie et al., 2009). The gene encoding the transporter was first cloned from the maize yellow stripe1 (*ys1*) mutant (Curie et al., 2009), which has defective Fe (III)-MA uptake (von Wirén et al., 1996). YS1 does only transports metal-MA complexes but also metal-nicotianamine (NA) complexes (Schaaf et al., 2004). Non-graminaceous plant YS1 transporters play important roles in metal homeostasis by transporting metal-NA complexes because these plants synthesise NA but not MAs (Colangelo and Guerinot, 2006). Although Fe is mainly present as oxidised  $\text{Fe}^{3+}$  compounds, poorly soluble in neutral to alkaline soil, chelation of MAs to Fe(III) dramatically increases the solubility of  $\text{Fe}^{3+}$  in the rhizosphere, making graminaceous plants capable of taking up Fe as Fe (III)-MA complexes (Douchkov et al., 2005). Biosynthesis and secretion of MAs markedly increase in roots in response to Fe deficiency (Takagi et al., 1984). The biosynthetic pathway of MAs in graminaceous plants has been identified through extensive biochemical and physiological studies (Ma and Nomoto, 1993; Ma et al., 1999). Methionine

is the precursor of MAs (Higuchi et al., 1994) and is known as strategy II i.e. the secretion of Fe-chelating is adenosylated by S-adenosylmethionine (SAM) synthetase (Takizawa et al., 1996) (Figure 1.2).

#### 1.2.3.2 *NAS*

Nicotianamine synthase (NAS) catalyses the trimerisation of S-adenosylmethionine (SAM) to NA (Higuchi et al., 1994). NA, is a chelator of Fe and other heavy metals that play a key role in Fe uptake, phloem transport and cytoplasmic distribution and ensures Fe solubility in the weakly alkaline environment of the cytoplasm (Hell and Stephan, 2003; Takahashi et al., 2003). Manipulation of cellular NA is another promising approach to increase Fe concentration *in planta* (Douchkov et al., 2005). The cloning of *NAS* genes (Ling et al., 1999; Higuchi et al., 1994) provides new tools for the modulation of endogenous NA concentrations in plant tissue. *NAS* genes were first isolated from barley (HvNAS1–7) through enzyme purification from Fe-deficient barley roots. Subsequently, *NAS* genes were also isolated from rice (OsNAS1–3; (Higuchi et al., 2001) and maize (ZmNAS1–3; (Mizuno et al., 2003).

#### 1.2.3.3 *DMAS*

Deoxymugineic acid synthase (DMAS) reduces the 300-keto form of NA to 2'-deoxymugineic acid (DMA) (Bashir et al., 2006). All the MAs share their biosynthetic pathway from methionine to DMA, which is then hydroxylated to form other MAs in barley (*Hordeum vulgare* L.) by the Fe-deficiency induced (*IDS*) dioxygenase genes *IDS2* and *IDS3* (Kobayashi et al., 2001). Various researchers have reported that rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* L.) and maize (*Zea mays* L.) secrete only small amounts of DMA, and thus are susceptible to low-Fe availability (Higuchi et al., 1994; Lee et al., 2009; Bashir et al., 2006). However, barley secretes large amounts of other MAs in addition to DMA under Fe deficiency, such as MA and 3-epihydroxymugineic acid, and is therefore more tolerant to Fe deficiency than other graminaceous plants (Nakanishi et al., 1993; Kanazawa et al., 1994; Ma et al., 1999).

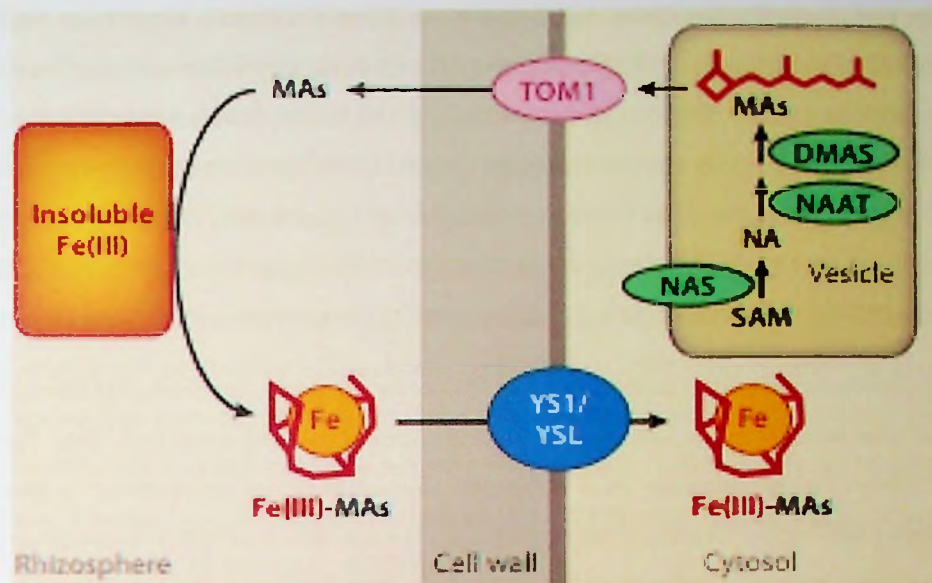


Figure 1.2: Chelation-based strategy for iron uptake in gramminacious monocots. Ovals represent the transporters and enzymes that play central roles in this strategy, all of which are induced in response to Fe deficiency. Abbreviations: DMAS, deoxymugineic acid synthase; MAs, mugineic acid family phytosiderophores; NA, nicotianamine; NAAT, nicotianamine aminotransferase; NAS, nicotianamine synthase; SAM, S-adenosyl-L-methionine; TOM1, transporter of mugineic acid family phytosiderophores 1; YS1/YSL, YELLOW STRIPE 1/YELLOW STRIPE 1-like. Adopted from Kobayashi and Nishizawa (2012).

#### 1.2.4 Fe redistribution with plants

During mineral uptake by the cells of the root tissues, minerals are transferred to the xylem vessels for translocation to the shoots (Briat et al., 1995). They are then transported in aqueous solution, either as free ions or complexed to low molecular weight organic molecules and their transport in xylem vessels is due to the transport of water, which is directed from the roots to the shoots and which ends in the leaf blades, where the water is released to the surrounding atmosphere by transpiration (Curie and Briat, 2003; Briat et al., 1995). The supply of minerals from roots to transpiring leaves is therefore high, as long as transpiration is high and acquisition by the roots is sufficient (Curie and Briat, 2003).

Transport of nutrients in the phloem follows the transport of photosynthetic carbohydrates, which is directed from photosynthetic leaves to plant tissues where carbohydrates are either consumed or stored for later use (Briat et al., 2007). Phloem transportation of minerals depends on the capacity of phloem loading. Phloem loading of Fe, which is in the form of Fe-NA complexes, is limited by the availability of this Fe-chelator rather than by the presence of Fe (Grusak, 1994). Fe and Zn are easily transported in the

phloem and may readily move from xylem to phloem vessels, suggesting that the distribution of Fe and Zn in plants is some-what flexible. However, the supply of Fe and Zn to the growing tissues requires a continuous uptake by the roots. Remobilisation and transfer of Fe and Zn from well supplied older tissues to deficient growing tissues does not occur before senescence of the older tissues is induced (Curie and Briat, 2003). Therefore, symptoms of Fe and Zn deficiency are first apparent in young growing leaves but these disappear during subsequent growth when the minerals become sufficient.

Homeostatic processes that control mineral uptake by the roots, translocation through the plant, and deposition in the various plant tissues appear to be strongly regulated (Grusak, 1999). Therefore, an increase in a nutrient's uptake may not necessarily enhance its content in edible tissues to the same degree. This is especially true for Fe transport where, for example, the levels of Fe chelates or phytoferritin are tightly balanced to minimise accumulation of toxic Fe at all points within the plant system (Bashir et al., 2010).

#### 1.2.5 Iron storage mechanisms in plants

Iron storage in plants takes place in the apoplastic space, in vacuoles and in ferritins (Briat and Lobréaux, 1997).

##### 1.2.5.1 *Ferritin*

Ferritins constitute a broad superfamily of Fe storage proteins, found in all organisms except yeast (Arosio et al., 2009). Three sub-classes of these proteins have been defined: haem-free ferritins present in pro- and eukaryotes; haem-containing bacterioferritins, found only in bacteria; and DNA binding proteins from starved cells (miniferritins), present only in prokaryotes (Briat et al., 2010).

Plant ferritins are located in the plastids (Harrison and Arosio, 1996), and leaves are a major sink for plant Fe storage, with 80% of Fe localised in chloroplasts (Theil and Briat, 2004). Most plant ferritins reside in non-green plastids (Theil and Briat, 2004), and Fe changes during leaf development as ferritin levels increase in developing leaves (Briat and Lobréaux, 1997). Ferritins have also been observed in the mitochondria, in similar fashion to some animal ferritins (Zancani et al., 2004).

Ferritin also occurs in other tissues such as cotyledons, roots and shoot apices, cell vessels, in vascular cambium, reproductive cells and senescing cells (Ravet et al., 2009b). In seeds, ferritins are widely believed to be the major Fe-storage form, where they provide Fe to Fe-containing proteins after germination. These ferritins are therefore essential for correct building of the photosynthetic apparatus (Lobreaux and Briat, 1991). In leaves, it is

hypothesized that ferritin, is an Fe source at early stages of development, for the synthesis of Fe-containing proteins involved in photosynthesis, thus indicating that ferritin synthesis in leaves is developmentally controlled (Briat and Lobréaux, 1997). Research reports show that ferritin degrades during germination (Theil and Hase, 1993), which suggested that post-transcriptional control is an important regulator of ferritin synthesis during plant development (Theil and Hase, 1993). When stored in ferritin, Fe cannot react with oxygen, suggesting an additional function of protecting cells against oxidative stress, in addition to both short and long term Fe homeostasis (Briat et al., 2007). This function of protecting cells against free Fe-induced oxidative stress has been demonstrated in *Arabidopsis* where the loss of function of four *Arabidopsis* ferritin genes (*Atfer* 1-4) not only led to high levels of reactive oxygen species but also increased the activity of enzymes that are involved in their detoxification (Ravet et al., 2009b).

#### 1.2.5.2 *Vacuolar iron transporter (VIT)*

Vacuolar sequestration is another important mechanism used in regulation of Fe homeostasis, and this has been reported to serve as a safe Fe storage strategy. Zhang et al. (2012b) isolated and characterized two rice vacuolar Fe transporters (VIT), *Oryza sativa* VIT1 (*OsVIT1*) and *Oryza sativa* VIT2 (*OsVIT2*), that are orthologs of the *Arabidopsis* VIT1 (*AtVIT1*). These transporters mobilise divalent elements like Fe, Zn and Mn. In rice they are highly expressed in the flag leaf blade and sheath respectively. Disruption of *OsVIT1* and *OsVIT2* led to increased Fe and Zn accumulation in rice seeds and a corresponding decrease in the source organ (flag leaves).

*AtVIT1* is highly expressed in developing seeds where it mediates vacuolar sequestration of iron ( $\text{Fe}^{2+}$ ), and manganese ( $\text{Mn}^{2+}$ ) but not cadmium ( $\text{Cd}^{2+}$ ) (Kim et al., 2006). However, *AtVIT1* affects the spatial Fe localisation rather than the level of its accumulation (Kim et al., 2006). These observations suggest that VIT orthologs may regulate various physiological processes depending on the organisms they originate from (Zhang et al., 2012a). Overall, this may indicate the possibility of enhanced Fe and Zn translocation between source and sink organs as a novel strategy that can have potential to be utilized in biofortification of Fe and Zn in staple foods like banana.

#### 1.2.6 **The intercellular and intracellular transport of Fe**

Fe redistribution, both between the plant organs and at the sub-cellular level has been identified as a crucial step for its proper storage and utilisation (Ivanov et al., 2012). Fe can be chelated and transported as Fe-NA and Fe-citrate complexes by transporters, such as the

Yellow Stripe-Like (YSL) family (Conte and Walker, 2011) and Ferric Reductase Deficient 3 (FRD3) proteins (Green and Rogers, 2004; Durrett et al., 2007) respectively, or as free Fe by various divalent metal transporters (Jeong and Connolly, 2009). When Fe has been transported across the plasma membrane of the epidermal cells in roots, several members of different transporter families have been implicated in the intracellular and intercellular transport of Fe, including the NRAMP (Natural Resistance-Associated Macrophage Protein) and the YSL (*Yellow Stripe-Like*) protein families (Grotz and Guerinot, 2006). The natural resistance amplified macrophage protein (NRAMP) family transports divalent cations and the YSL family members likely transport metal chelates.

#### 1.2.6.1 *Natural Resistance Associated Macrophage Protein (NRAMP)*

The NRAMP family is an evolutionarily conserved, ubiquitous metal transporter family, that is characterised in mammals and mediates the transport of metal ions into the cytoplasm and is responsible for dietary Fe absorption (Forbes and Gros, 2001). Plant NRAMP proteins were predicated to be ion transporters based on their sequence and structural resemblance to the NRAMP in organisms such as humans and yeast (Socha and Guerinot, 2014). These genes can also mediate the transport of a broad range of metals in addition to  $\text{Fe}^{2+}$ , such as  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Al}^{3+}$  (Thomine et al., 2000; Nevo and Nelson, 2006; Xia et al., 2010). Researchers have characterised NRAMP proteins in a number of plant species including *Solanum lycopersicum*, *Glycine max*, *Malus baccata*, *Thlaspi japonica* and *Thlaspi caerulescens* (Bereczky et al., 2003; Kaiser et al., 2003; Mizuno et al., 2005; Oomen et al., 2009). NRAMP transporter proteins identified in *Arabidopsis thaliana* and *Oryza sativa*, are six and seven respectively (Socha and Guerinot, 2014).

In *Planta*, *AtNRAMP1*, *AtNRAMP3* and *AtNRAMP4* mRNAs are reported to accumulate in response to Fe deficiency (Grotz and Guerinot, 2006). *AtNRAMP1* expression has been reported in roots (Grotz and Guerinot, 2006), and is also believed to be a high affinity Mn transporter in root due to the transcriptional up-regulation of *AtNRAMP1* under Mn deficiency (Cailliatte et al., 2010). *AtNRAMP1* over expression increases plant resistance to toxic Fe concentrations indicating a role in Fe distribution rather than uptake (Curie et al., 2000), and also greater resistance to Mn deficiency (Socha and Guerinot, 2014). *AtNRAMP1* has a plastid targeting sequence, and plastids are Fe storage sites in plants (Terry and Abadia, 1986). Therefore, *AtNRAMP1* may transport excess Fe into plastids to prevent toxicity.

Expression of *AtNRAMP3* and *AtNRAMP4* is believed to be restricted to the vascular system of the roots and shoots of *Arabidopsis* (Thomine et al., 2003; Lanquar et al., 2005), and localised to the vacuolar membrane (Lanquar et al., 2005). Therefore, these genes may be important for metal remobilisation from the vacuole in *planta* (Thomine et al., 2003). Both *AtNRAMP3* and *AtNRAMP4* transporters may be functionally redundant (Lanquar et al., 2005) as only *Atnramp3*, *nramp4* double mutants exhibit a strong phenotype during Fe deficiency (Socha and Guerinot, 2014); (Grotz and Guerinot, 2006). Therefore, this may indicate an inability of the double mutants to mobilize Fe stored within the vacuole early in development (Grotz and Guerinot, 2006). Further, both *AtNRAMP3* and *AtNRAMP4* are considered necessary for Fe remobilisation during early germination due to their high expression levels during this growth stage (Lanquar et al., 2005).

#### 1.2.6.2 *Yellow stripe like (YSL) family*

The YSL transporters belong to a family of oligopeptide transporters, which can transport amino acid containing compounds and their derivatives (Yen et al., 2001), are common only to plants, bacteria, fungi and archaea (Yen et al., 2001). In general, YSL proteins mediate cellular uptake of metals complexed to non-proteinogenic amino acids: phytosiderophores (PS) or their biosynthetic precursor NA (Yen et al., 2001). In graminaceous plants (grasses), PS and NA are mainly used in metal uptake and translocation (Palmer and Guerinot, 2009). While in the non-graminaceous plants (such as *A. thaliana*), PS are not produced (Socha and Guerinot, 2014). Therefore, YSLs in non-grasses probably use NA for intercellular and intracellular metal transport (Bashir et al., 2011)

In rice, 18 YSL genes have been identified (Koike et al., 2004; Divol et al., 2013; Conte et al., 2013). Many of these genes are said to be expressed in both the roots and shoots, namely *OsYSL6*, *OsYSL14*, *OsYSL16*, while others are expressed preferentially in the shoots such as *OsYSL13* and *OsYSL2* (Grotz and Guerinot, 2006; Koike et al., 2004). *OsYSL2* has been identified as a plasma membrane protein, and in *Xenopus*, *OsYSL2* has been reported to mediate the transport of Fe(II)-NA and Mn(II)-NA but not, Fe(III)-DMA or Mn(II)-DMA (Koike et al., 2004). *OsYSL2* mRNA increases more in Fe-deficient roots than in Fe-sufficient roots. Further, *OsYSL2* is expressed in the companion cells of the phloem suggesting a role for *OsYSL2* as a transporter of Fe and Mn in the phloem (Koike et al., 2004; Grotz and Guerinot, 2006).

In *Arabidopsis*, the YSL family consist of eight members which are thought to be metal-NA transporters (Curie et al., 2001). YSL1 from *A. thaliana* (AtYS1) has been linked to loading of

Fe and NA into *Arabidopsis* seed (Jean et al., 2005). However, AtYSL1 may also transport metals such as  $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Ni^{2+}$  and  $Cd^{2+}$  (Schaaf et al., 2004). It is thought that YSL proteins transport metal-NA complexes (Ishimaru et al., 2010), however, the identity of the AtYSL1 substrate was not identified in uptake experiments with yeast or *Xenopus laevis* oocytes (DiDonato et al., 2004).

### 1.2.7 Transgenic approaches to improve Fe content in edible plant parts

Transgenic approaches for improvement of micronutrient content and availability in crops focuses mainly on two most common mineral deficiencies, Fe and Zn (Poletti and Sautter, 2005). These approaches aim at improving the plant's ability to acquire Fe from the soil, and to transport and store it in edible parts. As discussed earlier, the proteins involved in the process of Fe uptake, transport and storage in plants have been identified and isolated. In addition, the molecular mechanisms affecting the accumulation of Fe in plants have been studied widely (Grusak, 1999). Therefore, several reports have been published rescinding the use of transgenic approaches based on application of this knowledge to increase Fe and Zn content in different plants.

Expression of the soybean ferritin gene has been used to increase Fe content in rice seed (Goto et al., 1999), tobacco leaves (Goto et al., 1998; Van Wuytswinkel et al., 1999) and in lettuce, where Mn content was also increased (Goto et al., 2000). Lucca et al. (2001), reported a two-fold increase in Fe content in the endosperm of Japonica rice using French bean ferritin under the control of the glutelin promoter. In addition, Vasconcelos et al. (2003), through over-expression of the soybean ferritin gene driven by the endosperm specific glutelin promoter, in Indica rice grains, reported an increase of both Fe and Zn concentration in brown grain as well as in polished grain.

Poletti and Sautter (2005), suggested that another approach to improve micronutrient content was to modify the uptake and transport pathways of micronutrients. Even if trace minerals are abundant in the soil, only a small amount is taken up by the plants and available for accumulation in storage organs. Therefore, a more efficient uptake and transport of trace elements would increase the presence of these elements in plants and also their storage in sink organs. Transgenic barley expressing an *Arabidopsis* zinc transporter (AtZIP1), showed higher Fe and Zn content than the wild type, although seeds from transgenic lines were significantly smaller than seeds from non-transformed plants.

Interestingly, over expression of a nicotianamine synthase (AtNAS1) also resulted in an increase in shoot Fe, Zn and Mn concentrations in transgenic tobacco (Klatte et al., 2009). While Johnson et al. (2011), through over-expression of *OsNAS1*, *OsNAS2* and *OsNAS3*, reported an increase in NA, Fe and Zn concentrations in unpolished rice of the three over expressed gene populations relative to the wild types, with the highest concentration in the *OsNAS2* and *OsNAS3* populations. Furthermore, Fe in polished grain from the *OsNAS2* over expression showed a four-fold increase relative to wild types while a two-fold increase in Zn content was also reported in the same lines. This therefore, shows that *OsNAS* genes, particularly *OsNAS2* have enormous potential for Fe and Zn biofortification in plants.

Masuda et al. (2012) and Wirth et al. (2009) reported the combination of three transgenic approaches that have been used to produce Fe biofortified rice: by enhancing Fe storage in grains *via* expression of ferritin using endosperm-specific promoters, enhancing Fe translocation through overproduction of NA, and enhancing Fe flux into endosperm by means of *OsYSL2* expression under the control of an endosperm-specific promoter and sucrose transporter promoter. Both the glasshouse and field experiments showed a six-fold and four-fold higher Fe levels respectively in polished rice seeds with no growth defects and 1.6 times Zn accumulation. However, despite using *HvNAS1* and *OsNAS2* genes in rice Fe biofortification, not much added advantage was observed compared with the earlier work of Johnson et al. (2011).

### 1.3 BANANAS AND THEIR ORIGIN

Bananas belong to the family *Musaceae*, within the order Zingiberales, subclass Liopsida division *Magnoliophyta* with two genera; *Musa* and *Ensete* (Stover and Simmonds, 1987). The genus *Musa* is comprised of all the edible bananas and plantains with over 50 species in five sections namely *Eumusa*, *Australimusa*, *Rhodachlamys*, *Callimusa* and *Ingentimusa*. Amongst these, *Eumusa* is the biggest and geographically most widely distributed section of the genus giving rise to the great majority of edible bananas worldwide (Stover and Simmonds, 1987). Almost all of the 300 or more cultivars arose from two seeded diploid species, *Musa acuminata* Colla and *M. balbisiana* Colla. These are diploid, triploid and tetraploid hybrids of these genomes (Ploetz et al., 2003) and the edible bananas are classified into different genomic groups: AA, AB, BB, AAA, AAB, AAAA, AAAB, and ABBB (Bakry et al., 2009). The botanical classification based on agro-morphological variations helps in the differentiation of land races of dessert bananas (AA, AAA, AAB), cooking bananas (AAA, AAB, ABB), and plantain cooking bananas (AAB). However, more complete

morpho-descriptors or molecular markers have led to classification of genomic subgroups for triploid bananas (Bakry et al., 2009). Bananas have developed various economic uses based on their different morphological characteristics.

### 1.3.1 Importance of bananas to Uganda's economy

In Uganda, bananas are one of the most important food sources. Moreover, most producers are small scale farmers growing the crop for both home consumption and sale at local markets (Tushemereirwe et al., 2001). The locally consumed bananas are of vital importance to food security for millions of people (Frison and Sharrock, 1998). In Africa, bananas provide food for approximately 70 million people, and are a staple for about 20 million people in the East African Highlands. This region alone produces nearly 15 million tonnes annually (Frison and Sharrock, 1998). Uganda has the highest per capita consumption of bananas in the world with a rate of 243 kg per capita per year (BARNESA, 2008). Further bananas are locally referred to as 'matooke' and are eaten daily, with the crop gathering great cultural and social significance (Frison and Sharrock, 1998). At present, banana utilisation in Uganda is largely limited to the fresh form with only rudimentary efforts to process the crop for storage. Four types are recognized according to use namely: cooking, dessert, juice and beer and roasting.

The East African Highland bananas (EAHBs, AAA) are important varieties of cooking banana (Karugaba and Kimaru, 1999; Tushemereirwe et al., 2001). They are harvested green, peeled and boiled or wrapped in banana leaves and steamed, before being prepared for consumption with sauce (Karugaba and Kimaru, 1999). The fruit of dessert bananas varieties are eaten raw when ripe, and have a very good aroma and flavour which remain relatively firm when ripe. The most popular cultivars are *Bogoya* and *Sukali Ndiizi* (Karugaba and Kimaru, 1999). Juice and beer banana fruit are harvested green, ripened and processed into juice, which can further be processed into a local beer popularly known as *tonto* (Gensi et al., 1994; Aked and Kyamuhangire, 1996). These cultivars include the East African Highland bananas which are mutants of the cooking bananas, *Kayinja*, *Kivuvu* and *Kisubi* varieties with the ABB, AB, AAA-EA genome groups, respectively (Karugaba and Kimaru, 1999). Roasting banana varieties, called *Gonja* (AAB), are popularly referred to as plantains. In Uganda, these bananas are often eaten when roasted after ripening but they may also be boiled or deep fried (Aked and Kyamuhangire, 1996). Plantain bananas have potential to be the most promising variety for banana value addition in terms of crisps because of the firm nature of the fruit pulp when ripe (Nowakunda et al., 2000).

### **1.3.2 Banana nutritional characteristics**

In general, bananas contain 23% carbohydrate, 1% protein and 0.3% fat and provide approximately 116 Kcal of energy per 100g of flesh (Chandler, 1995). Bananas are rich in other nutrients particularly potassium (3580 µg/g), vitamin C (8.7 mg/100g), and vitamin B in form of thiamine, riboflavin, niacin and pyridoxine (Morton, 1987). Available data for micronutrient content indicates that vitamin and mineral concentrations in various bananas may vary depending on the genotypes, geographical location of the fruit sampled, sampling procedure, sample processing, sample size and method of estimation (Hardisson et al., 2001b; Wall, 2006). For example, Fe content in Cavendish collected from three Hawaiian sites estimated Fe content in the range 0.62 to 1.0 mg/100 g of fresh weight (Wall, 2006), indicating that these particular bananas are a poor source of Fe. Bananas biofortification with Fe and Zn will provide additional benefits to consumers based on the different applications and cultivar diversity that will enhance the bananas to be utilised fully with additional nutritional benefits to end users.

### **1.3.3 Rationale for banana biofortification**

In regions such as Uganda, many banana cultivars in use have been selected overtime and have evolved deep cultural norms and values within communities such that farmers and consumers have entrenched food preferences that extend to specific varieties that are already integrated into the local farming system.

According to Carswell (2003), introduction of a new banana crop to a community that has been farming a specific crop is usually met with resistance, especially within subsistence cropping systems like those found in Uganda. Bananas, such as the East African highland bananas (EAHBs), besides being a staple crop are regarded as cash crops. Therefore, rather than introducing new crops, the nutrient quality of staple diets can be improved by increasing micronutrients in edible plant parts through biofortification of banana. A biofortified staple crop would enable the daily delivery of adequate micronutrients through a traditional diet without recurrent costs associated with strategies like supplementation, as well as minimising resistance from the recipients.

In light of this, banana biofortification through genetic engineering appears to be the most suitable option. Most popular cultivated varieties are essentially both male and female sterile making their conventional breeding impossible. Secondly, bananas are vegetatively propagated and therefore there is limited risk of gene flow into the environment. Advances

in banana transformation methods and the availability of a number of gene sequences, has made development of new banana varieties through transgenic approaches possible.

#### **1.3.4 Strategies for improving bananas for increased Fe content**

Since there are no reported Fe-rich banana varieties, Fe content improvement in banana fruit pulp can only rely on the application of biotechnological techniques. The existing knowledge of Fe metabolism and improvement of Fe content in a variety of crops (Chapter 1.2) have demonstrated the effectiveness of biofortification for mineral improvement in plants. This raises the possibility that such approaches can be applied to banana. Presently, several genes involved in Fe metabolism: FRO2, IRT1, OsNAS1, OsNAS2, FEA1 and plant ferritin, whose functions have been demonstrated in other crops, are also being tested in banana through genetic transformation. This work is being conducted by scientists at QUT using available gene and promoter sequences.

#### **1.3.5 Banana transformation and regeneration**

##### **1.3.5.1 Overview**

Genetic transformation in banana is important because the development of suitable biofortified bananas by conventional breeding is hampered by long generation times, various levels of ploidy, sterility of most edible EAHBs cultivars and lack of genetic variability (Bosque-Pérez et al., 1996; Vuylsteke, 1996; Sagi et al., 1998; Tripathi et al., 2005). The high degree of sterility and clonal mode of propagation mean that gene flow is a minor issue with this crop, making a transgenic approach even more appropriate (Tripathi et al., 2008). Genetic engineering of crops is a fast process (Vuylsteke, 1996), however, transformation and regeneration of true-to-type transgenic plants requires a well-established cell and tissue culture regeneration system. Since this is a difficult process, several methods have been developed to establish appropriate ways in generating cells from banana.

##### **1.3.5.2 Banana transformation techniques**

Different methods for gene delivery have been developed and successfully applied for the generation of transgenic plants. Techniques in routine use include: direct DNA transfer (Christou, 1992), *Agrobacterium*-mediated transformation (Hiei et al., 1994) and microprojectile bombardment (Christou, 1992).

Relative success in genetic engineering of banana has been achieved to enable the direct transfer of foreign genes into plant cells (Tripathi et al., 2005). Genetic transformation

using micro-projectile bombardment of embryogenic cell suspensions has been successfully applied to a wide range of banana cultivars (Becker et al., 2000; Sagi et al., 1998; Aquil et al., 2012). Genomic integration of a single, perfect transgene copy is most desirable for crop improvement. However, the majority of transgenic lines produced *via* micro-projectile bombardment have complex transgene loci composed of multiple copies of whole, truncated, and rearranged integrated sequences that are frequently organized as direct or inverted repeats (Svitashev et al., 2002). Therefore, *Agrobacterium*-mediated gene transfer is usually used to produce simpler integration patterns, fewer rearrangements within inserted transgenes, and reduced problems with transgene co-suppression and instability over generations compared to methods based on direct gene transfer (Trifonova et al., 2001). As such, *Agrobacterium*-mediated transformation of banana offers greater advantages over direct gene transfer methodologies such as particle bombardment and electroporation, (Ganapathi et al., 2001; Khanna et al., 2004; Maziah et al., 2007).

Previously, *Musa* spp. was generally regarded as recalcitrant for *Agrobacterium*-mediated transformation. However, compatibility was found between banana and *Agrobacterium tumefaciens* that lead to discovering of the potential of using this transformation technique (Pérez Hernández et al., 1999). Moreover, protocols for *Agrobacterium*-mediated transformation of banana embryogenic cell suspensions have since been developed (Ganapathi et al., 2001; Khanna et al., 2004).

#### 1.3.5.3 *Generating banana explants for transformation*

Currently, most of the transformation protocols for banana are based on cell suspension cultures (Ganapathi et al., 2001; Khanna et al., 2004; Becker et al., 2000; Sági et al., 1997). These embryogenic cell suspension cultures are established and regenerated through somatic embryogenesis from highly proliferating meristems (Sadik et al., 2010). This typically leads to reduction of transgene copy number, resulting in fewer problems relating to co-suppression and instability (Shibata and Liu, 2000). However, establishment of cell suspensions is lengthy and cultivar-dependent (Tripathi et al., 2005). This is a major barrier for transforming the majority of EAHB cultivars from cell suspensions (Namanya et al., 2004). In light of this, two methods using either apical meristems (Tripathi et al., 2005) or intercalary meristematic tissues (Tripathi et al., 2008) have been developed. These techniques have been reported to be applicable to a wide range of *Musa* cultivars irrespective of ploidy or genotype (Tripathi et al., 2008). The use of intercalary meristematic tissues relies on micro-propagation instead of disorganized cell cultures, which has the advantage of allowing regeneration of homogeneous populations of plants in a short period

of time (Tripathi et al., 2008). Transgenic banana plants generated for the current project in NARO and QUT have used cell suspensions and an *Agrobacterium*-mediated transformation protocol developed at QUT (Khanna et al., 2004).

#### 1.4 PROJECT AIMS

One of the major activities of the Bill and Melinda Gates Foundation-funded banana biofortification project at QUT and NARO is to generate banana lines that accumulate high bioavailable Fe levels in fruit tissue. Bananas as a staple crop in Uganda are reported to be deficient in Fe and Zn. The research contained in this thesis was aimed at producing transgenic bananas rich in Fe and Zn in order to alleviate the hidden hunger in Uganda and East Africa. This was achieved by testing various genes and promoters involved in Fe uptake, translocation and storage that have been generated and tested in both Australian and Ugandan banana genotypes under field and glasshouse conditions. The overall aim of this PhD project was to analyse, at both the molecular and biochemical level, transgenic banana lines containing Fe uptake, assimilation and storage enhancement genes. The objectives of this work were:

- Biochemical characterisation of a range of local banana cultivars from different districts to generate baseline data of soil attributes, and Fe and Zn content in fruit and leaf tissue.
- Molecular and biochemical characterisation of *Sukali Ndiizi* and *Nakinyika* banana cultivars transformed with the iron storage soybean ferritin (*Sfer*) gene and *Arabidopsis* iron uptake (*FRO2*, *IRT1*) genes under field conditions.
- Molecular and biochemical characterisation of Cavendish bananas transformed with iron chelator and transporter genes from rice (*OsNAS1*, *OsNAS2*, *OsYSL2*) and algae (*FEA1*) under field and glasshouse conditions.

## Chapter 2: General methods

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### 2.1 GENERAL METHODS FOR SAMPLING

#### 2.1.1 Soil sampling

Soil sampling was carried out in an area of approximately 0.25 acre in each Ugandan district under study, after sub division into three plots (replicates). In each plot, four sub-samples were collected in the 0 – 20 cm layer to form a composite sample. In the 20 – 40 cm layer, which presented greater uniformity, two sub-samples per plot were collected to form a composite sample. Soil sampling was carried out with a 10 cm open auger. The soil samples were air dried, finely ground, and passed through a 2 mm mesh sieve and stored at ambient temperature before analysis. The soil samples were analysed at the Soils and Agrometeorology department at the National Agricultural Research Laboratories in Uganda.

#### 2.1.2 Banana fruit sampling

In order to generate baseline data on Fe content in banana fruit the following cultivars: *Nakitembe* (AAA-EA), *Bogoya* (AAB-EA), *Gonja* (AAB-EA), *Kisansa* (AAA-EA), *Kivuvu* (ABB), *Mbwazirume* (AAA-EA), *Mpologoma* (AAA-EA), *M9* (AAA-EA hybrid), dwarf Cavendish and *Sukali Ndiizi* (AAB-EA) were used for the study. These cultivars were selected because of their preference by growers and their marketability and due to the fact that they are widely grown country wide within banana growing communities. Samples were obtained from the following districts of Uganda: Bushenyi, Kabarole, Kasese, Kapchorwa, Lira, Masaka, Mbale, Mbarara, Nakaseke, Sironko, Serere, Tororo, and Wakiso. Fruit samples were picked at the full green stage at 15 weeks post fruit emergence, when the fruit is fully developed but still green. Fruits were harvested from positions representing the top, middle and bottom sections of each bunch of fruit and combined to make a composite sample.

#### 2.1.3 Banana leaf sampling

In order to generate baseline data on Fe content in banana leaves, leaf samples were obtained from banana plants at three different stages of growth: vegetative (before the reproductive stage of plant development), flowering (when the male bud has not yet opened), and fruiting (after 12 weeks post fruit emergence). The furled leaf was sampled at the vegetative stage. Samples were taken from the flag leaf at both the flowering and fruiting stages as well as from the first, second and third leaf from the centre of the banana

plant. Leaf samples were obtained from the following cultivars mentioned in section 2.1.2 above. The leaf samples for analysis were obtained from the apex, middle and bottom then pooled together to represent a given leaf of sampled position.

## **2.2 SOIL SAMPLE ANALYSIS**

### **2.2.1 pH determination**

Soil pH was measured in suspension with a 1:2.5 soil to water ratio. Deionised water (50 mL) was added to 20 g of soil. The mixture was then stirred for 10 min, allowed to stand for 30 min and stirred for an additional 2 min. The pH was measured using a glass electrode (Orion 550 A Thermo Electron Corporation, USA) after calibration with pH meter solutions at pH 10 and 7, respectively.

### **2.2.2 Soil texture determination**

Soil particle size analysis was determined by the Bouyoucos (1962) method. The soil sample (50 g) was weighed into a 400 mL beaker, saturated with enough distilled water and 10 mL of 10 M sodium hexa-metaphosphate (BDH laboratory supplies, England) were added. The mixture was then allowed to stand for 10 min before overnight incubation on a reciprocating shaker (HS 501 D – IKA-Werke, Germany). The soil suspension was then transferred into a 1000 mL graduated cylinder. A Hydrometer (Gallenkamp, England) was then submerged in the suspension and distilled water was added until the 1000 mL mark was achieved. Next, the cylinder was sealed with a tight-fitting rubber bung and the soil suspension was vigorously mixed by inversion several times. Amyl-alcohol (two - three drops) was added to remove froth, and left to settle for 20 min before a density measurement was taken by gently placing the hydrometer (Gallenkamp, England) into the column. After 40 sec, initial hydrometer (Gallenkamp, England) and temperature readings of the suspension were taken, followed by another reading after 2 hr.

The hydrometer (Gallenkamp, England) and temperature readings taken after 40 sec were used to determine the sand content; while those taken after 2 hr signified that the silt had settled, reflecting the clay content. The silt was determined by subtracting the sum of the clay and sand content from overall soil constituents.

### **2.2.3 Soil iron determination**

Soil Fe content was determined using Mehlich 3 extractions; where 3g of a well ground and sieved soil sample was weighed and 30 mL of Mehlich 3 extractant added. The solution was shaken using a reciprocating shaker (HS 501 D – IKA - Werke, Germany) at 200 rpm for 5

min and then left to settle for 10 min before centrifugation at 2000 rpm for 5 min. To a 50 mL glass vial 30 mL of Mehlich 3 extractant was added. The Fe was then quantified on an Atomic absorption Spectrophotometer (Shimadzu, Japan) spectrophotometer at 213.9 nm.

## **2.3 BANANA SAMPLE PROCESSING**

During sample preparation, various precautions were taken in order to prevent sample contamination with other sources of Fe such as soil, metallic equipment or metallic surfaces.

### **2.3.1 Banana leaf processing**

Banana leaves were harvested at three growth stages as described in section 2.1.3 above. The harvested leaf tissue was washed with Milli-Q water. After washing, the leaf tissues were placed in a paper bag and oven dried (Gallenkamp, England) at 60 °C for 72 hr. The dried samples were finely ground using a porcelain mortar and pestle and then sieved using a plastic sieve mesh of 0.2 mm pore size (Kenpoly, Kenya). The fine powder was then packed in clean sterile 15 mL plastic polypropylene (PP) tubes with High density polyethylene (HDPE) screw caps (cat # 188271, Greiner Bio-One, Germany) and stored at room temperature prior to analysis.

### **2.3.2 Banana fruit processing**

Banana fruits were harvested at full green maturity stage (in section 2.1.2) from both cooking and dessert cultivars. Upon arrival, the fruits were washed under running tap water to remove field dust, peeled using a plastic knife, and placed on sterile plastic petri plates (prior to oven drying at 60 °C. The dried samples were ground into fine powder using a porcelain mortar and pestle and then sieved using a plastic sieve mesh of 0.2 mm pore size (Kenpoly, Kenya). The fine powder was then packed in clean sterile 15 mL plastic tubes with HDPE screw caps (cat # 188271, Greiner Bio-One, Germany) and stored at room temperature prior to analysis.

### **2.3.3 Banana leaf and fruit elemental analysis**

#### **2.3.3.1 Reagents, solutions and samples**

All the reagents used for analysis were of the highest commercially available purity grade. The  $\text{mQH}_2\text{O}$  obtained from a Milli-Q Plus water purification system (Millipore, Australia), was employed to prepare all standard and sample solutions. Trace Select<sup>®</sup>, for trace analysis,  $\geq 69\%$  (T)  $\text{HNO}_3$  (Sigma, Australia) and EMSURE ISO- $\text{H}_2\text{O}_2$  30 % (Perhydrol<sup>®</sup>) for analysis (Merck, Germany) were used for sample dissolution. Mono-elemental, high-purity

grade 1000 mg L<sup>-1</sup> stock 2 % HNO<sub>3</sub> solutions were purchased from Merck (Australia) for analysis of the following elements: Aluminium (Al), Boron (B), Barium (Ba), Calcium (Ca), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Iron (Fe), Potassium (K), Manganese (Mn), Nickel (Ni), Phosphorus (P), Titanium (Ti) and Zinc (Zn). The purity of the (ICP-OES) plasma torch argon was greater than 99.99 %.

#### 2.3.3.2 *Digestion procedure*

Sample digestion was performed according to Wheal et al. (2011). Given weights (approx. 0.3 g) of oven dried (Gallenkamp, England) materials were weighed by analytical balance (Mettler Toledo, Switzerland) into 50 mL polypropylene (PP) tubes with HDPE screw caps (cat # 227261, Greiner Bio-One, Germany). Maximum sample mass did not exceed 0.35 g as pressure build-up during initial heating risks rupture of the PP tubes and cause samples to dry out during digestion. Method blanks (MB) with no added plant material were also treated in the same manner. Digestion was initiated by adding 2 mL HNO<sub>3</sub> (Sigma, Australia) and 0.5 mL H<sub>2</sub>O<sub>2</sub> (Merck, Germany using calibrated Dispensette® bottle top dispensers. The caps were hand-tightened and the tubes vortexed (Vortex 2 genie, Scientific Industries, USA) to ensure entire sample was wetted. The samples were pre-digested overnight at room temperature (20 – 22 °C). The tubes were vortexed (Vortex 2 genie, Scientific Industries, USA) again before incubation in a DigiPREP digestion block system (Perkin-Elmer, Singapore) at 80 °C for 30 min. The pressure built up during the 30 min incubation was released by loosening each cap sufficiently to release pressure in the tube. The tubes were immediately retightened firmly and replaced in the digestion block. The temperature of the DigiPREP digestion block system (Perkin-Elmer, Singapore) was raised to 125 °C and samples were incubated for 120 min. No further sample handling took place until the program was finished. The digested samples were removed from digestion block and allowed to cool to room temperature. Samples were then made up to final volume in two stages to allow for cooling of diluted acid. Initially approximately 22 mL of mqH<sub>2</sub>O from a Dispensette® bottle-top dispenser were added. Then the remaining volume of water to make the total sample volume 25 mL was added using a fine tip HDPE wash bottle. The caps were sealed and the samples agitated by an orbital mixer (Ratek instruments, Australia) at 300 rpm for 5 min. Undissolved material (silicates) were allowed to settle for at least 60 min. Settled fruit sample extracts were decanted into 15 mL PP tubes with HDPE screw caps (# 227261, Greiner Bio-One, Germany). Settled leaf sample extracts were filtered into clean 15 mL PP tubes using 0.45 µm Millex® HV disposable syringe filters (Millipore®, Germany) to

ensure the removal of particulates. Tubes were stored at room temperature or immediately analysed by ICP-OES (Perkin-Elmer, Australia) as per Chapter 2.3.3.3.

#### 2.3.3.3 Instrumentation

Digested banana leaf and fruit samples were analysed using an Optima 8300 DV ICP-OES (Perkin-Elmer, Australia) with both axial and radial view configuration and equipped with a solid state detector, cyclonic spray chamber (cat # N0770605, Perkin-Elmer, Australia), and GemCone low-flow nebulizer (cat # N0770358, Perkin-Elmer, Australia). The operation conditions are summarized in Table 2.1. The emission lines that were used to measure the concentrations of specific elements of interest in analysed samples are listed in Table 2.2.

**Table 2.1:** Instrumental conditions of the ICP OES.

| Parameter                           | Value                     |
|-------------------------------------|---------------------------|
| Power reference (RF) incident power | 1.5 kW                    |
| Plasma argon flow rate              | 11 L min <sup>-1</sup>    |
| Auxiliary argon flow rate           | 0.6 L min <sup>-1</sup>   |
| Nebulizer argon flow rate           | 0.7 L min <sup>-1</sup>   |
| Pump                                | 1.00 mL min <sup>-1</sup> |
| Mist chamber                        | Cyclonic spray chamber    |
| Nebulizer                           | GemCone low-flow          |

**Table 2.2:** Optical emission lines used to analyse elements of interest

| Element | Emission line (nm) |
|---------|--------------------|
| Fe      | 239.52             |
| Zn      | 213.85             |
| Cu      | 327.39             |
| Mn      | 257.61             |
| Ni      | 231.60             |

#### 2.3.3.4 Validation studies

The accuracies of the measurements were assessed using both banana fruit and leaf reference materials IPE 929 and IPE 925 from Wagenigen Plant Analytical Laboratory (Netherlands). The precision of the method was defined in terms of the percentage of the variation of coefficient. The limit of detection (LOD) and quantification (LOQ) of each

analyte were calculated as the analyte concentration that corresponded to the standard deviation of seven independent measurements of the blank, divided by the slope of the analyte calibration curve multiplied by three and ten times, respectively.

## **2.4 GENERAL METHODS FOR NUCLEIC ACID PURIFICATION FROM PLANTS**

### **2.4.1 Overview**

The research work was conducted at the National Agricultural Research Organization (NARO, Kawanda, Uganda) and the Centre for Tropical Crops Biocommodities (CTCB, Brisbane, Australia). Banana cultivars, *Sukali Ndiizi* (ABB-EA, apple banana) and *Nakinyika* (AAA-EA, cooking) were used for transformation at the NARO, while Cavendish (AAA, dessert) was used for the experiments at the CTCB. The plants studied at the NARO were planted under field conditions while those analysed at the CTCB were grown under glasshouse conditions at the Queensland Crop Development Facility (QCDF; Brisbane, Australia). Embryogenic cell suspensions, transformation, selection, regeneration and acclimatisation of transgenes were conducted by the scientists in the tissue culture facilities of both institutions. The binary plant vectors used for transformation were created by scientists at the CTCB and provided to the NARO as part of collaborative work funded by the Bill and Melinda Gates Foundation.

### **2.4.2 DNA extraction**

Total genomic DNA was extracted from leaf tissue using a modified CTAB protocol based on Dellaporta et al. (1983). Approximately 1g of leaf tissue was ground in LN<sub>2</sub> and incubated in 700 µL of extraction buffer (2% CTAB, 200 mM Tris-HCL [pH 8], 0.14 M NaCl, 1.4% (v/v) β-mercaptoethanol, 20 mM EDTA) at 65 °C for 30 min with occasional mixing. Plant material was removed by centrifugation (Centrifuge 5424R, Germany) at 9000 × g for 5 min at room temperature and the supernatant extracted twice with an equal volume of chloroform: isoamyl-alcohol (24:1 v/v) at room temperature. The aqueous phase was collected and 10 µL of RNase A (Invitrogen, Germany) (1 mg/mL) was added before incubation (Water bath, OLS 200, Great instruments, England) at 37 °C for 60 min. The sample was extracted again with chloroform: isoamyl-alcohol (Sigma, Germany) (24:1 v/v), before precipitation with an equal volume of isopropanol for 10 min. The DNA was collected by centrifugation (Centrifuge 5424R, Germany) at 9000 × g for 10 min at room temperature and the pellet washed with 70% ethanol (Merck, Germany). The DNA pellet was dried under vacuum and re-suspended in 50 µL of mqH<sub>2</sub>O. The yield and quality of the DNA were assessed by spectrophotometry using a Nano drop Spectrophotometer (Thermo Scientific, Australia)

with absorbance readings at 260 and 280 nm and agarose gel electrophoresis using molecular grade agarose (cat# Bio-41025, Bioline, USA), and the DNA was either used immediately or stored at -20 °C. The quantified DNA was used for PCR and Southern hybridisation analysis.

### 2.4.3 Purification of total RNA from banana leaf tissue

#### 2.4.3.1 RNA isolation

Total RNA was obtained according to the procedures and buffers described by Valderrama-Cháirez et al. (2002) with minor modifications. Essentially, 150 mg of banana leaf tissue was ground in LN<sub>2</sub> and 1 mL of RNA extraction buffer containing 2% β-mercaptoethanol (Sigma, Germany) was added and contents mixed by vortex (Vortex -2 genie, Scientific Industries, USA) for 1 min. Total RNA was recovered by centrifugation (Eppendorf 5424R, Germany) at 15,000 × g for 5 min at room temperature. The polysaccharides were precipitated with  $\frac{1}{30}$  volume of 5 M Sodium acetate (pH 5.2) and 0.2 volume of 100 % ethanol (Merck, Germany) and recovered by centrifugation (Eppendorf 5424R, Germany) at 15,000 × g for 5 min at room temperature. The mixture was extracted thrice with chloroform: isoamyl-alcohol (Sigma, Germany) (49:1, v/v) and total RNA precipitated with  $\frac{1}{4}$  volume of 12 M LiCl (Fisher, England) to the supernatant followed incubation at 4 °C overnight. Total RNA was recovered by centrifugation (Eppendorf 5424R, Germany) at 15,000 × g for 45 min at 4 °C. The pellet was washed twice with 70 % ethanol (Merck, Germany) dried under vacuum (Eppendorf Concentrator 5301, Germany) and resuspended in DEPC-treated mqH<sub>2</sub>O, total RNA was stored at -80 °C. The quality of RNA was analysed by agarose electrophoresis and RNA concentration was determined by spectrophotometry (Thermo Scientific, Australia) at A<sub>260nm</sub> and A<sub>280nm</sub>.

#### 2.4.3.2 DNase treatment of RNA

The obtained RNA was treated with RNase-free DNase (Promega, USA) according to the manufacturer's instructions. Briefly, the DNase reaction contained about 1 µg of RNA, 1 U of DNase and 1 × DNase reaction buffer in a total volume of 10 µL. The reaction was carried out at 37 °C for 30 min, and terminated with 1 µL of DNase stop solution after which DNase was inactivated at 65 °C for 10 min. The quality of RNA was analysed by agarose gel electrophoresis use 2 % molecular grade agarose (cat# Bio-41025, Bioline, USA) and RNA concentration was determined using a Nanodrop (Thermo Scientific, Australia) at A<sub>260nm</sub> and A<sub>280nm</sub>.

#### 2.4.4 cDNA synthesis

Preparation of cDNA was prepared using the AMV RT-Kit (Promega, USA) following manufacturer's instructions. Reagents were prepared in two master mixes (1 and 2). Master mix 1 contained 10 pmoles of Oligo DT primer, 1 µg of RNA and mqH<sub>2</sub>O water to a total volume of 11 µL. Master mix 2 consisted of RT 10 × buffer, 25 mM MgCl<sub>2</sub>, 10 mM dNTPs, 1 µL reverse transcriptase, 15 U RNase inhibitor and mqH<sub>2</sub>O to a total volume of 9 µL. Master mix 1 was denatured at 70 °C for 5 min and placed on ice for 5 min before being combined with master mix 2 to make the 20 µL reaction mixture. The reaction mixture was incubated at 45 °C for 45 min to achieve cDNA synthesis, followed by inactivation of reverse transcriptase at 95 °C for 5 min. The synthesised cDNA was then either stored at -20 °C or used for RT-PCR with gene specific primers.

### 2.5 GENERAL METHODS IN NUCLEIC ACID AMPLIFICATION

#### 2.5.1 PCR amplification

For all PCRs, thin walled 0.2 mL tubes were used to amplify products in a DNA-Engine® Peltier thermal cycler (Biorad, USA). Annealing temperatures were dependant on primers and are specified in individual chapters. Oligonucleotide (primers) were acquired from Sigma-Aldrich at a concentration of 100 µM. Primers were diluted to 10 µM for PCR.

##### 2.5.1.1 *Standard DNA Amplification*

Genomic DNA from putative transgenic lines was analysed using PCR to detect the presence of *IRT1*, *FRO2*, *Sfer*, *FEA1*, *OsNAS1* and *OsNAS2* gene sequences in all transgenic plants. All PCR reactions were performed in a DNA Engine Peltier thermal cycler (PTC-0200, Biorad, USA). The PCR products were amplified from DNA template (0.3 to 1 µg) using Go Taq® Green Master Mix (Promega, USA) and each reaction included 1× Go Taq Green® (Promega, USA) master mix, 7.5 pmol of each primer, and mqH<sub>2</sub>O to a total volume of 20 µL. Templates were initially denatured at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing temperatures of 55 °C for 30 sec and extension at 72 °C for 1 min per Kb of expected product. A final extension step of 72 °C for 4 min was included. Plasmid vectors were included as a positive control while water and untransformed plant DNA were used as negative controls.

##### 2.5.1.2 *RT-PCR*

RNA (amounts specified in each chapter) was used as template in RT-PCR. During RNA handling, all materials and buffers were treated with DEPC-treated mqH<sub>2</sub>O (Fermentas,

England) to remove RNase. Reaction mixtures were subjected to an initial denaturation step 95 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing temperature of 55 °C for 30 sec; 72 °C for 2 min and a final extension step of 72 °C for 4 min.

### 2.5.2 Agarose gel electrophoresis

With exception of PCR reactions performed with Go Taq® green (Promega, USA), DNA fragments from PCR or restriction digestion analysis included 1 × agarose gel loading dye (0.04 % bromophenol blue; 1.7 mM Tris-HCL, pH 8.0; 0.17 mM EDTA; 0.08 % glycerol) before agarose gel electrophoresis. DNA fragments from PCR or restriction digestion analysis were separated for 1 hr in 1 % agarose (for DNA) or 2 % agarose gels (for RNA or DNA fragments less than 500 bp) made with molecular biology grade agarose (cat# Bio-41025, Bioline, USA) in 1 × TAE buffer (10 mM Tris-acetate; 0.5 mM EDTA, pH 7.8) containing 0.25 % SYBR® Safe stain solution in DMSO (Invitrogen, USA) at 4 V/cm on a B1 EasyCast™ Mini Gel System (Owl Separation System, USA). Nucleic acids were visualized on a Safe Imager™ blue light transilluminator (Invitrogen, USA) and images recorded using a G-Box (Syngene, USA) gel documentation system with Gene-Snap v6.07 and GeneTool v6.07 software.

### 2.5.3 Purification of DNA from agarose gels

Bands were excised from agarose gels and the DNA purified using the Mo Bio UltraClean™ GelSpin™ DNA purification Kit (Mo Bio Laboratories inc., USA) according to the manufacturer's instructions. All purified DNA was eluted in a final volume of 20 µL of 10 mM TE buffer and stored at -20 °C until required.

### 2.5.4 Restriction enzyme digestion of DNA

Up to 1 µg of pDNA or plant gDNA was incubated with 10 U of the appropriate restriction enzyme (Roche Applied Sciences, USA) in compatible 1 × reaction buffer at 37 °C for 16 hr.

### 2.5.5 Southern hybridisation analysis

To determine the copy number of *IRT1*, *FRO2*, *Sfer*, *FEA1*, *OsNAS1* and *OsNAS2* genes in the genomes of transgenic lines, Southern blot analysis was performed (Southern, 1975). The PCR DIG probe synthesis kit (Roche Applied Sciences, USA), positively charged nylon membrane, hybridisation buffer (Roche Applied Sciences, USA), blocking reagent (Roche Applied Sciences, USA), Anti-DIG-AP antibody (Roche Applied Sciences, USA), DIG labelled molecular weight marker (Roche Applied Sciences, USA) and the chemi-luminescent films

used were procured from Roche Applied Sciences (Roche Applied Sciences, USA) and the manufacturer's procedures were followed during the analysis.

#### 2.5.5.1 *Probe labelling*

DIG-labelled probes (specific for *IRT1*, *FRO2*, *Sfer*, *FEA1*, *OsNAS1* and *OsNAS2*) were prepared using the PCR DIG probe synthesis kit. 50  $\mu$ L PCR reactions contained 5  $\mu$ L dNTP mix, 1  $\mu$ L Taq polymerase, 1  $\mu$ L of pDNA and 0.5  $\mu$ M of each primer. The reaction was subjected to an initial denaturation step of 95 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec; annealing at 55 °C for 30 sec, extension at 72 °C for 2 min and final extension step of 72 °C for 5 min.

#### 2.5.5.2 *DNA electrophoresis and gel processing*

10  $\mu$ g of plant gDNA from selected transgenic lines and non-transgenic lines and 10  $\mu$ g of positive control pDNA were used for Southern blotting. The 50  $\mu$ L restriction digestion reaction contained 10  $\mu$ g of gDNA, 1  $\mu$ L *EcoRI* restriction enzyme and 1  $\times$  *EcoRI* buffer. The digestion mix was incubated overnight at 37 °C. The digested DNA was run on a 0.8 % agarose gel in a 1  $\times$  TAE buffer (Sigma, Germany) at 60 V for 4 hr. The quality of restriction digestion was determined after staining the gel in SYBR® safe (Invitrogen, USA) and visualisation under UV. Uniform smearing of DNA confirmed complete restriction digestion. The gel was rinsed in  $\text{mqH}_2\text{O}$  and processed at room temperature by depurination at 35 rpm for 20 min. The gel was then rinsed with  $\text{mqH}_2\text{O}$  neutralised twice at 35 rpm for 15 min with three changes of buffer. After the final rinse in  $\text{mqH}_2\text{O}$ , the gel was equilibrated in 2  $\times$  SSC buffer for 5 min before DNA transfer.

#### 2.5.5.3 *DNA transfer*

Transfer of the DNA fragments from the gel to a positively charged nylon membrane was done by upward capillary motion. The transfer pyramid was constructed as described by Sambrook et al. (2001) and left for 12 hr to allow DNA blotting on the membrane.

#### 2.5.5.4 *Hybridisation*

DNA was fixed on the membrane using UV cross-linking (Bibby Stuart Scientific, England) and the membrane was rinsed twice in 2  $\times$  SSC buffer for 2 min before hybridisation. The membrane was pre-hybridised in 15 mL hybridisation buffer at 42 °C 7 rpm for 1 hr. After 1 hr incubation, the pre-hybridisation buffer was replaced by 10 mL of warm hybridisation buffer containing probes for genes of interest. Probes were prepared by denaturation at

100 °C for 1 min and rapid cooling on ice for 5 min. The hybridisation was then done overnight at 42 °C.

#### 2.5.5.5 *Detection*

The membrane was washed as per manufacturer's instructions and incubated with antibody (Anti-Dig-AP) (Roche Applied Sciences, USA) diluted at 1:10 in buffer 2 for 30 min at 30 rpm. The membrane was washed twice in buffer 1 (with Tween 20) for 30 min at 45 rpm and equilibrated with buffer 3 for 2 min. For detection, the membrane was placed on cling film and 15 drops of CDP-Star® were added onto the membrane. After spreading of the solution, the membrane was wrapped in the cling film and incubated at room temperature for 5 min. The membrane was exposed to Lumi-Film chemiluminescent detection film (Roche Applied Sciences, USA) for 10 min in dark before it was developed.

# Chapter 3: Determining the mineral content of banana and soil samples collected from different regions of Uganda

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## 3.1 INTRODUCTION

The importance of the optimal intake of essential minerals to maintain good health is widely recognised. Inadequate intake of mineral elements has been observed to be a major nutritional problem in developing nations (Aremu and Udoessien, 1990). Fruits and vegetables are an important source of essential elements (Ekholm et al., 2007). Minerals play a vital role in the proper development and good health of the human body, and fruits are considered to be the chief source of minerals needed in the human diet (Wall, 2006). The risks associated with mineral deficiencies depend on a number of factors such as daily dietary intake, the chemical form of minerals contained in the food consumed, presence of substances that limit or increase the bioavailability of minerals, and the physiological state of the consumer (Hardisson et al., 2001a).

Banana (*Musa* spp.) is the most important food crop in the tropical and sub-tropical zones of the world. Most bananas produced in Africa are used as a staple food and source of cash income for small holder farmers. In some African countries, such as Uganda, the daily consumption of bananas is 1 kg per person (FAOSTAT, 2001), the highest in the world. East Africa is the largest banana producing and consuming region in Africa while Uganda is the world's second largest producer (FAOSTAT, 2004). Banana (dessert and cooking) and plantain fruits belong to the *Eumusa* section of the genus *Musa*. Edible bananas are classified into different genomic groups: AA, AB, BB, AAA, AAB, ABB, AAAA, AAAB and ABBB (Bakry et al., 2009). Dessert bananas are consumed raw when ripe at full maturity. Cooking bananas are prepared for consumption at various stages of maturity and are disliked when raw because of the absence of sweetness, an unpleasant texture, which feels hard and granular in the mouth (Gibert et al., 2009).

The East and Central Africa sub-regions produce nearly 20 million tons of bananas annually, of which approximately 10.5 million tons is produced in Uganda (BARNESA, 2008). More than 85% of the bananas grown in Uganda are comprised of the East African Highland Bananas (*Musa* spp. AAA genotype; EAHBs), cooking (AAA), juice (ABB), and dessert (AB,

AAA) bananas. This region represents 10% of the worlds' plantain and banana production (FAOSTAT, 2010). The EAHBs (AAA-EA) are special types of banana grown in the Great Lake zone of East Africa, and are used in preparation of 'matooke,' a popular local Ugandan dish.

Although the majority of banana varieties are high in carbohydrates, fibre, vitamins C and B, they have low amounts of Fe, I and vitamin A (Frison and Sharrock, 1998). Promoting the consumption of bananas with enriched mineral content, can alleviate severe deficiencies in developing countries like Uganda, where diets are banana-based. Furthermore, increasing the nutritional value of bananas through biofortification will enhance health and well-being of the people. However, little is known about the micronutrient variations in the East African Highland bananas grown in different districts of Uganda.

Although bananas are widely cherished in East Africa, little information is available about the effect of soil and plant mineral status in different parts of Uganda. The relationship between fruit Fe, Zn and the contributions of soil pH, soil texture, and soil Fe on the different cultivars obtained from different banana growing districts was assessed in order to determine whether environment plays a role in banana mineral uptake. A statistical study ANOVA, correlation and cluster analysis was carried out on soil texture, pH, soil Fe fruit Fe and Zn in order to separate the banana samples into homogeneous groups. This study aimed to investigate the effect of soil pH, texture, soil Fe on the fruit and leaves Fe and Zn composition from the different banana growing regions in Uganda. This was in order to ascertain the degree to which the Fe and Zn accumulation in the banana cultivars studied was affected, if at all, by genetic or environmental factors. These findings should contribute to the understanding of the relationship existing between soil properties and fruit mineral accumulation, as well as being helpful for the on-going Bill and Melinda Gates banana biofortification project at QUT and NARO. In order to provide base line data on existing soil Fe content, pH, soil texture as well the effect of genotype and environmental interaction on mineral content of the different banana cultivars prior to biofortification were analysed.

The specific objectives of this chapter were to:

1. Determine soil properties and Fe content in selected banana growing Ugandan districts
2. Determine Fe and Zn content in fruit pulp from different banana varieties collected from selected Ugandan districts
3. Determine the effect of genotype and environmental interaction between different banana cultivars

4. Determine Fe and Zn content in banana leaves at different positions and stages of plant development

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Sample collection sites and sampling

The soil, banana fruits and leaf samples were collected from the Bushenyi, Gulu, Kabalore, Kasese, Kapchorwa, Lira, Masaka, Mbale, Mbarara, Nakaseke, Sironko, Serere, Tororo and Wakiso districts in Uganda (Figure 3.1 and Table 3.1). These districts have a tropical climate with a bimodal rainfall pattern characterised by two rainy seasons, with dry spells occurring between January to March and July to August. The sampling procedures were as per Chapter 2.1.1 – 2.1.3.

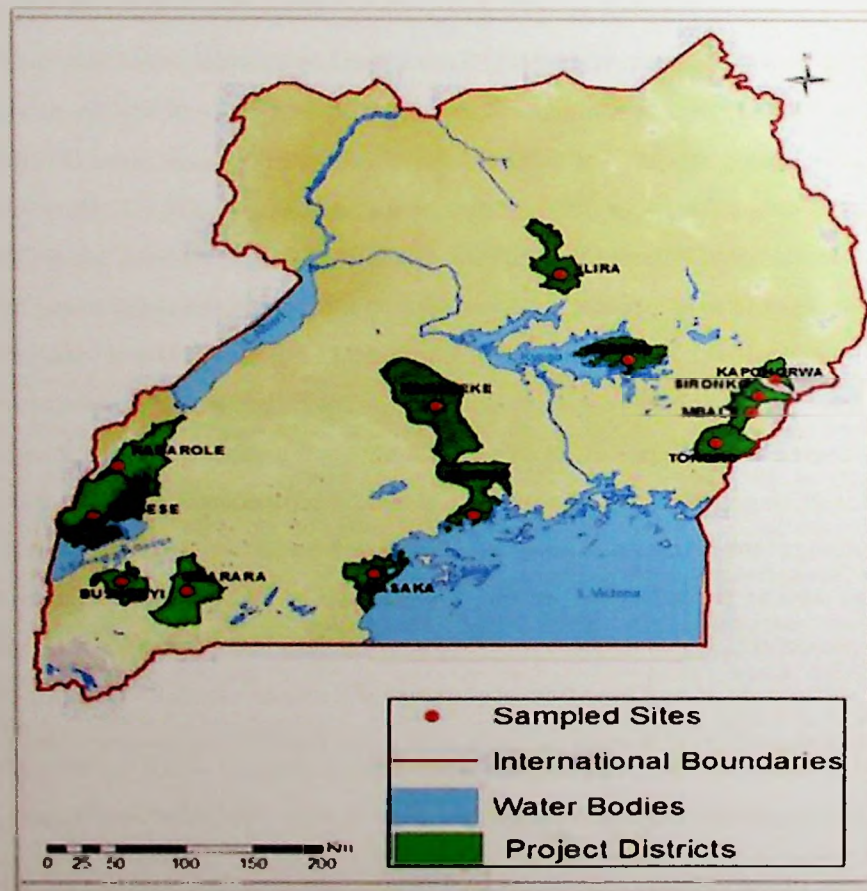


Figure 3.1: Map of Uganda showing sample collection

**Table 3.1:** Selected districts for banana sample collection and analysis in Uganda

| District  | Subcounty   | Village    | Geographical position | Elevation (m) |
|-----------|-------------|------------|-----------------------|---------------|
| Wakiso    | Namutumba   | Namutumba  | 0° 47"N 32° 27"E      | 1124          |
| Nakaseke  | Nakaseke    | Nakaseke   | 0° 72"N 32° 47"E      | 1113          |
| Masaka    | Lwengo      | Ssenke     | 0° 36"S 31° 54"E      | 1232          |
| Mbarara   | Kakoba      | Kakoba     | 0° 59"S 30° 60"E      | 1412          |
| Bushenyi  | Nyabubare   | Nyakinengo | 0°54"S 30° 10"E       | 1177          |
| Kasese    | Bugoye      | Ibanda     | 0° 32"S 30° 07"E      | 1433          |
| Kabalore  | Fort Portal | Nyabukalu  | 0° 67"S 30° 28"E      | 1560          |
| Mbale     | Bumboni     | Luki       | 0° 98"N 34° 16"E      | 1128          |
| Tororo    | Kwapa       | Amagoro    | 0° 68"N 34° 19"E      | 1251          |
| Sironko   | Musene      | Mutufu     | 1° 20"N 34° 32"E      | 1234          |
| Kapchorwa | Buginyanya  | Bulambuli  | 1° 28"N 34° 38"E      | 2161          |
| Serere    | Serere      | Serere     | 1° 53"N 33° 45"E      | 1135          |
| Lira      | Ngetta      | Ngetta     | 2° 06"N 32° 95"E      | 1103          |

### 3.2.2 Soil characterisation

Soil samples were collected from the locations described in Chapter 3.2.1 as per Chapter 2.1.1. Analysis of the pH, texture and Fe content of the soil samples was carried out according to the procedures described in Chapter 2.2.

### 3.2.3 Banana leaf and fruit analysis

#### 3.2.3.1 *Banana leaf and fruit sample preparation*

Banana leaf samples were taken at different stages of development from leaves at different positions (indicating leaf maturity) (Figure 3.2). Banana leaves were then processed as described in Chapter 2.3.1. Banana fruits were harvested at full green maturity stage (described in Chapter 2.1.2) from both cooking and dessert cultivars. The procedure for

sample preparation is described in Chapter 2.3.2 (Figure 3.3). Leaf and fruit samples were digested according to Wheal et al. (2011) (described in section 2.3.2). Prepared sample digests were either stored at room temperature or immediately analysed by ICP-OES as per Chapter 2.3.3.

### 3.2.3.2 *Statistical and chemometric analysis*

Data were subjected to analysis of variance using One-way ANOVA (XLSTAT, 2014). The parameters of interest were expressed as mean  $\pm$  standard deviation (SD). The means were separated by least significant difference (LSD) test and subsequent parameters of soil pH, texture, soil Fe, and fruit Fe were also subjected to Pearson correlation analysis, principal component analysis (PCA), cluster analysis, and agglomerative hierarchical clustering using the XL-stat statistical package (XLSTAT, 2014).



**Figure 3.2:** Banana leaves at different stages of development **A)** vegetative, **B)** flowering and **C)** Fruiting stage. The leaves marked in **B)** are i) Flag leaf, ii) first leaf, iii) second leaf and iv) third leaf.



**Figure 3.3:** Banana fruit processing procedure prior to mineral analysis. A) Banana bunch B) Fruit finger washing, C) Sample peeling using plastic knife, D) Sample drying, E) Drying oven, F) Sample grinding using ceramic mortar and pestle .

### 3.3 RESULTS

#### 3.3.1 Analysis of soil properties

The data from soil analysis shows that the soil pH ranged from 5.1 to 7.2, which is strongly acidic to neutral in nature. Moderately acidic soils, whose pH ranged from 5.1 to 5.5, were observed in the districts of Bushenyi, Buginyanya, Mbarara and Tororo. Soils from the districts of Lira, Mbale, Nakaseke and Serere were moderately acidic with a pH range of 5.6 to 6.0. In contrast soils from Wakiso and Sironko districts were slightly acidic with pH ranging from 6.0 to 6.5, while soils from Masaka, Kabalore and Kasese districts were neutral with pH ranging from 6.6 to 7.3.

Soil Fe content ranged from  $101.4 \pm 10.5$  to  $260.0 \pm 15.5$  ppm (Table 3.2). The eastern districts of Mbale, Sironko and Tororo had the lowest soil Fe content while the central districts of Nakaseke and Wakiso had the highest soil Fe content. The soil texture was mainly characterised by sandy clay loam soils in majority of the districts except for Bushenyi and Kabalore with sandy clay, while Kapchorwa and Masaka had clay soils.

**Table 3.2: Soil properties of different districts**

| District  | Soil properties |               |       | Soil texture |       |
|-----------|-----------------|---------------|-------|--------------|-------|
|           | Soil pH         | Soil Fe (ppm) | Sand% | Clay%        | Silt% |
| Wakiso    | 6.4             | 260.0±15.5    | 67.0  | 23.0         | 10.0  |
| Nakaseke  | 5.9             | 210.8±18.5    | 59.0  | 31.0         | 10.0  |
| Masaka    | 7.2             | 113.9±17.8    | 41.7  | 46.8         | 11.5  |
| Mbarara   | 5.4             | 136.2±10.7    | 64.3  | 24.7         | 11.0  |
| Bushenyi  | 5.1             | 162.2±26.4    | 48.8  | 38.8         | 12.4  |
| Kasese    | 6.7             | 181.9±20.5    | 52.5  | 31.2         | 16.3  |
| Kabalore  | 7.2             | 191.8±13.2    | 46.2  | 37.2         | 16.6  |
| Kapchorwa | 5.3             | 154.0±14.6    | 46.2  | 43.2         | 10.6  |
| Sironko   | 6.2             | 113.1±10.8    | 62.2  | 25.2         | 12.6  |
| Mbale     | 5.9             | 101.4±10.5    | 70.2  | 23.2         | 6.6   |
| Tororo    | 5.5             | 127.3±9.80    | 72.2  | 23.2         | 4.6   |
| Serere    | 5.8             | 154.0±10.5    | 72.2  | 23.2         | 4.6   |
| Lira      | 5.9             | 142.3±11.8    | 62.2  | 25.2         | 12.6  |

### 3.3.2 Mineral content of cooking bananas

The amounts of Fe and Zn in the cooking cultivars *Kisansa*, *Mbwazirume*, *Mpologoma*, *M9* and *Nakitembe* were determined by ICP-OES (Figure 3.4). The average Fe content in the cooking types was 10 mg/kg DW and the Zn content was 7 mg/kg DW. *Mpologoma* had the lowest and *Kisansa* had the highest Fe content. While *M9* hybrid had the lowest concentration of Zn (6 mg/kg DW) and *Nakitembe* had the highest (8 mg/kg DW). However, with exception of *Mpologoma* from Nakaseke and Buginyanya; *Nakitembe* from Mbale; *Mbwazirume* from Wakiso, Mbale and Buginyanya; and *M9* hybrid from Sironko districts, there was no significant difference in Fe content at  $P \leq 0.05$  within the cultivars from the different districts. Zn content was significantly different at  $P \leq 0.05$  for cultivars *Mpologoma* from Nakaseke; *Nakitembe* from Nakaseke and Mbale; *Mbwazirume* from Wakiso, Mbale and Lira; *Kisansa* from Nakaseke, Mbale and Serere; and *M9* from Mbale and Tororo districts.

### 3.3.3 Mineral content of dessert bananas

Analysis of Fe and Zn levels in the fruit pulp of the dessert cultivars *Bogoya*, *Dwarf Cavendish*, *Gonja* (*Plantain*), *Kivuvu* (*Bluggoe*) and *Sukali Ndiizi* was performed by ICP-OES and results are shown (Figure 3.5). The average Fe content in the dessert cultivars was 12 mg/kg DW. *Sukali Ndiizi* had the lowest (8 mg/kg DW) and *Dwarf Cavendish* had the highest (11 mg/kg DW). The average Zn content of the dessert bananas was 7 mg/kg DW. The lowest Zn content was observed in *Bogoya* and *Sukali Ndiizi* with 5 mg/kg DW and the highest was in *Dwarf Cavendish* and *Kivuvu* (*Bluggoe*).

The Fe content was significantly different ( $P \leq 0.05$ ) for *Gonja* from Kabalore and Kasese districts; *Bogoya* from Wakiso and Nakaseke districts; and *Sukali Ndiizi* from Nakaseke and Masaka districts. However, for the *Bluggoe* and *Dwarf Cavendish* cultivars, there was no significant difference across the different districts. The Zn content was significantly different at  $P \leq 0.05$  for *Gonja* obtained from Mbarara and Lira; *Bogoya* from Nakaseke and Mbale; *Sukali Ndiizi* from Bushenyi; and *Bluggoe* from Mbarara and Kabalore districts, while the Zn content of the *Dwarf Cavendish* cultivar did not vary significantly.

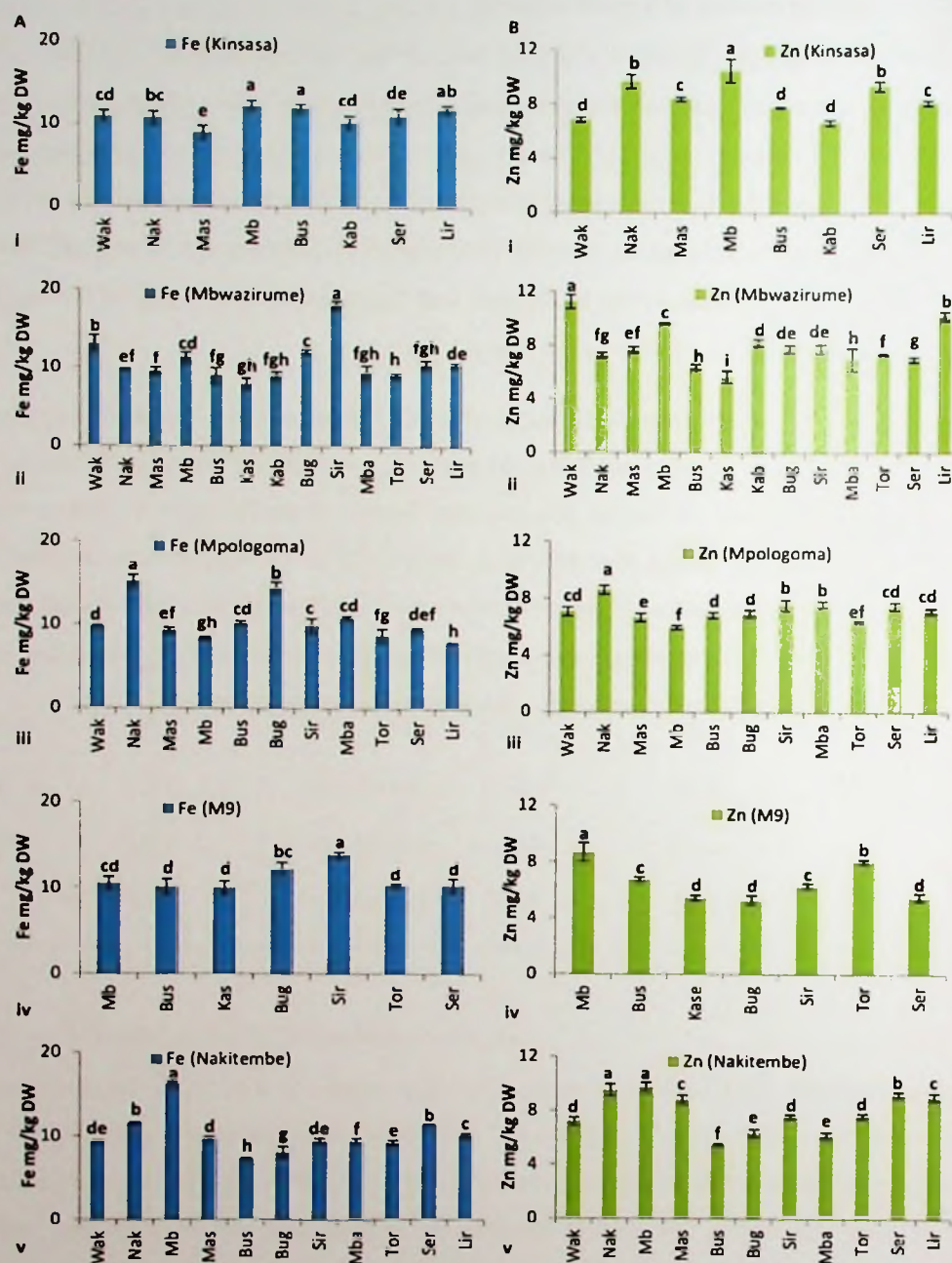
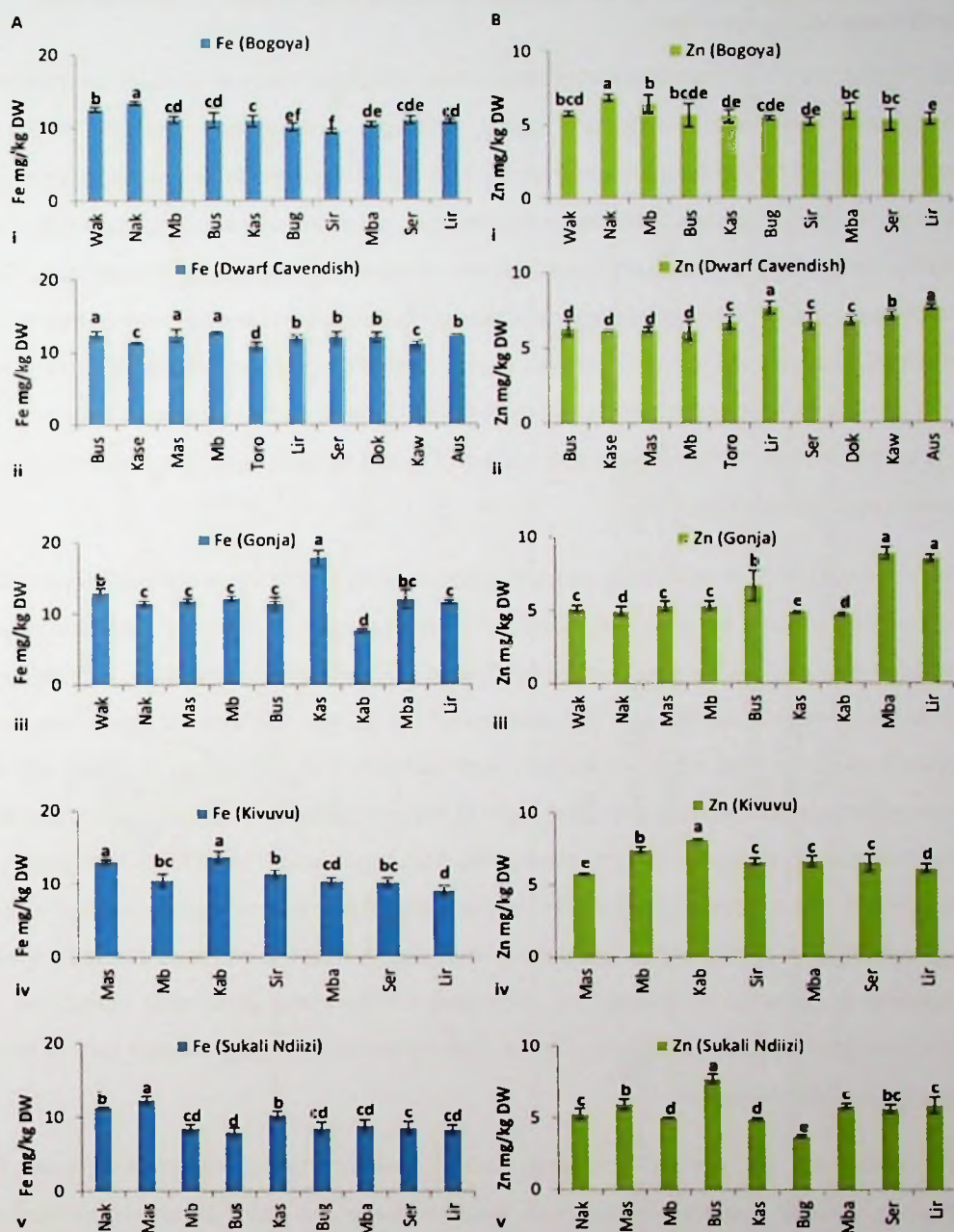


Figure 3.4: Mineral content of fruit from cooking banana cultivars from selected Ugandan districts. A) Fe and B) Zn content was determined for the cultivars i) *Kinsasa* ii) *Mbwazirume*, iii) *Mpologoma*, iv) *M9*, and v) *Nakitembe*. Data are mean  $\pm$  SD (n=5). Superscript letters indicate groups that are not significantly different at  $P \leq 0.05$ . The names of districts where samples were collected are abbreviated as follows: Wakiso (Wak), Nakaseke (Nak), Masaka (Mas), Mbarara (Mb), Bushenyi (Bus), Kasese (Kas), Kabororere (Kab), Buginyanya (Bug), Sironko (Sir), Mbale (Mba), Tororo (Tor), Serere (Ser), Lira (Lir).



**Figure 3.5:** Mineral content of fruit from dessert banana cultivars from selected Ugandan districts. A) Fe and B) Zn content were determined for i). Bogoya, ii) Dwarf Cavendish, iii) Gonja, iv) Kivuvu v). Sukali Ndiizi. Data are mean  $\pm$  SD (n=5). Superscript letters indicate groups that are not significantly different at  $P \leq 0.05$ . The names of districts where samples were collected are abbreviated as follows: Wakiso (Wak), Nakaseke (Nak), Masaka (Mas), Mbarara (Mb), Bushenyi (Bus), Kasese (Kas), Kabalorere (Kab), Buginyanya (Bug), Sironko (Sir), Mbale (Mba), Tororo (Tor), Serere (Ser), Lira (Lir).

### **3.3.4 Investigating the relationships between fruit mineral content and environmental properties**

The impact of environmental parameters such as soil properties and altitude on fruit Fe and Zn accumulation were assessed using principal component analysis (PCA) and agglomerative hierarchical clustering (AHC). PCA is a bilinear modelling method which gives an interpretable overview of the main information in a multi-dimensional data table while cluster analysis is an unsupervised pattern recognition technique in statistics. These techniques involve trying to determine relationships between samples without using prior information about these relationships. In this study PCA (Figure 3.6) and AHC (Figure 3.7) were used to further understand what factors influence Fe and Zn uptake in banana plants. This was achieved by combining soil pH, soil Fe, soil texture, altitude, banana Fe and Zn contents of different cultivars.

The results of the PCA modelling and AHC analysis show that several variables contribute to the cooking banana cultivar characteristics. The PCA only plotted the first two principal components (PCs), which accounted for 55% of the variability in the data, PC1 accounted for 34.1% of the variables and PC2 accounted for 21.4%. The first PC shows the largest possible variance that accounts for as much variability in the data as possible and each succeeding component in turn has the highest variance possible under the constraint that it is orthogonal to the preceding components. PC1 represents the effects of altitude, soil texture and pH properties while PC2 represents the effects soil mineral elements Fe and Zn and fruit mineral composition. The data further shows that the cultivars did not segregate according to genetic similarity but according to the areas of sample collection. This therefore implies that environmental or climatic conditions play a major role in banana growth or mineral uptake.

Data shows that soil pH and soil loam content are correlated with cooking types from Kabalore, Kasese and Masaka districts, while altitude and clay soils are correlated with cooking types from Bushenyi and Kapchorwa districts. Soil Fe, soil sand content, and fruit Fe and Zn content are correlated with cooking types from Nakaseke, Wakiso, Mbale, Lira, Sironko and Serere districts. Cooking cultivars from Tororo and Mbarara districts had a weak contribution to the cooking cultivars from Tororo and Mbarara districts.

PCA Biplot (axes PC1 and PC2: 54.03 %) after Varimax rotation

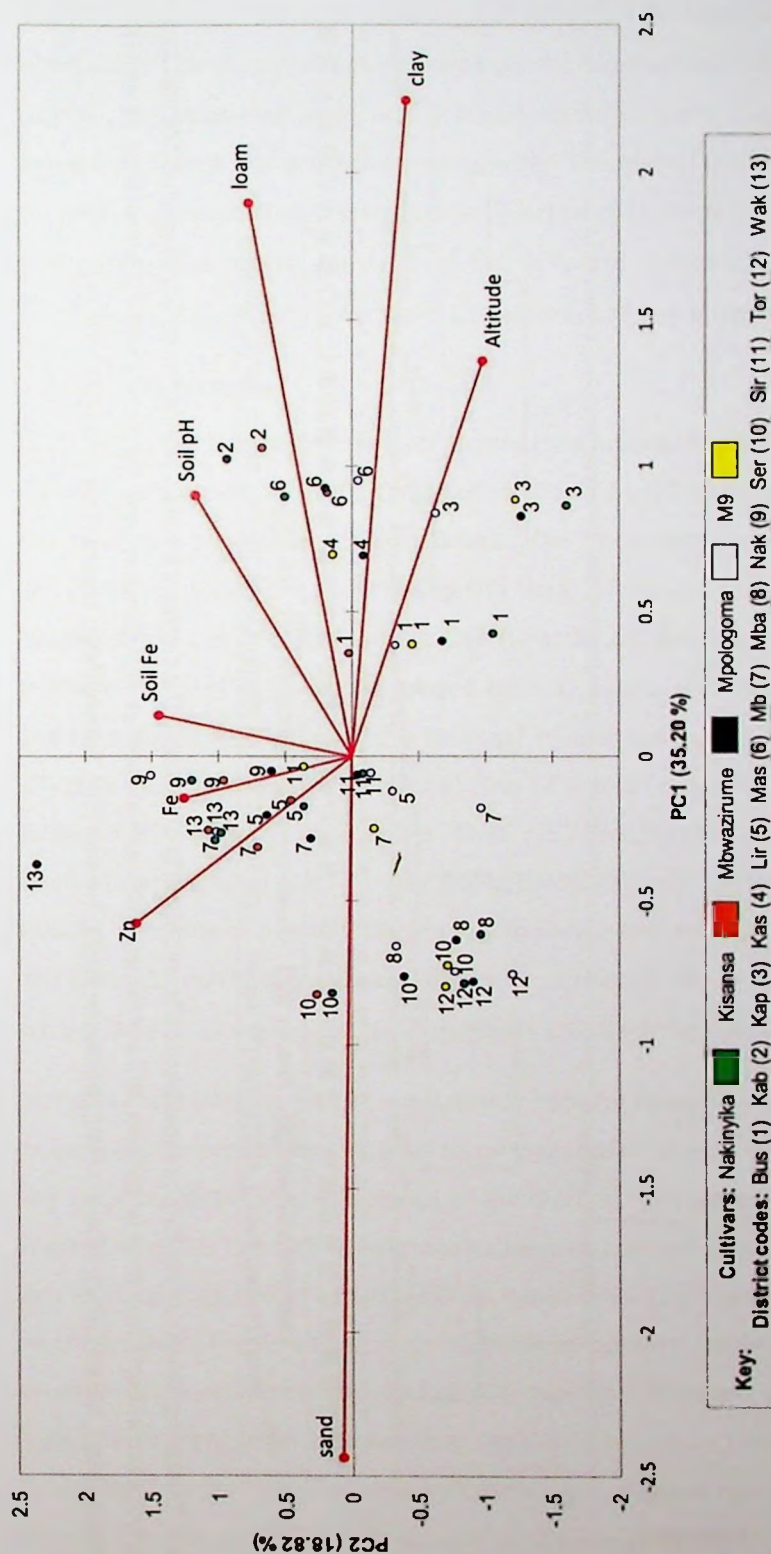
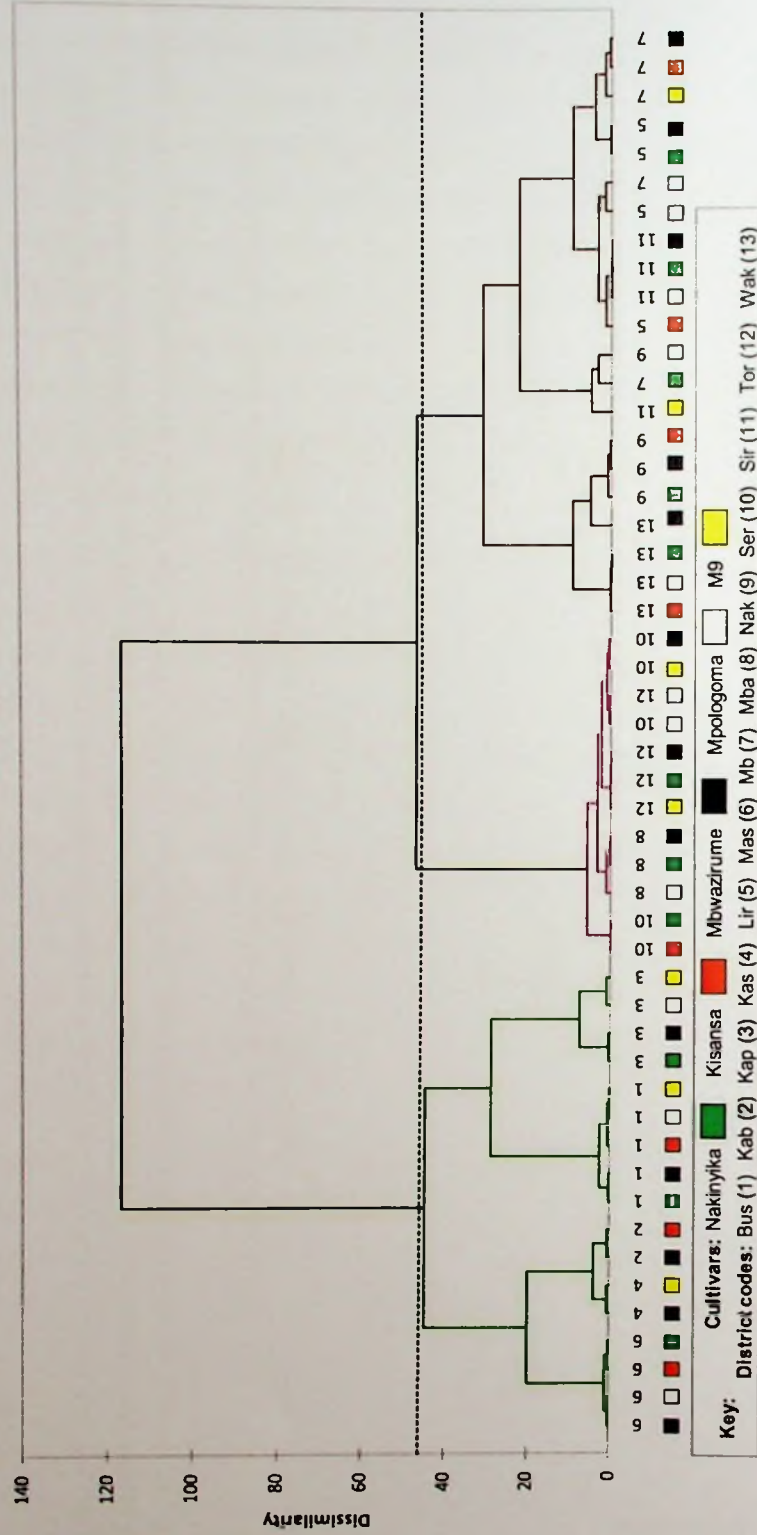


Figure 3.6: PCA plot of different cooking banana cultivars from different districts of Uganda. The cultivars are: Mpologoma, Nakitembe, Mbwazirume, Kisansa, and M9 were analysed according to fruit mineral content, soil and climatic (altitude) properties. District names are: Bushenyi (Bus), Kabalore (Kab), Kasese (Kas), Lira (Lir), Masaka (Mas), Mbale (Mba), Mbarara (Mb), Nakaseke (Nak), Sironko (Sir), Serere (Ser), Tororo (Tor) and Wakiso (Wak).

AHC Dendrogram



**Figure 3.7:** Clustering analysis of cooking bananas from different districts of Uganda. The cultivars *Kisansa*, *Mbawazirume*, *Mpologoma*, *M9* and *Nakitembe* were analysed according to fruit mineral content, soil and climatic (altitude) properties. District names are: Bushenyi (Bus), Kabalore (Kab), Kasese (Kas), Lira (Lir), Masaka (Mas), Mbale (Mba), Mbarara (Mb), Nakaseke (Nak), Sironko (Sir), Serere (Ser), Tororo (Tor) and Wakiso (Wak).

### 3.3.5 Biochemical analysis of banana leaf tissue

Biochemical data (Fe and Zn content) was collected (as described earlier in Chapter 2) for leaves at different stages of plant development namely: Vegetative, flowering and fruiting. At both the flowering and fruiting stages, samples were collected from the flag, first, second and third leaf positions respectively. The aim of this study was to determine the mineral accumulation and distribution in different leaf positions at development. The Fe and Zn analysis results for each of the different cultivars, in the different leaf types throughout plant development, are shown in subsequent figures below.

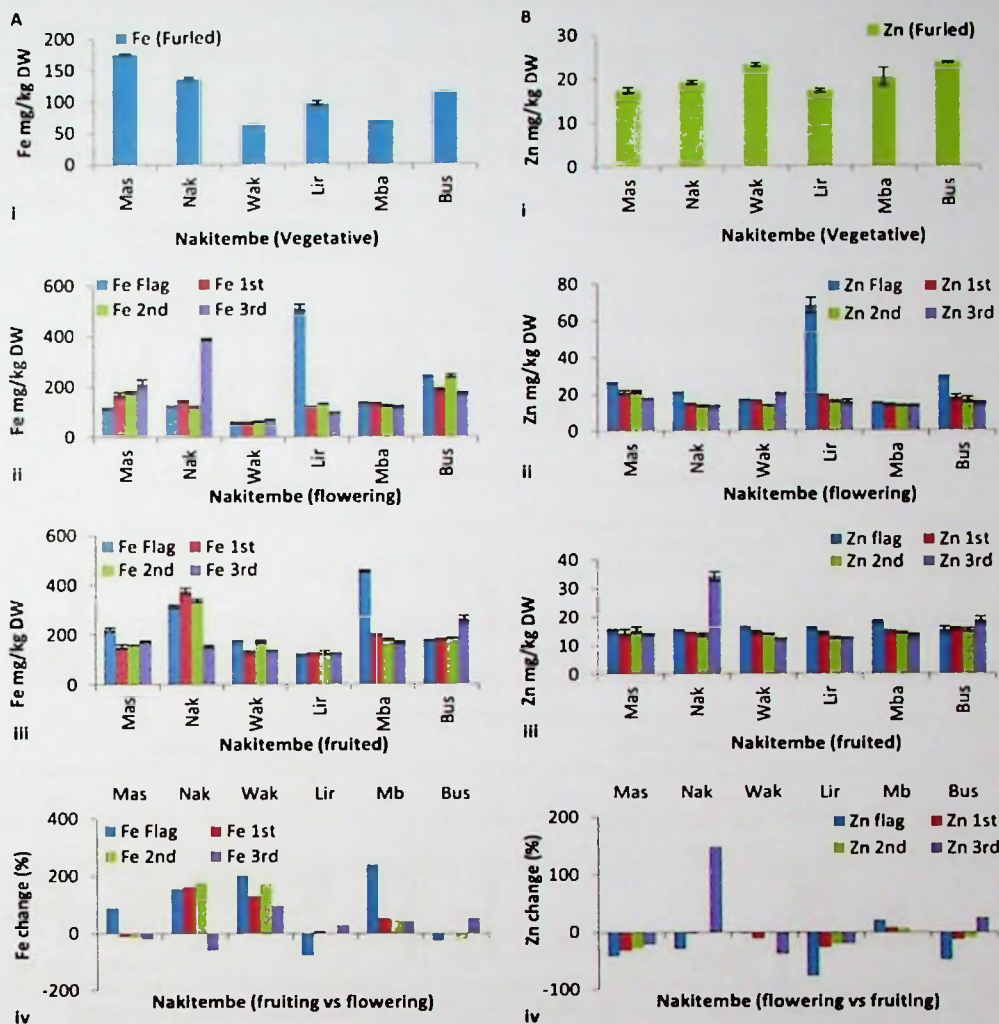
#### 3.3.5.1 *Nakitembe*

Analysis of the Nakitembe cultivar showed that at the vegetative stage (Figure 3.8 a i) Fe content ranged from  $63.58 \pm 1.01$  (Wakiso) to  $176.45 \pm 0.47$  mg/kg DW (Masaka). At flowering and fruiting in the flag leaf (Figure 3.8 a ii), the Fe content ranged from  $57.54 \pm 0.66$  mg/kg DW (Wakiso) to  $511.2 \pm 13.70$  mg/kg DW (Lira), while at fruiting the flag leaf Fe content ranged from  $119.75 \pm 0.37$  mg/kg DW (Lira) to  $455.8 \pm 3.00$  mg/kg DW (Nakaseke); Fe content first leaf at flowering ranged from  $57.54 \pm 0.66$  (Wakiso) to  $185.99 \pm 0.99$  mg/kg DW (Bushenyi); at fruiting in the first leaf Fe content ranged from  $126.13 \pm 0.93$  (Lira) to  $375.02 \pm 13.37$  mg/kg DW (Nakaseke). The Fe levels second leaf at flowering ranged from  $62.36 \pm 1.83$  mg/kg DW (Wakiso) to  $239.46 \pm 5.74$  mg/kg DW (Bushenyi), while at fruiting Fe content ranged from  $128.57 \pm 7.92$  mg/kg DW (Lira) to  $337.42 \pm 5.6$  mg/kg DW (Nakaseke). Finally, Fe content of the third leaf at flowering ranged from  $68.86 \pm 1.00$  mg/kg DW to  $387.89 \pm 3.57$  mg/kg DW (Nakaseke), while at fruiting the Fe content in third leaf ranged from  $124.51 \pm 0.3$  mg/kg DW (Lira) to  $263.33 \pm 12.8$  mg/kg DW.

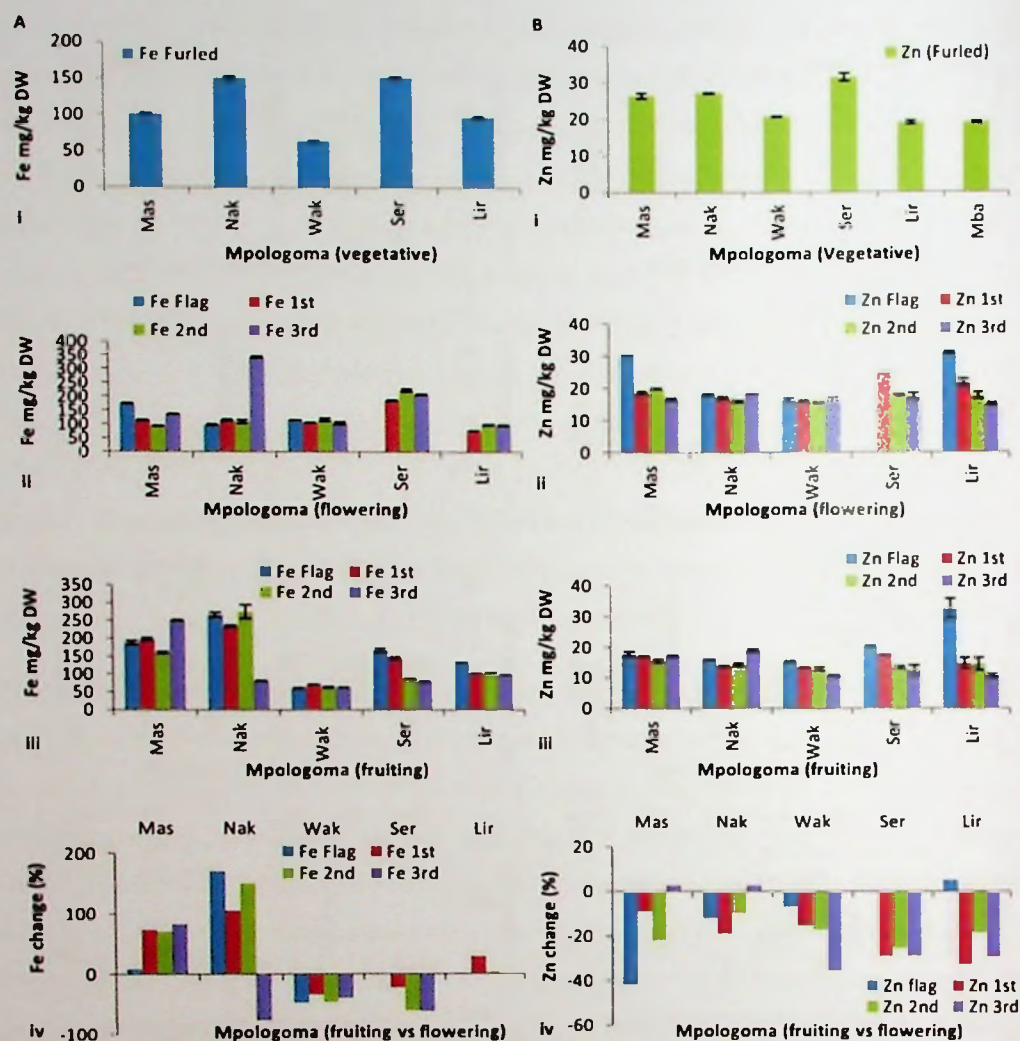
Samples from Wakiso district consistently had the lowest Fe content in all leaf positions analysed at flowering. Overall, at all three stages of Nakitembe cultivar plant development, the majority of the leaves from all of the districts that were analysed had less than 200 mg/kg DW of Fe. The furled leaf stage had less Fe content compared to both the flowering and fruiting stages in the banana cultivar Nakitembe. The fruiting stage accumulated more Fe compared to the flowering stage of leaf development. However, the Fe trends varies at all stages of development as seen in Nakitembe from Nakaseke district where Fe content is higher at fruiting than at flowering. Data on percentage change in Fe levels between flowering and fruiting (Figure 3.8iv) shows that banana leaves accumulate more Fe in various districts at fruiting with exception of a few districts.

The Zn content in the furled leaf at the vegetative stage (Figure 3.8 b i) ranged from  $17.23 \pm 0.43$  mg/kg DW (Lira) to  $23.82 \pm 0.14$  mg/kg DW (Bushenyi). At flowering (Figure 3.8 b ii), flag leaf Zn content ranged from  $15.15 \pm 0.4$  mg/kg DW (Mbarara) to  $29.59 \pm 0.94$  mg/kg DW (Lira); At fruiting stage the flag leaf Zn content ranged from  $15.15 \pm 0.21$  mg/kg DW (Nakaseke) to  $18.27 \pm 0.28$  mg/kg DW (Mbarara); first leaf at flowering (Figure 10 b iii) ranged from  $13.93 \pm 0.55$  mg/kg DW (Mbarara) to  $21.21 \pm 0.93$  mg/kg DW (Lira); at fruiting first leaf stage Zn content ranged from  $14.37 \pm 0.44$  mg/kg DW (Lira) to  $15.78 \pm 0.27$  mg/kg DW (Bushenyi); 2<sup>nd</sup> leaf at flowering (Figure 3.8 b iv) ranged from  $13.64 \pm 0.42$  mg/kg DW (Mbarara) to  $21.50 \pm 0.62$  mg/kg DW (Masaka); at fruiting second leaf Zn content ranged from  $12.86 \pm 0.32$  mg/kg DW (Lira) to  $15.55 \pm 1.04$  mg/kg DW (Masaka); third leaf at flowering (Figure 3.8 b v) ranged from  $13.65 \pm 0.19$  mg/kg DW (Mbarara) to  $20.23 \pm 0.13$  mg/kg DW (Wakiso). At the fruiting stage third leaf Zn content ranged from  $10.93 \pm 0.08$  mg/kg DW (Serere) to  $34.15 \pm 1.61$  mg/kg DW (Nakaseke).

Overall, the vegetative stage had more Zn compared to the flowering stage and there was no observable difference in Zn content at flowering except for the flag leaf from Lira district that had a significant amount of Zn in all leaves at flowering. At flowering stage of development in Nakitembe cultivar, the flag leaf tended to have higher Zn content compared to other leaf positions in samples from each district. At fruiting with exception of the third leaf at fruiting from Nakaseke district, the Zn content reduced from flag leaf to third and was significantly lower at the fruiting stage than at the flowering stage. This therefore, suggests that at fruiting probably Zn is remobilized to the fruit pulp, however, probably all the leaves do contribute to this effect. In summary, the Zn levels (Figure 3.8iv b) in Nakitembe show a reduction in the majority of the leaves with exception of the third leaf from Nakaseke and Bushenyi, and the leaves from Mbarara districts.



**Figure 3.8:** Mineral content of banana leaves from Nakitembe cultivar during plant development. A) Fe and B) Zn content was measured at the i) vegetative ii) flowering and iii) fruiting stages of development. iv) Percentage change in mineral content between flowering and fruiting. Data is mean  $\pm$  SD (n= 3). The names of the districts of Uganda from which samples were taken are abbreviated as follows: Masaka (Mas), Nakaseke (Nak), Wakiso (Wak), Lira (Lir), Mbarara (Mbar), Bushenyi (Bus).



**Figure 3.9:** Mineral content of banana leaves from *Mpologoma* cultivar during plant development. A) Fe and B) Zn content was measured at the i) vegetative ii) flowering and iii) fruiting stages of development. iv) percentage change in mineral content between flowering and fruiting. Data is mean + SD n=3. The names of the districts of Uganda from which samples were taken are abbreviated as follows: Masaka (Mas), Nakaseke (Nak), Wakiso (Wak), Serere (Ser), Lira (Lir).

### 3.3.5.2 *Mpologoma*

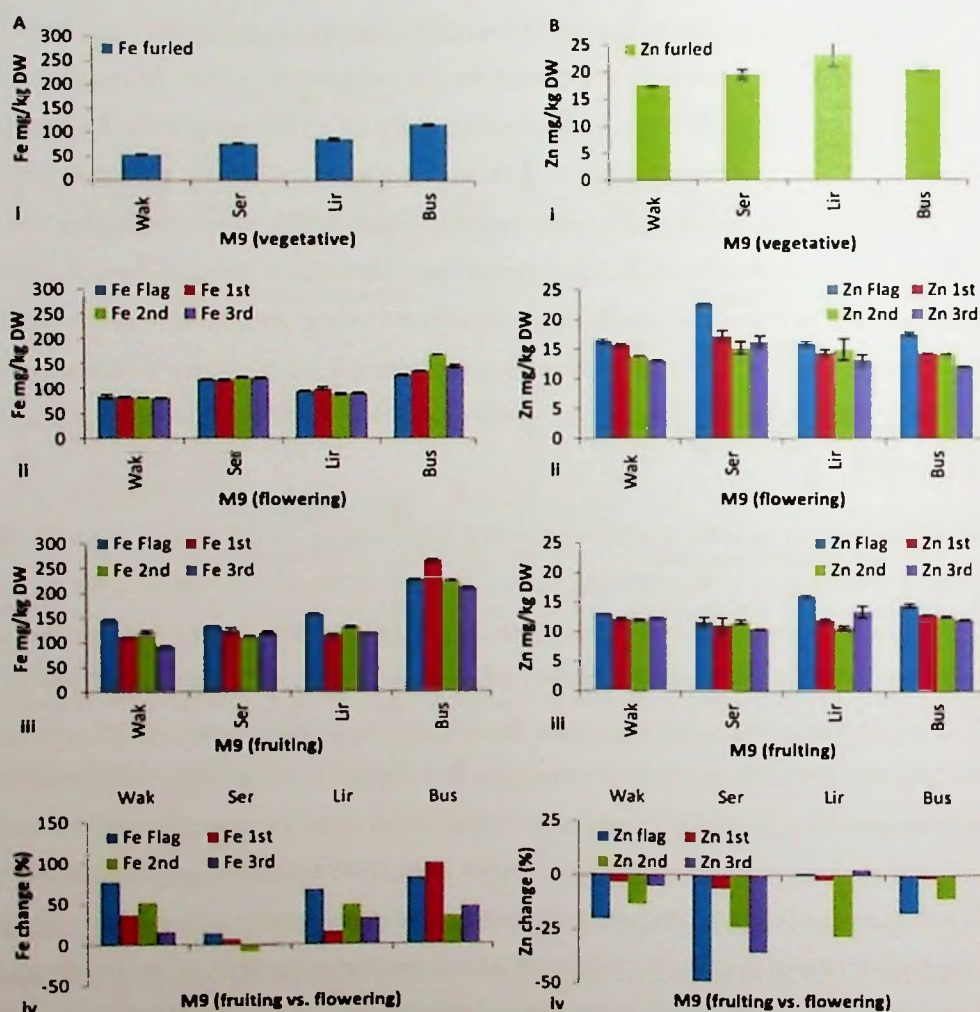
In the *Mpologoma* cultivar Fe content at the vegetative stage ranged from  $64.41 \pm 0.39$  mg/kg DW (Wakiso) to  $152.22 \pm 1.33$  mg/kg DW (Serere). At flowering and fruiting in the flag leaf (Figure 3.9 a ii), the Fe content in the flag leaf ranged from  $98.71 \pm 2.38$  mg/kg DW (Nakaseke) to  $173.46 \pm 13.40$  mg/kg DW (Masaka); at fruiting in flag leaf Fe content ranged from  $61.07 \pm 0.97$  mg/kg DW (Wakiso) to  $267.03 \pm 6.99$  mg/kg DW (Nakaseke); first leaf at flowering (Figure 3.9 a iii) ranged from  $78.97 \pm 1.71$  (Lira) to  $186.07 \pm 1.33$  mg/kg DW (Serere); at fruiting in the first leaf Fe content ranged from  $70.25 \pm 0.81$  (Wakiso) to  $235.16$

$\pm 3.29$  mg/kg DW (Nakaseke); second leaf at flowering (Figure 3.9a iv) ranged from  $93.98 \pm 1.53$  mg/kg DW (Masaka) to  $223.25 \pm 3.36$  mg/kg DW (Serere); at fruiting Fe content ranged from  $63.04 \pm 0.81$  mg/kg DW (Wakiso) to  $274.79 \pm 3.3$  mg/kg DW (Nakaseke); third leaf at flowering ranged from  $98.56 \pm 2.15$  mg/kg DW (Lira) to  $342.88 \pm 3.44$  mg/kg DW (Nakaseke); at fruiting Fe content in third leaf ranged from  $62.77 \pm 0.20$  mg/kg DW (Wakiso) to  $251.83 \pm 0.95$  mg/kg DW (Masaka).

The furled leaf stage has less Fe content compared to both the flowering and fruiting stages in the banana cultivar Mpologoma. However, the Fe trends varies at all stages of development as seen in Mpologoma from Nakaseke district where Fe content is higher at fruiting than at flowering. The Fe content at flowering increased in some districts from flag leaf stage to third leaf stage with Lira district having consistently lower Fe content than other districts in all the positions. At the fruiting stage of development the Fe content varied with no consistent trend, however, Wakiso district had the lowest Fe content in all leaves in Mpologoma cultivar and the majority of the leaves had less than 200 mg/kg DW of Fe. The banana leaves in cultivar Mpologoma have varying Fe levels in all stages of development but with greater reduction in amounts at fruiting compared to other stages. The percentage change in Fe shows varying trends (Figure 3.9 a iv) with an increase in Fe content at fruiting in districts of Masaka, Nakaseke and Lira and a reduction in Wakiso and Serere districts.

The Zn content in the furled leaf at the vegetative stage (Figure 3.9 b i) ranged from  $18.94 \pm 0.47$  mg/kg DW (Lira) to  $31.43 \pm 1.07$  mg/kg DW (Serere). At flowering (Figure 3.9 b ii), flag leaf Zn content ranged from  $16.33 \pm 0.17$  mg/kg DW (Wakiso) to  $30.95 \pm 0.30$  mg/kg DW (Lira); At fruiting, flag leaf stage Zn content ranged from  $15.22 \pm 0.17$  mg/kg DW (Wakiso) to  $32.49 \pm 3.24$  mg/kg DW (Lira); first leaf at flowering (Figure 3.9 b iii) ranged from  $15.77 \pm 0.45$  mg/kg DW (Wakiso) to  $24.69 \pm 0.04$  mg/kg DW (Serere); at fruiting 1<sup>st</sup> leaf stage Zn content ranged from  $13.36 \pm 0.11$  mg/kg DW (Wakiso) to  $17.51 \pm 0.13$  mg/kg DW (Serere); second leaf at flowering (Figure 3.9 b iv) ranged from  $15.46 \pm 0.27$  mg/kg DW (Wakiso) to  $19.92 \pm 0.13$  mg/kg DW (Masaka); at fruiting second leaf Zn content ranged from  $12.81 \pm 0.64$  mg/kg DW (Wakiso) to  $15.53 \pm 0.53$  mg/kg DW (Masaka); third leaf at flowering (Figure 3.9 b v) ranged from  $14.95 \pm 0.38$  mg/kg DW (Lira) to  $18.41 \pm 0.19$  mg/kg DW (Nakaseke). At the fruiting stage third leaf Zn content ranged from  $10.60 \pm 0.63$  mg/kg DW (Wakiso and Lira) to  $18.92 \pm 0.44$  mg/kg DW (Nakaseke).

Overall, the vegetative stage had more Zn compared to the flowering stage and there was no observable difference in Zn content at flowering except for the flag leaf from Lira district that had a high amount of Zn in all leaves. At the flowering stage of development in Mpologoma cultivar, the flag leaf tended to have higher Zn content compared to other leaf positions in samples from each district. At fruiting the Zn content reduced from flag leaf to 3<sup>rd</sup> and was lower at the fruiting stage than at the flowering stage. This therefore, indicates that at fruiting Zn is probably remobilized to the fruit pulp, and it is likely that all the leaves contribute to this effect. This is further confirmed in Figure 3.9 b iv) where there is a clearly observable reduction in Zn con in leaf tissue at fruiting.



**Figure 3.10:** Mineral content of banana leaves from M9 cultivar during plant development. A) Fe and B) Zn content was measured at the i) vegetative ii) flowering and iii) fruiting stages of development. iv) percentage change in mineral content between flowering and fruiting. Data is mean + SD n=3. The names of the districts of Uganda from which samples were taken are abbreviated as follows: Wakiso (Wak), Serere (Ser), Lira (Lir), Bushenyi (Bus).

### 3.3.5.3 M9

Leaf Fe content analysis in the M9 cultivar shows that at the vegetative stage Fe content ranged from  $53.94 \pm 0.63$  mg/kg DW (Wakiso) to  $119.54 \pm 1.93$  mg/kg DW (Bushenyi). At flowering and fruiting in the flag leaf (Figure 3.10a ii), the Fe content in the flag leaf ranged from  $83.38 \pm 3.62$  mg/kg DW (Wakiso) to  $126.74 \pm 1.26$  mg/kg DW (Bushenyi); at fruiting in flag leaf Fe content ranged from  $134.14 \pm 0.52$  mg/kg DW (Serere) to  $227.75 \pm 2.34$  mg/kg DW (Bushenyi); first leaf at flowering (Figure 3.10a iii) ranged from  $82.38 \pm 0.95$  (Wakiso) to  $135.03 \pm 0.85$  mg/kg DW (Bushenyi); at fruiting in the first leaf Fe content ranged from  $111.79 \pm 0.87$  (Lira) to  $266.42 \pm 2.39$  mg/kg DW (Lira); second leaf at flowering (Figure 3.10a iv) ranged from  $80.64 \pm 0.15$  mg/kg DW (Wakiso) to  $168.15 \pm 0.31$  mg/kg DW (Bushenyi); at fruiting Fe content ranged from  $112.5 \pm 0.83$  mg/kg DW (Serere) to  $225.01 \pm 0.66$  mg/kg DW (Bushenyi); third leaf at flowering ranged from  $80.65 \pm 0.78$  mg/kg DW (Wakiso) to  $146.63 \pm 2.73$  mg/kg DW (Nakaseke); at fruiting Fe content in third leaf ranged from  $93.04 \pm 1.09$  mg/kg DW (Wakiso) to  $210.34 \pm 1.76$  mg/kg DW (Bushenyi). Samples from Lira district consistently had the lowest Fe content in all leaf positions analysed at flowering.

Overall, at all three stages of M9 cultivar plant development, the majority of the leaves from all of the districts that were analysed had less than 200 mg/kg DW of Fe. The furled leaf stage has less Fe content compared to both the flowering and fruiting stages in the banana cultivar M9. However, the Fe content increased at fruiting compared to the flowering stage in M9 from all the districts.

The Fe content at flowering increased in some districts from flag leaf stage to second leaf but decreased in the third leaf in all the districts. Wakiso district had consistently less Fe while Bushenyi had the highest Fe in all the leaves at flowering. At the fruiting stage of development the Fe content varied with no consistent trend, however, Wakiso and Serere districts had the lowest Fe content in all leaves in M9 cultivar. Further, the majority of the leaves had less than 200 mg/kg DW of Fe at fruiting stage. The banana leaves in cultivar M9 have varying Fe levels in all stages of development but with greater reduction in amounts at fruiting especially in the 3<sup>rd</sup> leaf compared to other leaf positions. This probably, signifies that Fe is remobilized from the leaves to fruit pulp during fruiting especially in the first to third leaves but not in flag leaves. However, the percentage Fe change analysis (Figure 3.10a iv) shows that in cultivar M9 banana leaves accumulate more Fe at fruiting stage than at flowering stage of development.

The Zn content in the furled leaf at the vegetative stage (Figure 3.10 b i) ranged from  $17.52 \pm 0.19$  mg/kg DW (Wakiso) to  $22.91 \pm 2.18$  mg/kg DW (Lira). At flowering (Figure 3.10 b ii), flag leaf Zn content ranged from  $15.97 \pm 0.32$  mg/kg DW (Lira) to  $22.65 \pm 0.04$  mg/kg DW (Serere); At fruiting flag leaf stage Zn content ranged from  $11.77 \pm 0.71$  mg/kg DW (Serere) to  $16.10 \pm 0.35$  mg/kg DW (Lira); first leaf at flowering (Figure 3.10 b iii) ranged from  $14.21 \pm 0.01$  mg/kg DW (Bushenyi) to  $17.16 \pm 0.87$  mg/kg DW (Serere); at fruiting first leaf stage Zn content ranged from  $11.17 \pm 1.22$  mg/kg DW (Serere) to  $12.94 \pm 0.07$  mg/kg DW (Bushenyi); second leaf at flowering (Figure 3.10 b iv) ranged from  $13.94 \pm 0.01$  mg/kg DW (Wakiso) to  $15.24 \pm 1.00$  mg/kg DW (Serere); at fruiting second leaf Zn content ranged from  $10.73 \pm 0.29$  mg/kg DW (Lira) to  $12.61 \pm 0.18$  mg/kg DW (Bushenyi); third leaf at flowering (Figure 3.10 b v) ranged from  $12.06 \pm 0.38$  mg/kg DW (Bushenyi) to  $16.18 \pm 0.92$  mg/kg DW (Serere). At the fruiting stage third leaf Zn content ranged from  $10.51 \pm 0.07$  mg/kg DW (Serere) to  $13.47 \pm 0.96$  mg/kg DW (Lira).

Overall, the vegetative stage had more Zn compared to the flowering stage and there was no observable difference in Zn content at flowering except for the flag leaf from Serere district that had a significant amount of Zn in all leaves at flowering. At flowering stage of development in M9 cultivar, the flag leaf tended to have higher Zn content compared to other leaf positions in samples from each district. The Zn content tended to decrease in most of the leaves when comparing levels at fruiting and flowering (Figure 3.10 b iv), suggesting that leaf tissue may supply Zn to the developing fruit.

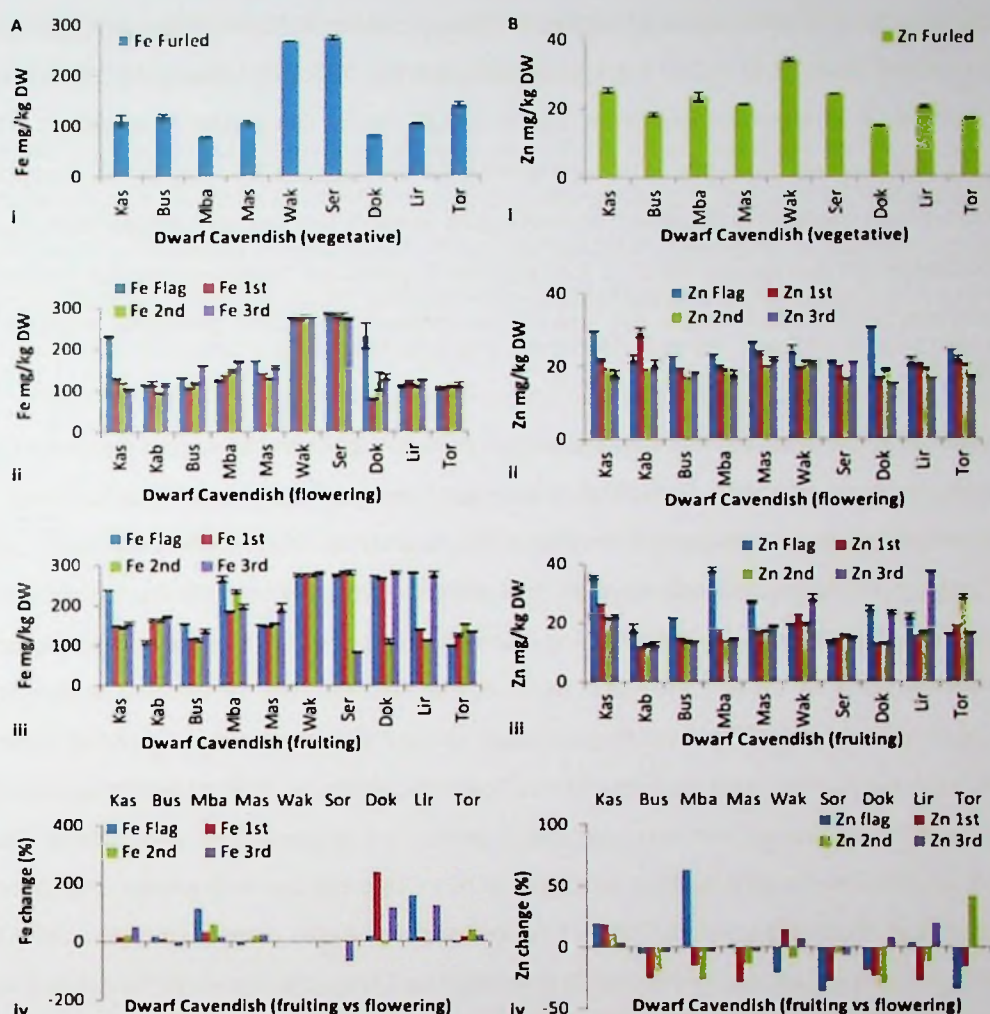


Figure 3.11: Mineral content of banana leaves from Dwarf Cavendish cultivar during plant development . A) Fe and B) Zn content was measured at the i) vegetative ii) flowering and iii) fruiting stages of development. iv) percentage change in mineral content between flowering and fruiting. Data is mean  $\pm$  SD n=3. The names of the districts of Uganda from which samples were taken are abbreviated as follows: Kasese (Kas), Kabalore (Kab), Bushenyi (Bus), Mbarara (Mba), Wakiso (Wak), Serere (Ser), Dokolo (Dok), Lira (Lir), Tororo (Tor).

### 3.3.5.4 Dwarf Cavendish

The results of Fe content in the Dwarf Cavendish cultivar show that at the vegetative stage (Figure 3.11 a i) Fe content ranged from  $78.11 \pm 0.61$  mg/kg DW (Mbarara) to  $266.26 \pm 0.10$  mg/kg DW (Wakiso). At flowering, the Fe content in the flag leaf (Figure 3.11 a ii) ranged from  $104.21 \pm 3.98$  mg/kg DW (Tororo) to  $285.06 \pm 2.03$  mg/kg DW (Serere); at fruiting in flag leaf Fe content ranged from  $98.50 \pm 0.66$  mg/kg DW (Tororo) to  $279.90 \pm 1.30$  mg/kg DW (Lira); first leaf at flowering (Figure 3.11 a iii) ranged from  $79.29 \pm 1.46$  (Dokolo) to  $278.58 \pm 5.80$  mg/kg DW (Serere); at fruiting in the first leaf Fe content ranged from  $116.88$

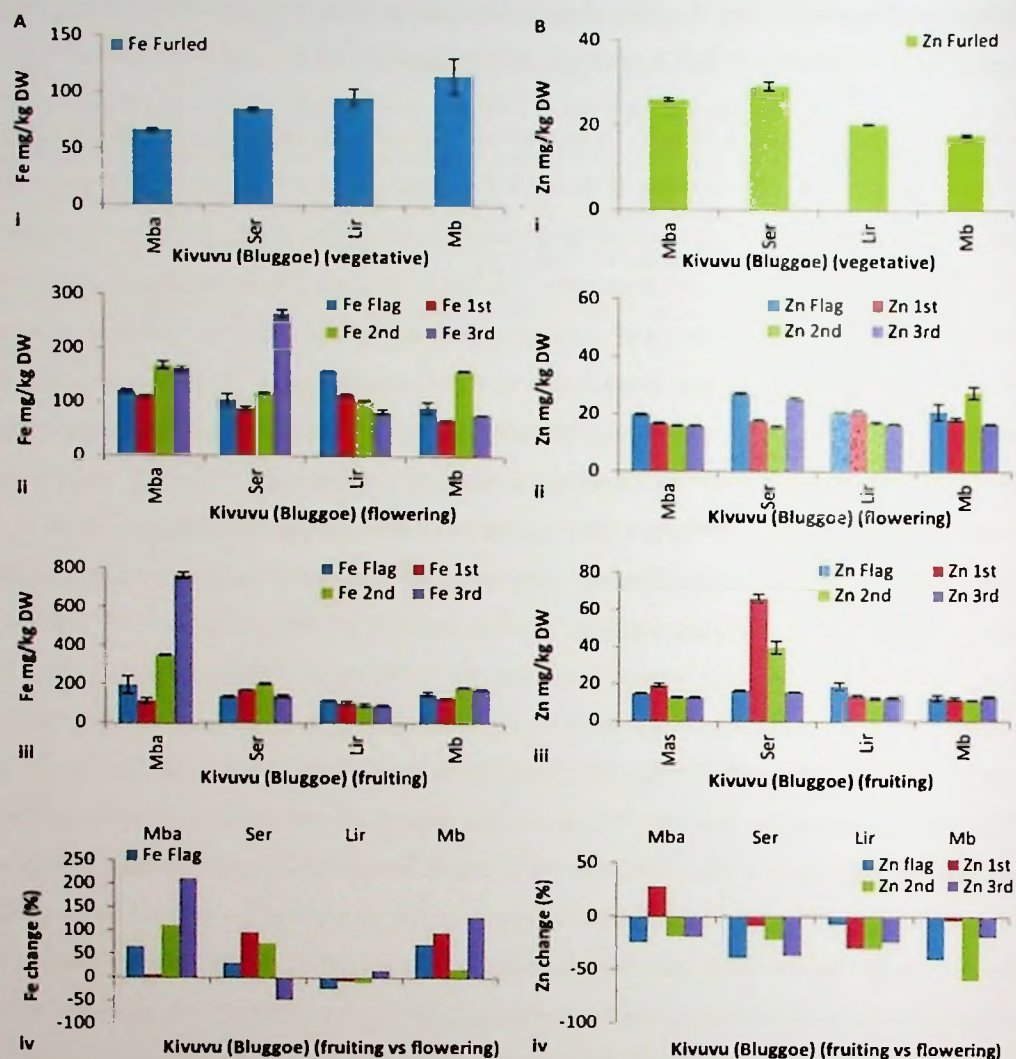
$\pm 0.03$  (Bushenyi) to  $282.23 \pm 3.31$  mg/kg DW (Serere); second leaf at flowering (Figure 3.11 a iv) ranged from  $93.61 \pm 0.62$  mg/kg DW (Kabalore) to  $278.15 \pm 5.24$  mg/kg DW (Serere); at fruiting Fe content ranged from  $108.99 \pm 5.88$  mg/kg DW (Dokolo) to  $280.86 \pm 5.24$  mg/kg DW (Serere); third leaf at flowering ranged from  $102.28 \pm 1.94$  mg/kg DW (Kasese) to  $276.71 \pm 0.64$  mg/kg DW (Wakiso); at fruiting Fe content in third leaf ranged from  $83.30 \pm 1.09$  mg/kg DW (Serere) to  $280.37 \pm 3.27$  mg/kg DW (Wakiso). Samples from Tororo district consistently had on average the lowest Fe content in all leaf positions analysed at flowering.

Overall, at all three stages of Dwarf Cavendish cultivar plant development, the majority of the leaves from all of the districts that were analysed had less than 150 mg/kg DW of Fe. The furled leaf stage except for Serere and Wakiso districts had less Fe content compared to both the flowering and fruiting stages in the banana cultivar Dwarf Cavendish. However, the Fe content varied with an increasing and decreasing trend at fruiting compared to the flowering stage in Dwarf Cavendish from all the districts. The Fe content at flowering decreased in some districts from flag leaf stage to third leaf. There was no general trend on which district had the least amount of Fe at flowering. At the fruiting stage of development the Fe content varied with no consistent trend. The banana leaves in cultivar Dwarf Cavendish had varying Fe levels in all stages of development but with greater reduction in amounts at fruiting especially in the first and second leaves compared to the third leaf position. This probably, signifies that Fe is remobilized from the leaves to fruit pulp during fruiting especially in the first to third leaves but not in flag leaves. However, the percentage Fe change analysis (Figure 3.11 a iv) shows that cultivar Dwarf Cavendish accumulates more Fe at fruiting than at flowering in all the leaf positions with a few exceptions.

The Zn content in the furled leaf at the vegetative stage (Figure 3.11 b i) ranged from  $14.98 \pm 0.15$  mg/kg DW (Dokolo) to  $33.95 \pm 0.56$  mg/kg DW (Dokolo). At flowering (Figure 3.11 b ii), flag leaf Zn content ranged from  $21.07 \pm 0.36$  mg/kg DW (Serere) to  $30.61 \pm 0.25$  mg/kg DW (Dokolo); In fruiting flag leaf, Zn content ranged from  $13.48 \pm 0.44$  mg/kg DW (Serere) to  $35.50 \pm 0.53$  mg/kg DW (Kasese); first leaf at flowering (Figure 3.11 b iii) ranged from  $16.63 \pm 0.20$  mg/kg DW (Dokolo) to  $29.22 \pm 1.30$  mg/kg DW (Kabalore); at fruiting first leaf stage Zn content ranged from  $11.31 \pm 0.52$  mg/kg DW (Kabalore) to  $26.06 \pm 0.27$  mg/kg DW (Kasese); second leaf at flowering (Figure 3.11 b iv) ranged from  $16.42 \pm 0.23$  mg/kg DW (Serere) to  $21.36 \pm 0.05$  mg/kg DW (Wakiso); at fruiting second leaf Zn content ranged from  $12.57 \pm 0.49$  mg/kg DW (Kabalore) to  $28.71 \pm 0.74$  mg/kg DW (Tororo); third leaf at flowering (Figure 3.11 b v) ranged from  $15.20 \pm 0.07$  mg/kg DW (Dokolo) to  $21.97 \pm 0.50$

mg/kg DW (Masaka). At the fruiting stage third leaf Zn content ranged from  $12.82 \pm 0.82$  mg/kg DW (Kabalore) to  $37.35 \pm 0.15$  mg/kg DW (Lira).

Overall, vegetative stage had less Zn compared to the flowering stage and there was no notable difference in Zn content at flowering except for the flag leaf from Kasese and Dokolo districts that had a high amount of Zn in all leaves at flowering. At flowering stage of development in Dwarf Cavendish cultivar, the flag leaf tended to have higher Zn content compared to other leaf positions in samples from each district. The Zn content reduced from flag leaf to third and was comparable to the flowering stage. This therefore, shows that at fruiting probably Zn is remobilized to the fruit pulp, however, probably all the leaves do contribute to this effect. The Dwarf Cavendish cultivar showed a varying trend in the percentage Zn change in different leaf positions with exception of Kasese district with consistently high Zn content other districts showed a reduction and increase in some positions as observed in Figure 3.11b iv.



**Figure 3.12:** Mineral content of banana leaves from Kivuvu (Bluggoe) cultivar during plant development. A) Fe and B) Zn content was measured at the i) vegetative ii) flowering and iii) fruiting stages of development. iv) Percentage change in mineral content between flowering and fruiting. Data is mean  $\pm$  SD n=3. The names of the districts of Uganda from which samples were taken are abbreviated as follows: Mbarara (Mba), Serere (Ser), Lira (Lir), Mbale (Mb)

### 3.3.5.5 *Kivuvu*

The results of Fe content in the *Kivuvu (Bluggoe)* cultivar show that at the vegetative stage. Fe content ranged from  $67.09 \pm 0.94$  mg/kg DW (Mbarara) to  $166.05 \pm 0.10$  mg/kg DW (Mbale). At flowering and fruiting in the flag leaf (Figure 3.12 a ii), the Fe content in the flag leaf ranged from  $90.66 \pm 11.24$  mg/kg DW (Mbale) to  $159.82 \pm 0.36$  mg/kg DW (Lira); at fruiting in flag leaf Fe content ranged from  $123.50 \pm 3.77$  mg/kg DW (Lira) to  $200.60 \pm 4.50$  mg/kg DW (Mbarara); first leaf at flowering (Figure 3.12 a iii) ranged from  $67.81 \pm 0.71$  (Mbale) to  $111.43 \pm 0.53$  mg/kg DW (Mbarara); at fruiting in the first leaf Fe content ranged

from  $107.29 \pm 10.01$  (Lira) to  $173.78 \pm 0.70$  mg/kg DW (Serere); second leaf at flowering (Figure 3.12 a iv) ranged from  $104.79 \pm 1.38$  mg/kg DW (Lira) to  $168.09 \pm 6.48$  mg/kg DW (Mbarara); at fruiting Fe content ranged from  $95.49 \pm 8.39$  mg/kg DW (Lira) to  $356.03 \pm 2.45$  mg/kg DW (Mbarara); third leaf at flowering ranged from  $78.20 \pm 0.08$  mg/kg DW (Mbale) to  $264.73 \pm 7.66$  mg/kg DW (Serere); at fruiting Fe content in third leaf ranged from  $96.07 \pm 4.16$  mg/kg DW (Lira) to  $505.5 \pm 15.2$  mg/kg DW (Mbarara). Samples from Mbale district consistently had on average the lowest Fe content in all leaf positions analysed at flowering except for the second leaf.

Overall, at all three stages of *Bluggoe* cultivar plant development, the majority of the leaves from all of the districts that were analysed had less than 200 mg/kg DW of Fe. The furled leaf stage had less Fe content compared to both the flowering and fruiting stages. There was no general trend on which district had the least amount of Fe at flowering. At the fruiting stage of development the Fe content varied with no consistent trend. The banana leaves in cultivar *Bluggoe* had varying Fe levels in all stages of development but with a slight increase in amounts at fruiting especially in the first and second leaves compared to the third leaf position. The percentage change in Fe content in most leaves at fruit development compared to flowering (Figure 3.12 a iv), except for Lira district, shows that *Bluggoe* leaves accumulated more Fe during development.

The Zn content in the furled leaf at the vegetative stage (Figure 3.12 b i) ranged from  $17.89 \pm 0.28$  mg/kg DW (Mbale) to  $29.37 \pm 1.08$  mg/kg DW (Serere). At flowering (Figure 3.12 b ii), flag leaf Zn content ranged from  $19.92 \pm 0.19$  mg/kg DW (Mbarara) to  $27.22 \pm 0.28$  mg/kg DW (Serere); At fruiting flag leaf stage Zn content ranged from  $12.61 \pm 1.45$  mg/kg DW (Mbale) to  $19.10 \pm 2.10$  mg/kg DW (Lira); first leaf at flowering (Figure 3.12 b iii) ranged from  $16.72 \pm 1.80$  mg/kg DW (Mbarara) to  $20.77 \pm 0.56$  mg/kg DW (Lira); at fruiting first leaf stage Zn content ranged from  $12.13 \pm 0.80$  mg/kg DW (Mbale) to  $19.50 \pm 1.08$  mg/kg DW (Mbarara); second leaf at flowering (Figure 3.12 b iv) ranged from  $15.51 \pm 0.42$  mg/kg DW (Serere) to  $27.68 \pm 2.09$  mg/kg DW (Mbale); at fruiting second leaf Zn content ranged from  $11.43 \pm 0.04$  mg/kg DW (Mbale) to  $13.10 \pm 0.28$  mg/kg DW (Mbarara); third leaf at flowering (Figure 14 b v) ranged from  $15.96 \pm 0.13$  mg/kg DW (Mbarara) to  $25.22 \pm 0.59$  mg/kg DW (Serere). At the fruiting stage third leaf Zn content ranged from  $12.63 \pm 0.45$  mg/kg DW (Lira) to  $16.01 \pm 0.04$  mg/kg DW (Serere).

Overall, vegetative stage had more Zn compared to the flowering and fruiting stage and there was no significant difference in Zn content at flowering. At flowering stage of

development in Bluggoe cultivar, the flag leaf tended to have higher Zn content compared to other leaf positions in samples from each district. The Zn content reduced from flag leaf to third and was comparable to the flowering stage. There was a reduction in Zn content at fruiting this therefore, shows that at fruiting probably Zn is remobilized to the fruit pulp, however, probably all the leaves do contribute to this effect. This is further observed in (Figure 3.12 b iv) where the percentage change in Zn content in different leaves during development show a reduction in Zn content with exception of the first leaf from Mbarara district.

### **3.4 DISCUSSION**

#### **3.4.1 Introduction**

Bananas are a very popular fruit of high economic value to the livelihoods of the people in the East African region, and of great importance in Uganda. They are an important source of nutrients for many people and eaten either as cooked or raw as dessert when ripe (Turner, 1997). They play a pivotal role in the diets of Ugandans, however, little is known about the Fe and Zn content of the Ugandan bananas because most of the reference data is based on Food and Agriculture Organisation food composition tables (Hardisson et al., 2001a). However, little is known about the mineral content of different banana cultivars or the interaction between the soil components found in the different agro-ecological zones of Uganda. According to Tahvonen (1993), many factors affect the elemental content of plants, including variety, state of maturity, soil types, soil condition, fertilization, irrigation and weather. The aim of this study was to generate data on the different factors that influence mineral uptake mechanism in different banana cultivars grown in Uganda in order to understand the mechanism that could generate results to guide future banana biofortification research. To achieve this aim, analysis was performed on the different soils and their properties based on the agro ecological zone where banana fruits were obtained. The Fe and Zn content in the fruit and leaf of both the cooking and dessert bananas grown in different parts of Uganda was also determined.

#### **3.4.2 Status of fruit mineral content**

This study, therefore, aimed to determine the Fe and Zn content in the fruit pulp of both the cooking and dessert bananas grown in different parts of Uganda. Overall, characteristic differences in cultivars that were analysed, showed variations in Fe and Zn levels in all the districts from which samples were taken (Figure 3.4 and Figure 3.5). This was observed regardless of cultivar in both cooking and dessert bananas, where the range of Fe and Zn in

fruit pulp was approximately 10 to 15 mg/kg DW and 5 to 10 mg/kg DW, respectively. Therefore, the results indicate that one kg of fresh Ugandan bananas would contain approximately 3 to 4.5 mg of Fe, given a water content of around 70% (Chandler, 1995), confirming that even high levels of banana consumption cannot meet daily human Fe requirements. This is a concern for high risk groups such as children and lactating mothers, who require 18 and 27 mg Fe per day. The DRI for Fe is 18 mg/day (female) and 8 mg/day (males) while the DRI for Zn is 8 mg/day (female) and 11 mg/day (males) (Wall, 2006). These finds are similar to those reported by Fungo et al. (2010). The low mineral content in the East African Highland cooking bananas may explain the high prevalence of mineral deficiencies in the banana growing regions of East Africa (Kikafunda et al., 2009).

A range of mineral concentrations have been reported for different bananas (Forster et al., 2002a; Fungo et al., 2010; Hardisson et al., 2001a; Wall, 2006; Leterme et al., 2006). However, there is no data available for a majority of the East African cooking and dessert banana of Uganda. The fruit Fe and Zn content of banana studied by Davey et al. (2007) varied between 10 to 15 mg/kg DW and 4.5 to 6.0 mg/kg DW, respectively, and agree well with the results of this study. The average values for Fe and Zn in this study were higher than those previously reported (Fungo et al., 2010; Wall, 2006; Goswami and Borthakur, 1996; Leterme et al., 2006). Further, the data included quality assurance using a certified reference material (WEPAL 195) and analysis of Titanium (Ti) (data not shown), a known indicator of environmental contamination (Cherney and Robinson, 1983; Cook et al., 2009), confirmed that the sample analysis was reproducible and free of potential contamination.

Differences between cultivars are difficult to assess from the literature since these studies were undertaken using a range of different cultivars grown at different times under different climatic and agronomic conditions. The little data that there is available suggests that varietal differences are important, especially in relation to critical concentrations (Martin-Prével, 1980), although these differences have sometimes been ignored. Analysis of the leaf tissue of 30 banana cultivars suggested that cultivars with a *balbisiana* (B) genome had lower concentrations of most elements than other species or than *acuminata* (A genome) cultivars (Turner, 1984). Furthermore, in cultivars containing both A and B genomes, the level of B genome content seemed to correlate with lower nutrient content such that AAA genome cultivars had higher levels than those with the AAB genomes, and the latter were higher than the ABB cultivars. This suggests that mineral uptake of banana cultivars is genome based rather than cultivar based as observed in the data where cooking cultivars with AAA genome had more Fe and Zn than dessert or plantain cultivars that have

a B genome. However, comprehensive assessment of different cultivars across multiple locations in Uganda revealed considerable variability in Fe and Zn content, which has also been observed in previous work (Davey et al., 2007). This makes it difficult to determine if genotype plays a role without analysing the impact of environmental factors. Therefore, it was necessary to investigate what effect geographic factors might have on Fe and Zn accumulation.

#### **3.4.3 Contribution of environmental factors (external factors) to fruit mineral content**

This study analysis was performed on the different soils and their properties based on the agro-ecological zone where banana fruits were obtained. According to Hue et al. (2000), environment plays a major role in nutrient uptake and crop development, the sufficient ranges given for bananas in terms of soil Fe is 75 - 300 ppm. In this study the results in Table 3.2 demonstrated that the soil Fe was sufficient to support banana plant growth, ranging between 101 and 260 ppm. This therefore showed that soil Fe was not a limiting factor in the sampled locations in Uganda. Bruce and Rayment (1982), reported that soil pH is classified as follows: moderately acidic (pH 6.0 to 5.6), slightly acidic (pH 6.5 to 6.1) and neutral (pH 6.6 to 7.3) pH. Soil pH measurements in this study ranged from 5.1 to 7.2 (Table 3.2). Therefore, the data has shown that the Ugandan soils are moderately acidic or neutral but not alkaline, meaning that poor mineral solubility is not a problem with regards to mineral dissolution and uptake. The soil pH affects the availability of various nutrients, toxic elements and chemical species to plant roots, and is therefore, a good predictor of nutrient deficiencies and toxic effects (McKenzie et al. 2004). According to Turner (1997), soil pH ranging from 4.7 to 8.0 has been reported to give good yields in bananas although they prefer neutral to moderately acid conditions (Martin-Prével, 1989). Taken together, the results indicate that both the pH and Fe content of Ugandan soils should be ideal for good banana growth.

In addition, the majority of the soils are sandy clay loams characterized as orthic and humic ferrasols. Based on the data obtained from this study the soils that were sampled are characterized as orthic and humic ferrasols (sand loams, and sandy clay loams) which are deep, thoroughly weathered, leached, fine textured, well drained and are usually red, this is similar to the study conducted by Oduol and Aluma (1990). The most important soil properties governing mineral availability are soil pH, redox conditions, soil structure, organic matter and water content (Frossard et al., 2000; Bronick and Lal, 2005). However,

soil structure is reported to influence soil water movement and retention, erosion, crusting, nutrient recycling, root penetration and crop yield (Bronick and Lal, 2005).

In order to examine the effect of environmental influences on Fe and Zn accumulation in banana fruit, PCA and clustering analysis was performed on the data obtained from fruit mineral content (Figure 3.4 and 3.5) and environmental analysis (Table 3.1 and 3.2). This is because banana mineral composition at harvest may be explained by several factors including cultivar, maturity, climate, soil type, agricultural practices and fertility (Forster et al., 2002b; Tahvonen, 1993). The principle components (PCs) seem to represent the influences of factors grouped by soil texture and climatic variables (PC1, Figure 3.5) and the mineral content of soil and fruit (PC2, Figure 3.6). Interestingly, pH seems to be positively correlated with both PC1 and PC2, while sand composition seems to be negatively correlated with PC1.

In addition, the PCA shows that the cultivars are grouped geographically by the region from which the samples were taken rather than by cultivar genotype (Figure 3.4). This trend is more clearly observed in Figure 3.6. This indicates how climatic conditions play a role in mineral deposition and confirms the trends observed when looking at fruit mineral content alone (Figures 3.4 and 3.5). These findings are similar to those observed by Forster et al. (2002a), where agro-climatic conditions are reported to be the main factors influencing the mineral content in vegetable foods reported in different regions of the world. Furthermore, the mineral content of fruits and vegetables reflects the trace mineral composition of the soil and environment in which the plants grow (Forster et al., 2002a). In addition, plant tissue mineral micronutrient content are strongly dependent on the soil availability of Fe and Zn, which itself depends on factors such as the soil pH, redox status, cation exchange capacity, water content, plant root architecture, and the presence of mycorrhizal fungi (Davey et al, 2007).

#### **3.4.4 Contribution of banana leaves mineral (internal factors) to fruit mineral content**

Leaves may play a significant role in banana mineral content during fruit development and maturation. Therefore, banana leaves from different cultivars at key stages of banana plant development were analysed. This was in order to observe any changes in leaf micronutrient levels during development, and to determine whether the leaves are a significant source of Fe and Zn transported to the developing fruit. Previously, a study by Goswami and

Borthakur (1996) showed how most minerals such as Fe were higher during earlier stages of development and declined towards maturity.

At the vegetative stage Fe and Zn levels were less than that at flowering stage (furled, 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> leaf) but as the leaf grows the Fe and Zn levels increase, but reduce at fruiting although there were variations as well in the mineral content. This study was unable to conclusively determine which leaf contributes to Fe and Zn deposition in fruit, possibly meaning that all leaves contribute significantly to mineral deposition in the banana fruit pulp. Apart from the basis of the genome, Lacoëuilhe and Martin-Prevel (1971) reported that the accumulation or depletion of leaf mineral accumulation is influenced by the physiological and chronological age of the leaf as well as by nutrient supply. Changes of nutrient concentrations with leaf position have been explored (Lahav, 1995). In healthy plants the concentrations of elements such as nitrogen, phosphorus, potassium, copper and sodium decrease as the leaf ages, the concentrations of calcium, magnesium, iron, manganese and zinc increase, while those of sulphur, boron and chlorine are reasonably stable. Changes in nutrient composition with leaf position occur. The nutrients in lamina 3 which decrease with increasing plant size are phosphorus, manganese, copper and zinc, while nitrogen, potassium and magnesium show higher concentrations in medium-large suckers (Turner and Barkus, 1974).

The effect of leaf position in different banana cultivars at vegetative, flowering and fruiting stages of development was analysed. The data obtained in this study showed varying levels of Fe and Zn in different banana cultivars as well as within cultivars from different regions of origin. The Fe and Zn content reduced gradually from flowering to fruiting stages in the different cultivars from flag leaf to fruiting stage of development. According to the data of (Twyford and Walmsley, 1974; Walmsley and Twyford, 1976), in their study the banana leaves showed varying amounts of Fe and Zn that were in line with this work, where Fe content increased with the maturity of the leaves. Similarly, in the study conducted by Leterme et al. (2006), among the fruits studied the leaves appeared to have high amounts of minerals especially Fe, however, high variability in amounts of Fe in different plant leaves was also observed. Interestingly, high mineral content has been observed in other plants. For example, Fe availability in African and Indian green vegetable leaves, for example, ranges from 2.5% to 27% (Agte et al., 2000; Kumari et al., 2004), whereas those of Zn and Cu range from 11% to 26% and 18% to 47%, respectively, in Indian green vegetables (Agte et al., 2000).

In this work, it was found that elements Fe increases for some of the cultivars and Zn decrease as the banana fruit matures, however, more Fe and Zn was observed in the leaves of the cooking cultivars than in the dessert cultivars studied. Previous studies have reported that after bunch emergence mobile nutrients can be redistributed within the plant and nutrients can move from the leaf system to the growing fruit. A decrease in nutrient concentrations in the lamina is often observed between bunch emergence and harvest (Martin-Prével et al., 1987; Twyford and Walmsley, 1974). According to (Langenegger and Plessis, 1977), the magnitude of the change depends upon the nutrient concerned, the external nutrient supply, dry matter accumulation, and climatic changes from bunch emergence to harvest.

#### 3.4.5 Conclusion: Future directions and summary statement

This study set out to identify the different factors that influence mineral uptake in different banana cultivars grown in Uganda. Variability was observed in the Fe and Zn content in the fruit and leaf tissue within the broad range of cultivars that were examined, which seemed to be more influenced by environmental factors than by genotype. In addition, during banana fruit development, leaf tissue may serve as an important source of minerals such as Fe and Zn.

Further avenues of exploration may include getting more Fe and Zn data from cultivars that were not investigated in this study to try to get a more complete picture of how these elements accumulate in leaf during development. It may also be useful in future to obtain Fe and Zn content from other cultivars that were not available for this study.

Broader analysis of more environmental and climatic conditions in future PCA analysis, assess the influence of more climatic variables and the inclusion of more minerals, such as magnesium and calcium, may yield more trends and relationships that were not possible to investigate given the current scope of the research, which was limited to Fe and Zn as they are important targets for micronutrient biofortification (Saltzman et al., 2013; White and Broadley, 2009; White and Broadley, 2005).

Finally, this work demonstrates that Fe biofortification of bananas to improve nutritional properties of fruit is needed and may need to be achieved via enhancement of Fe uptake from the soil via the roots or by more effective redistribution from source tissues such as leaves to the fruit pulp. Further, this analysis indicates that there is no high Fe banana

germplasm from which to obtain genes for banana Fe biofortification, as no banana cultivars with nutritionally significant levels of Fe were identified.

# Chapter 4: Molecular and biochemical characterisation of transgenic bananas containing iron uptake and storage transgenes

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## 4.1 INTRODUCTION

Access to a healthy diet is a fundamental right of all human beings. Deficiencies of minerals and vitamins affect a large proportion of the world's population, particularly in the developing world (Stein, 2010). Micronutrient malnutrition affects more than two billion people, mostly among resource poor countries like Uganda with Fe, Zn and vitamin A deficiencies most prevalent (Kennedy et al., 2003). In South Asia and Sub-Sahara Africa a large number of disability adjusted life years are lost due to selected nutritional deficiencies (Caulfield et al., 2006). IDA in Uganda is more prevalent in children and women of child-bearing age (Khush et al., 2012; Acham et al., 2012). The major causes of anaemia in developing countries, including Uganda are mainly linked to low bioavailability of Fe from plant based diets such as banana and limited use of Fe-fortified infant foods and cereals (Kikafunda et al., 2009).

In Uganda, progress in tackling anaemia in general and nutritional anaemia has been slow despite its reflection in international goals and resolutions as well as various policies (MOH, 2002). Furthermore, data obtained from our baseline study (Chapter 3) showed that there are no high Fe and Zn bananas in Uganda and available germplasm does not seem to have candidate genes useful for biofortification, and the soil environment does not limit Fe availability to banana plants. Banana (*Musa ssp*) is an important source of calories and a staple crop for more than 300 million people in sub-Saharan Africa. A banana staple diet, however, does not provide adequate source of Fe, Zn, pro-vitamin A and vitamin E (Robinson and Saúco, 2010). Previous work in this thesis demonstrated that the typically Fe concentrations in the fruit pulp of Ugandan banana cultivars ranged between 7 to 12 mg/kg DW (Chapter 3), requiring the consumption of between 1.5 and 3 kg (dry weight) of banana per day to meet the required daily allowance (RDA) of 18 mg Fe for an adult woman.

Naturally, such consumption levels are too high to be a practical solution for IDA without further improvement.

To address this problem, biofortification could be advantageous for people unable to change their dietary habits because of financial, cultural, regional or religious restrictions. It can also be advantageous to governments because of its potential cost effectiveness and sustainability compared to nutritional supplement programs (Mayer et al., 2008). Banana is a particularly suitable target for biofortification because IDA is a serious problem in Uganda where banana is a major staple crop (UDHS, 2011). Further, in the EAHBs there is insufficient genetic variability to achieve Fe biofortification target levels using traditional breeding approaches (Chapter 3). When breeding approaches for enhanced nutrient content are limited, the use of recombinant DNA strategies to engineer enhanced micronutrient accumulation in plants may be required to insure adequate nutrient balance, as is the case in staple crops like banana.

While Fe is abundant in the earth's crust (5%), the ferric form of Fe is very insoluble, particularly in calcareous or high pH soils (White and Broadley, 2005). Plants like banana increase Fe solubility by reducing the local pH around root hairs (Marschner and Römheld, 1994) by actively pumping protons via proton transducing ATPase. Reduction of ferric Fe to ferrous Fe via ferric chelate reductase also substantially increases Fe solubility. Ferrous Fe is then transported into root hairs via various transporters (Jeong and Connolly, 2009).

Several biotechnological strategies have been used to enhance Fe accumulation in edible plant parts. These include increasing Fe uptake using both FRO2 and IRT1 proteins (Connolly et al., 2002; Vasconcelos et al., 2006) and over-expression of the Fe storage protein ferritin (Ravet et al., 2009b). Such approaches to increasing the Fe uptake and storage capacity may potentially be used to create bananas with elevated Fe content. The approach taken by researchers at the Queensland University of Technology (QUT, Australia), in collaboration with the National Agricultural Research Organization (NARO, Uganda), to address this problem is to generate consumer acceptable banana varieties with significantly increased levels of Fe in the fruit pulp using genetic engineering. Promising genes for application include the *IRT1* and *FRO2* Fe assimilation genes from *Arabidopsis thaliana*, and the *ferritin* gene from Soybean (*Sfer*).

Such an approach also requires well characterized promoters for targeted transgene expression. Several studies have reported the identification and isolation of transcripts from genes associated with banana (Holdsworth et al., 1987; Medina-Suárez et al., 1997;

Trivedi and Nath, 2004). The banana expansin1 (Exp1) promoter is a promising candidate for use in banana biofortification as expansin proteins that have been reported to participate in fruit ripening in addition to cell wall expansion, organogenesis, and seed germination (Trivedi and Nath, 2004). Further, transcripts of expansin1 isolated from banana fruit (Cavendish, cv Robusta) were shown to be ethylene regulated during ripening (Cosgrove, 2000; Trivedi and Nath, 2004). Promoters from the cauliflower mosaic virus (CaMV) and banana bunchy top virus (BBTV) are also promising candidates. The constitutive CaMV 35S promoter has been used successfully in banana transformation (Sagi et al., 1995; Becker et al., 2000; Khanna et al., 2004), and BBTV promoter sequences have been shown to direct reporter gene expression in banana vascular tissue (Dugdale et al., 2000; Dugdale et al., 2001).

The aim of the research described in this thesis was to characterise transgenic banana lines developed with Fe uptake and storage genes using molecular and biochemical tools.

The specific objectives of this chapter were to:

1. Characterise transformed lines using PCR, RT-PCR and Southern hybridisation.
2. Biochemical analysis of banana fruit pulp from transgenic banana lines.
3. Biochemical analysis of banana leaves from transgenic banana lines.
4. Evaluate potential effects of transgenes on banana phenotypes developed under field conditions.

## 4.2 MATERIALS AND METHODS

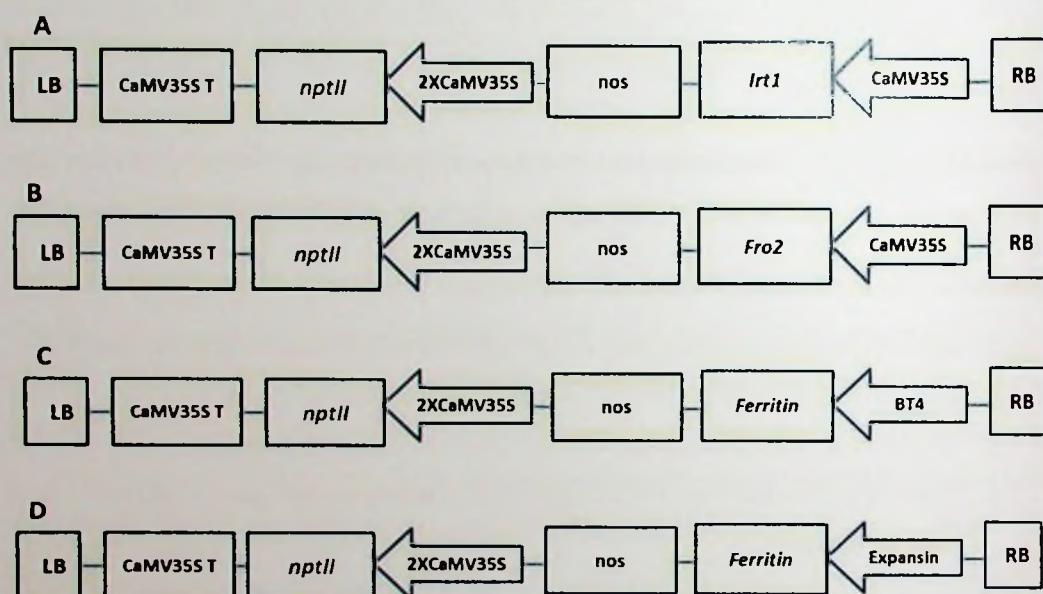
### 4.2.1 Production of transgenic plants

Transgenic banana plants harbouring the iron uptake (*FRO2* and *IRT1*) and storage enhancing (*Sfer*) genes were generated at the NARO (Uganda) as part of the research described in Chapter 2.4.1. These transgene constructs were cloned into the plant expression vector, pCAMBIA 2300 (pCAM). Iron uptake genes were under the control the constitutive CaMV35S promoter whereas storage enhancing gene, *Sfer*, was driven by the banana bunchy top virus (BT4) and banana fruit expansin (Exp1) promoters (Table 4.1 and Figure 4.1). This was aimed at determining which gene combination is appropriate to enhance Fe uptake and storage in the fruit pulp.

Transgenic plants were weaned and acclimatized in a biosafety glass house level II for 3 months and later transferred to confined field environment under the natural conditions favourable for banana plant growth.

**Table 4.1:** Description of plant expression vectors used in the study

| Description    | Transgene   | Origin                      |
|----------------|-------------|-----------------------------|
| pCAM-35S-IRT1  | <i>IRT1</i> | <i>Arabidopsis thaliana</i> |
| pCAM-35S-FRO2  | <i>FRO2</i> | <i>Arabidopsis thaliana</i> |
| pCAM-Exp1-Sfer | <i>Sfer</i> | <i>Glycine soja</i>         |
| pCAM-BT4-Sfer  | <i>Sfer</i> | <i>Glycine soja</i>         |



**Figure 4.1:** Schematic representation of binary expression vectors used for transformation of banana. A) pCAM-35S-IRT1 and B) pCAM-35S-FRO2 vectors contain Arabidopsis IRT1 and FRO2 genes, respectively. The C) pCAM-BT4 and D) pCAM-Exp vectors both contain the soybean ferritin (*Sfer*) transgene. The figure illustrates the T-DNA region between the right and the left borders of each vector.

## 4.2.2 Molecular analysis of transgenic plants

### 4.2.2.1 Genomic DNA isolation and PCR analysis

Genomic DNA was isolated from leaves of putative transgenic plants and untransformed banana plants as described in Chapter 2.4.2. Screening of gDNA for the presence of respective transgenes was carried out by PCR in 20  $\mu$ L reactions as described (Chapter 2.5.1) using gene-specific primers (Table 4.2). The gDNA template integrity was confirmed by PCR using a primer pair designed to amplify banana *Actin*. The plant transformation vector pDNA containing either the *IRT1*, *FRO2* or *Sfer* genes was used as template for the

respective positive control reactions. DNA extracted from untransformed plants of each cultivar and water were used as negative and non-template controls, respectively.

**Table 4.2:** List of primers used for PCR analysis of transgenic banana plants

| Primer  | Primer sequence (5' to 3')     | Tm (°C) | Expected size (bp) |
|---------|--------------------------------|---------|--------------------|
| F-35S   | AAGCTTGGTACCTCATGGAGTCAAAGATTC | 59      | 520                |
| R-IRT1  | TCTAGATTAAGCCCATTTGGCGAT       | 63      |                    |
| F-35S   | AAGCTTGGTACCTCATGGAGTCAAAGATTC | 59      | 580                |
| R-FRO2  | TCACTGAGACTATTCCTAATGGC        | 53      |                    |
| F-BT4   | GACATCACGTGCGTCAACCAAATGC      | 54      | 840                |
| R-Sfer  | GCAGTTGGAACAGCAAGCTCGC         | 58      |                    |
| F-Exp1  | GCCACCAACTTGTCTTTCC            | 54      | 368                |
| R-Sfer  | GCAGTTGGAACAGCAAGCTCGC         | 58      |                    |
| F-Actin | CTGGTGATGGTGTGAGCCAC           | 56      | 640                |
| R-Actin | CATGAAATAGCTGCACAAATGGTATAAGG  | 55      |                    |

#### 4.2.3 RT-PCR analysis

RNA was extracted from leaf tissue of putatively transformed banana plants along with controls as previously described (Chapter 2.4.3), and was eluted in 50 µL of RNase-free water. The RNA was treated with DNase enzyme RQ1 (Promega) to remove the traces of DNA. One µg of RNA was used for first strand cDNA synthesis using AMV (Promega) cDNA synthesis kit. The absence of DNA in the RNA extracts was confirmed by standard PCR using β-actin primers before RT-PCR (Chapter 2) with gene-specific primers (Table 4.3).

**Table 4.3:** List of primers used for RT-PCR analysis of transgenic banana plants

| Primer    | Primer sequence (5' to 3') | Tm (°C) | Expected size (bp) |
|-----------|----------------------------|---------|--------------------|
| F-IRT1    | ATGGCACTCGTGGATCTTCT       | 64.1    | 211                |
| R-Nos     | ATGTGATAATCATCGCAAGACC     | 62.6    |                    |
| F-FRO2    | GACCGTCCATCTCCAACATC       | 64.4    | 357                |
| R-Nos     | ATGTGATAATCATCGCAAGACC     | 62.6    |                    |
| F-Sfer    | GAAGGGTTGGAAAGGGTCA        | 63.9    | 159                |
| R- Nos    | ATGTGATAATCATCGCAAGACC     | 62.6    |                    |
| F-β Actin | CAACAATACTTGGGAAAACGG      | 66.4    | 150                |
| R-β Actin | TGGCTGACACTGACGACATTC      | 62.9    |                    |

#### 4.2.4 Southern hybridisation

To confirm the stable integration of *IRT1*, *FRO2* and *Soy ferritin* genes and copy number in transgenic banana plants the plants that were PCR positive for the gene of interest and an untransformed plant (randomly selected) were analysed. The genomic DNA was isolated as described in Chapter 2.4.2 and the quality of the gDNA was assessed by electrophoresis through 0.8% agarose gels and quantified in a Nano drop 2000. Genomic DNA (10 µg) was digested using EcoR1 enzyme (Roche), which cuts once within the T-DNA of the pCAMBIA2300 binary vector. The digested genomic DNA, along with control plasmid, was separated on 0.8% (w/v) agarose gel before Southern hybridization as per Chapter 2.5.5. Probe was prepared by labelling PCR product of promoter and gene (Chapter 2.5.5.1).

#### 4.2.5 Banana leaf and fruit analysis

Banana leaf and fruit sample preparation was carried out as per Chapter 3.3.1. Statistical analysis first performed by testing the continuous variable data from the control and test groups for normality using the Kolmogorov-Smirnov (K.S.) test at  $P < 0.05$  and for homogeneity of variance using Bartlett's test at  $p < 0.01$ . After confirming normality and homogeneity of variance, the control and test groups were compared using ANOVA (XLSTAT 2014) and the means were separated by the Least Significant difference (LSD).

### 4.3 RESULTS

#### 4.3.1 PCR screening of transgenic lines

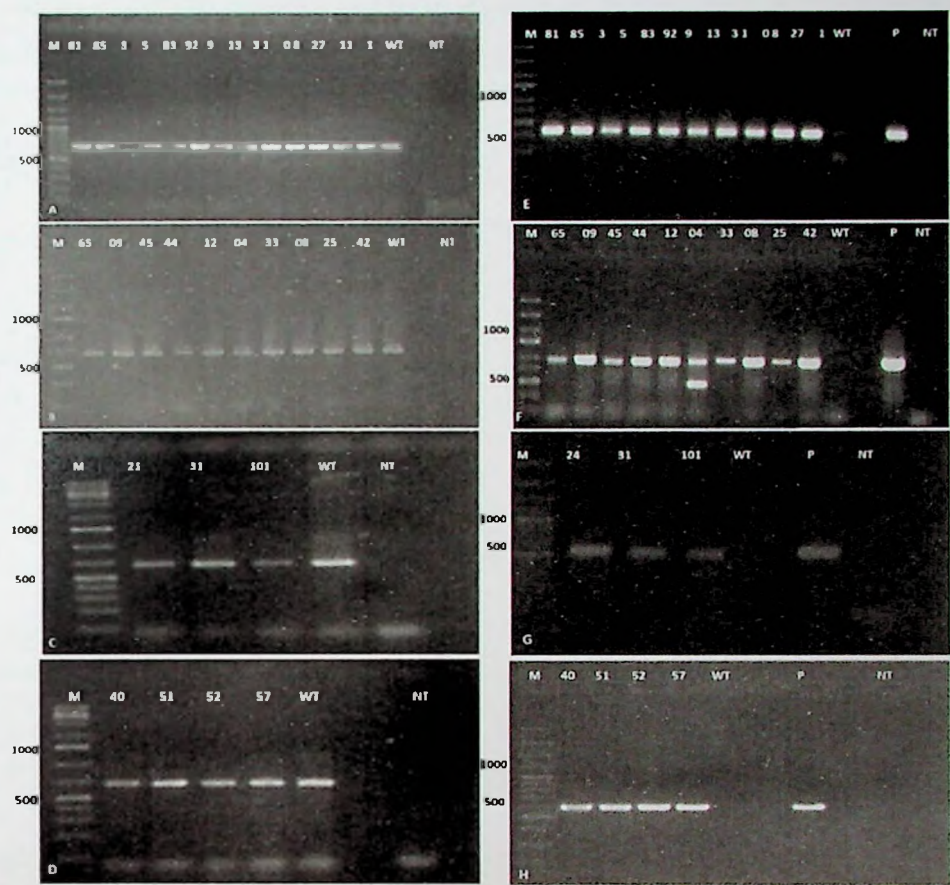
After transformation, selection, regeneration, and acclimatization a total of 73 putative transgenic lines were available for molecular characterization, including 2 non-transgenic lines. These plants were first screened by PCR to confirm the presence of the transgene as described in Chapter 4.2.2 (Table 4.4), before subsequent analysis by RT-PCR and Southern hybridization.

**Table 4.4:** PCR screening results summary

| Transgene            | 35S-FRO2 | 35S-IRT1 | BT4-Sfer | Exp-Sfer |
|----------------------|----------|----------|----------|----------|
| Total lines screened | 15       | 15       | 21       | 22       |
| Actin (+)            | 15       | 15       | 21       | 22       |
| Transgene (+)        | 4        | 3        | 21       | 22       |

The amplification of the *Actin* gene, the internal control for DNA quality, was observed for all plants, including non-transgenic plants (Figure 4.2). Distinct amplicons of the expected

size (640 bp) and 380 bp were obtained (Figure 4.2 A, C,E and G), confirming that the gDNA quality was high.

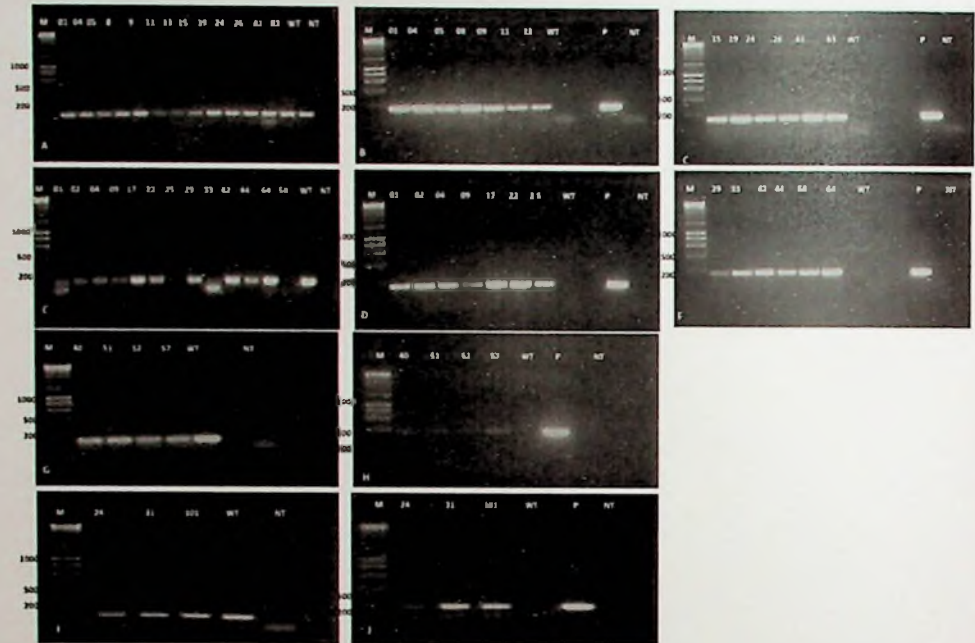


**Figure 4.2:** PCR analysis of genomic DNA from different transgenic and non-transgenic banana plants using gene specific primers. A), B), C), and D) are banana Actin-specific gels. Transgenic banana lines transformed with *Exp-Sfer* (E; Lanes 81-27), *BT4-Sfer* (F; Lane 65-42), *35S-FRO2* (G; Lane 40-57) and *35S-IRT1* (H; Lane 24-101) were screened for the presence of transgene integration. The following lanes were also present: Fermentas Ladder 1 (M); wild type (WT); vector DNA (P); non-template control (NT).

PCR analysis on DNA extracted from lines transformed with *Exp-Sfer*, *BT4-Sfer*, *35S-FRO2* and *35S-IRT1* (Figure 4.2) yielded the expected products of size 368 bp, 840 bp, 580 bp and 520 bp, respectively. The obtained products were consistent with the sizes of the plasmid DNA control. This indicated that transformation of plants with transgenes *Exp-Sfer*, *BT4-Sfer*, *35S-FRO2* and *35S-IRT1* was succesful for the genes of interest and these PCR primers appear to be specific to each gene of interest. Transgenes amplified well in all transgenic lines and pDNA control and no amplification in non-transgenic or non-template controls.

### 4.3.2 RT-PCR analysis

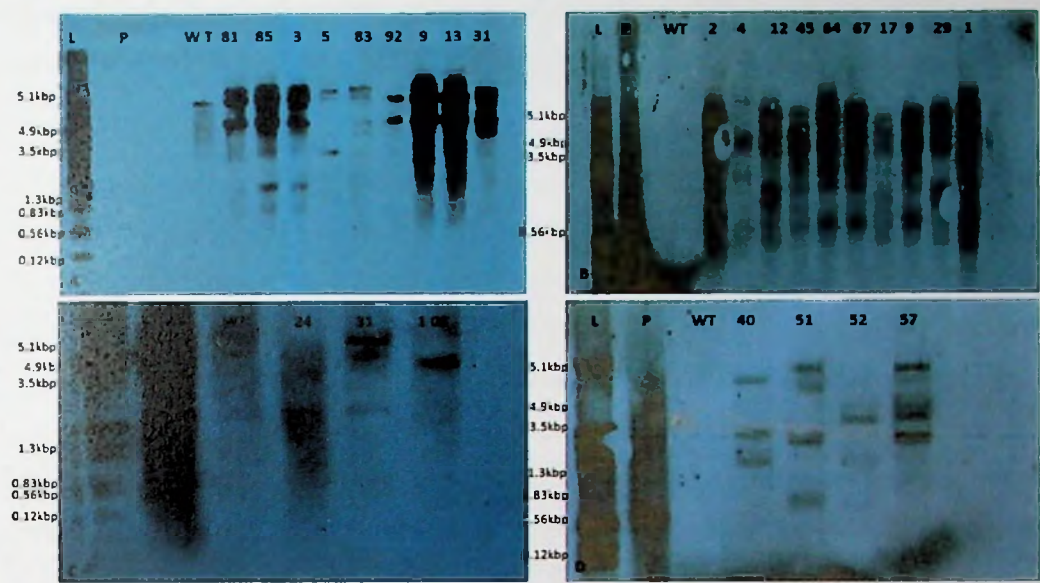
Though stable integration of transgenes into plant host genomes was indicated by PCR analyses, expression of these transgenes needed to be confirmed by RT-PCR. Therefore, semi-quantitative RT-PCR was carried out (Figure 4.3) at vegetative leaf stage on selected transgenic banana lines screened previously (Figure 4.2) as described in Chapter 4.2.3.



**Figure 4.3:** RT-PCR analysis of transgenic banana lines to determine gene expression. The banana Actin gene was analysed for A) Exp1-Sfer, D) BT4-Sfer, G) 35S-FRO2 and I) 35S-IRT1 transgenic lines. Lines transformed with Exp1-Sfer (B and C; lanes 01 – 83), BT4-Sfer (E and F; lanes 01 – 64), 35S-FRO2 (H; lanes 40 – 51) and 35S-IRT1 (J; lanes 24 – 101) also were screened for transgene expression. The following lanes were also present: Hyper Ladder 1 (M); Wild type (WT); Genomic DNA (P); non-template control (NT).

RT-PCR expression analysis showed the amplification products were of the expected size of 150 bp (*Actin*), 159 bp (*Sfer*), 357 bp (*FRO2*) and 211 bp (*IRT1*) compared to the respective positive control pDNA, indicating expression of the transgenes. As expected, the non-template RT-PCRs yielded no product. Actin primers specific for RT-PCR analysis indicated the absence of gDNA contamination and confirmed good quality RNA, giving strong amplifications in all reactions except for 25, 33 and 58 (Figure 4.3, panel D). In these cases, the expected Actin bands were faint or not observed. However, strong amplification of the transgene in the respective samples suggests that the issue was poor RT-PCR of these particular samples, and not the template quality. Overall, RNA quality was sufficient to determine the presence of transgene expression by RT-PCR. Interestingly, RT-PCR products

of the transgenic lines transformed with *35S-FRO2* and of *35S-IRT1* line 24 were faint, indicating weak transcription.



**Figure 4.4:** Southern blot analysis on selected transgenic banana lines. The gels show analysis of selected transgenic events transformed with **A)** *BT4-Sfer* (lanes 1 – 10), **B)** *Exp1-Sfer* (lanes 1 – 10), **C)** *35S-IRT1* (lanes 1 – 3) and **D)** *35S-FRO2* (lanes 1 – 4). Unique cutter restriction enzyme *EcoR1* and *nptII* probe was used. The following lanes were present in all gels: molecular marker ladder (L), plasmid (P); wild type (WT).

#### 4.3.3 Southern analysis of transgenic lines transformed with Exp-Sfer, BT4-Sfer, 35S-FRO2 and 35S-IRT1 transgenes

Integration patterns of the transgenes into the genome of banana lines expressing *BT4-Sfer*, *Exp1-Sfer*, *35S-FRO2* and *35S-IRT1* were analysed by Southern blot analysis (Figure 4.4). The mobility of the bands differed in most transgenic lines, indicating that these lines represent different events. Number of integrations varied between one and seven. The lowest integration numbers (one and two) were detected in *BT4-Sfer* (lanes 6 and 8; Figure 4.4 A), *Exp1-Sfer* (lanes 3 and 10; Figure 4.4 B), *35S-IRT1* (lane 3; Figure 4.4 C) and *35S-FRO2* (lane 3; Figure 4.4). *BT4-Sfer* and *Exp1-Sfer* had lines with the highest integration patterns (greater than 3).

#### 4.3.4 Biochemical analysis of transgenic banana lines

In this experiment, selected transgenic banana lines and the non-transformed controls were analysed for mineral content. Levels of Fe and Zn were of particular interest as these minerals are important biofortification targets. The results of the analysis are shown below.

#### 4.3.4.1 Analysis of mineral accumulation in fruit of Nakinyika and Sukali Ndiizi

##### 4.3.4.1.1 Fruit analysis: Nakinyika Cultivar

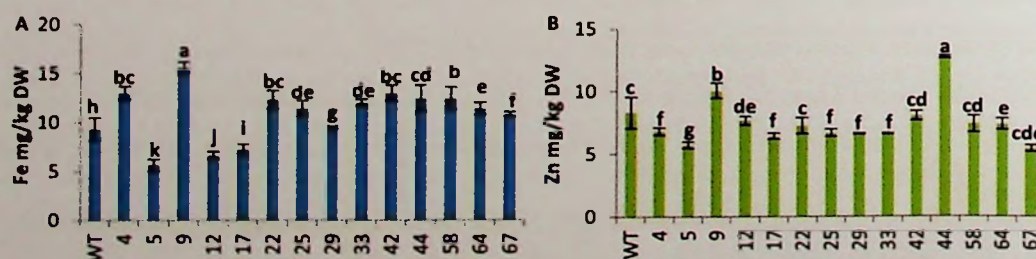
In this experiment out of 20 transgenic lines (*BT4-Sfer*), 14 lines were chosen that were showing a given trend of low, moderate and high expression levels of the respective transgenes (Figure 4.5).

The results show that the Fe content of the wild type was  $8.30 \pm 1.13$  mg/kg DW, and that of the transgenic banana lines transformed with *BT4-Sfer* ranged from  $5.64 \pm 0.56$  to  $15.63 \pm 0.54$  mg/kg DW. Transgenic line 5 had the lowest and line 9 had the highest Fe content, which was 1.7 times more than wild type. With the exception of transgenic lines 5, and 12 that had the lowest Fe content, the transgenic lines accumulated significantly more Fe than wild type at  $P \leq 0.05$ . The Zn content of the wild type was  $8.28 \pm 1.26$  mg/kg DW, and that of the transgenic lines ranged from  $4.75 \pm 0.11$  to  $10.01 \pm 0.62$  mg/kg DW. The Zn content of the transgenic lines was significantly different from the wild type at  $P \leq 0.05$ . The transgenic lines had less Zn compared to wild type.

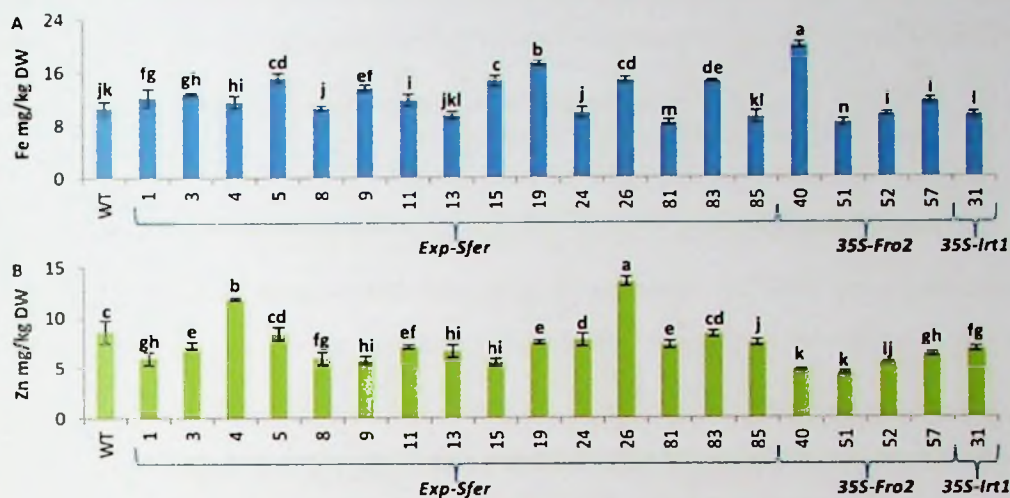
Overall, compared to wild type plants, *BT4-Sfer* expression in banana fruit accumulated slightly more Fe but less Zn in the fruit pulp. This shows that ferritin overexpression leads to accumulation of Fe but not Zn in banana fruit pulp of cooking cultivar Nakinyika.

##### 4.3.4.1.2 Fruit analysis: Sukali Ndiizi Cultivar

*Sukali Ndiizi* lines transformed with *35S-IRT1*, *35S-FRO2* and *Exp1-Sfer* were analysed to determine Fe and Zn content in the fruit pulp (Figure 4.6). The transgenic lines for *Exp1-Sfer* were selected based on RT-PCR expression analysis.



**Figure 4.5:** Mineral content of transgenic Nakinyika lines. A) Fe and B) Zn content was measured in the fruit of wild type (WT) plants and lines transformed with *BT4-Sfer* (lines 4 – 67). Data are mean  $\pm$  SD,  $n = 6$  replicates from one plant. Statistical differences were calculated by one-way ANOVA. Different letters indicate that means were statistically different by LSD method ( $P \leq 0.05$ ).



**Figure 4.6:** Mineral content of transgenic Sukali Ndiizi lines. **A)** Fe and **B)** Zn content was measured in the fruit of wild type (WT) plants and lines transformed with *Exp1-Sfer* (lines 1 – 85), *35S-FRO2* (lines 40 – 57) and *35S-IRT1* (line 31). Data are mean  $\pm$  SD,  $n = 6$  replicates from one plant. Statistical differences were calculated by one-way ANOVA. Different letters indicate that means were statistically different by LSD method ( $P \leq 0.05$ ).

The results in Figure 4.6 A show that the average Fe content of wild type was  $10.64 \pm 0.96$  mg/kg DW and the Fe content of transgenic lines transformed with *Exp1-Sfer* ranged from  $8.31 \pm 0.46$  mg/kg DW (line 81) to  $17.26 \pm 0.44$  mg/kg DW (line 19). The Fe content of transgenic lines transformed with *35S-FRO2* ranged from  $8.22 \pm 0.51$  mg/kg DW (line 51) to  $19.92 \pm 0.48$  mg/kg DW (line 40). There was no significant difference between Fe content of wild type and *35S-IRT1* line 31. Transgenic lines 19 and 40 had Fe contents 1.6 and 1.8 times more than the wild type. Some transgenic lines transformed with *Exp1-Sfer* and *35S-FRO2* accumulated more Fe than wild type. The Zn content of the wild type (Figure 4.6 B) was  $8.57 \pm 1.16$  mg/kg DW and that of all the transgenic lines was less than the wild type except for *Exp1-Sfer* lines 04 and 26 that had Zn content of  $11.82 \pm 0.14$  and  $13.52 \pm 0.45$  mg/kg DW. This indicates that the transgenes *Exp1-Sfer*, *35S-FRO2* and *35S-IRT1* probably do not improve Zn accumulation in banana fruits. In addition, plants that had multiple transgene copy numbers (more than 3) did not in any way impact on the amount of Fe accumulated but probably affected the level of Zn accumulation as per Figures 4.4 and 4.6 respectively.

#### 4.3.4.2 Biochemical analysis of transgenic banana leaves

In this experiment the banana leaves at different stages of development, transformed using different transgenes were collected from the field trial at NARO (Uganda) and analysed for various mineral elements to determine whether transformation has had an effect on their content. The results of mineral content are shown in subsequent figures. In analysis of the

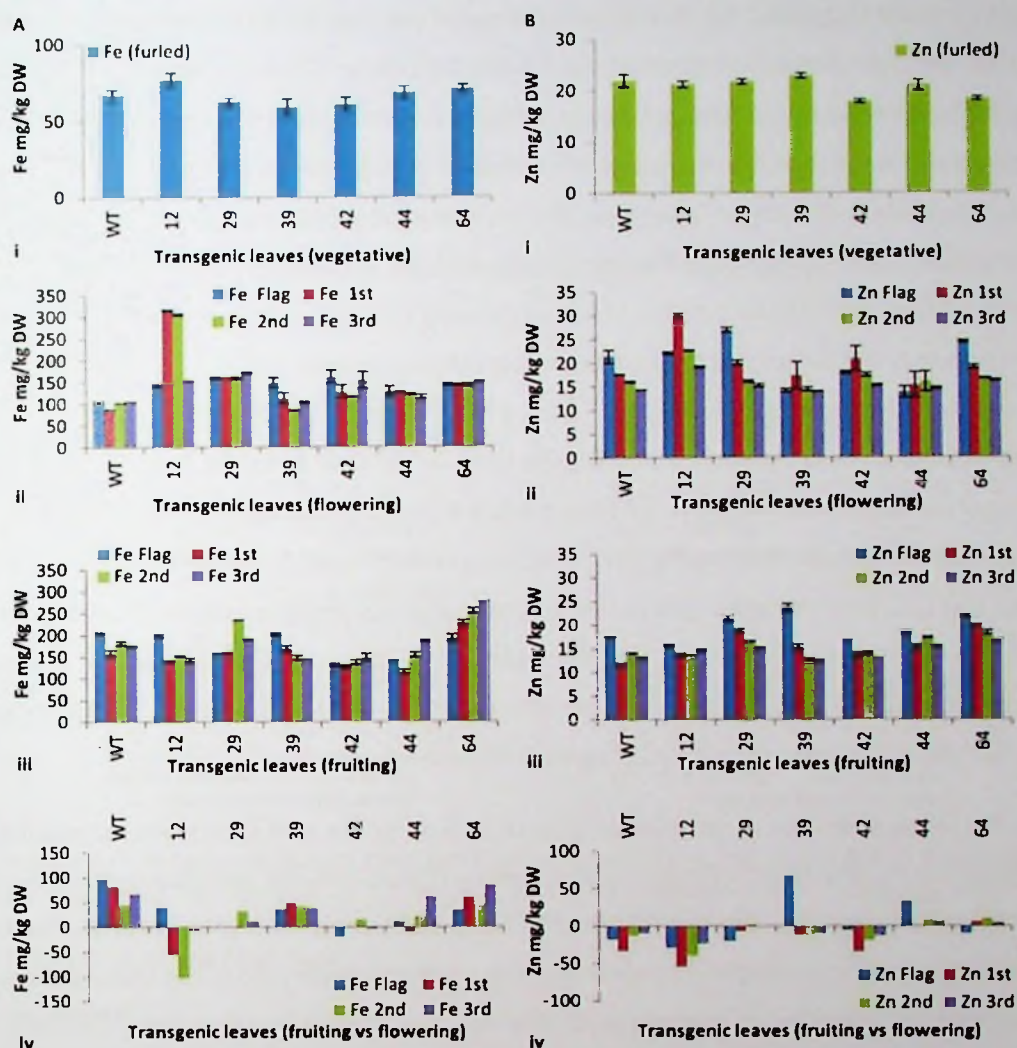
leaves of Sukali Ndiizi, only vegetative and flowering stages were reported as wild type samples for comparison of the transgenic lines at the fruiting stage data were not available.

#### 4.3.4.3 *Analysis of mineral accumulation in Nakinyika leaves of BT4-Sfer transformed lines at different stages of leaf development*

##### *Fe content*

The effect of leaf position and mineral accumulation was determined in banana leaves transformed with *BT4-Sfer* transgene to determine the mineral accumulation at both vegetative and flowering stage of fruit development (Figure 4.7).

In plants at the vegetative stage, the Fe content in the cultivar Nakinyika show that the furled leaf wild type (Figure 4.7 Ai) was  $67.08 \pm 4.00$  mg/kg DW and that of the transgenic lines ranged from  $58.99 \pm 5.24$  (line 39) mg/kg DW to  $77.14 \pm 4.40$  (line 12) mg/kg DW. In the flag leaf (Figure 4.7Aii & iii), Fe content of transgenic lines was approximately 1.2 (line 44) to 1.6 (line 42) fold and 0.6 (line 42) to 0.99 (line 39) fold higher than the respective wild type levels at flowering ( $103.26 \pm 3.44$  mg/kg DW) and fruiting ( $204.50 \pm 1.77$  mg/kg DW). In the first leaf (Figure 4.7 Aii & iii), transgenic lines had 1.3 (line 39) to 3.6 (line 12) fold more Fe compared to WT level of  $87.55 \pm$  mg/kg DW at flowering, while at fruiting these lines were 0.7 (line 44) to 1.4 (line 64) fold higher than wild type Fe levels ( $158.60 \pm 6.75$  mg/kg DW). In the second leaf (Figure 4.7 Aii & iii), transgenic lines had 0.8 (line 39) to 3 (line 12) fold and 0.8 (line 42) to 1.4 (line 64) fold higher Fe content compared to wild type levels at flowering ( $101.15 \pm 4.46$  mg/kg DW) and fruiting ( $180.63 \pm 4.46$  mg/kg DW), respectively. The third leaf Fe content of transgenic lines (Figure 4.7 Aii & iii) was approximately 1 (line 39) to 1.6 (line 29) fold and 0.8 (line 12) to 1.6 (line 64) fold higher than the wild type levels at flowering ( $104.74 \pm 0.49$  mg/kg DW) and fruiting ( $174.35 \pm 1.61$  mg/kg DW), respectively.



**Figure 4.7:** Mineral content of banana leaves cultivar Nakinyika transformed with BT4-Sfer during development. The A) Fe and B) Zn content of wild type (WT) and transgenic (lines 12-64) plants were analysed at the i) vegetative(furled leaf), ii) flowering and iii) fruiting stages. The percentage change iv) in Fe and Zn content was compared between ii) and iii).

Analysis of the percentage change in Fe between flowering and fruiting (Figure 4.7Aiv) showed that all leaves in the wild type had greater Fe content at fruiting. Transgenic lines 39 and 64 followed a similar trend to the wild type. However, the remaining BT4-Sfer were not as consistent as the wild type and some leaves had less Fe at the fruiting stage compared to flowering. This data suggested that in some transgenic lines there is reduced Fe content during development compared to the wild type, possible due to Fe being translocated to other parts of the banana plant.

#### Zn content

The furled leaf (Figure 4.7 b i) Zn content of the wild type was  $21.90 \pm 1.24$  mg/kg DW, and that of the transgenic lines ranged from  $17.73 \pm 0.37$  mg/kg DW (line 42) to  $22.77 \pm 0.45$  mg/kg DW (line 39). In the flag leaf (Figure 4.7 b ii & iii), Zn content of transgenic lines was approximately 0.6 (line 42) to 1.1 (line 64) fold and 0.9 (line 12) to 1.3 (line 39) fold higher than the wild type levels at flowering ( $21.43 \pm 1.34$  mg/kg DW) and fruiting ( $17.61 \pm 0.13$  mg/kg DW), respectively. In the first leaf (Figure 4.7 b ii & iii), transgenic lines had 0.9 (line 42) to 1.7 (line 12) fold and 1.2 (line 12) to 1.7 (line 64) fold higher Zn content compared to wild type levels at flowering ( $17.56 \pm 0.08$  mg/kg DW) and fruiting ( $11.79 \pm 0.11$  mg/kg DW), respectively. In the second leaf (Figure 4.7 b ii & iii), transgenic lines had 0.9 (line 39) to 1.4 (line 12) fold more Zn compared to wild type level of  $16.04 \pm 0.24$  mg/kg DW at flowering, while at fruiting these lines were 0.9 (line 39) to 1.3 (line 64) fold higher compared to wild type Zn levels ( $14.08 \pm 0.12$  mg/kg DW). With exception of line 12, the Zn content in other lines was less than the wild type in the second leaf at flowering. In the third leaf (Figure 4.7b ii & iii), transgenic lines had approximately 1 (line 39) to 1.3 (line 12) fold and 1 (line 39) to 1.3 (line 64) fold Zn content relative to wild type levels at flowering ( $14.45 \pm 0.01$  mg/kg DW) and fruiting ( $13.07 \pm 0.22$  mg/kg DW), respectively.

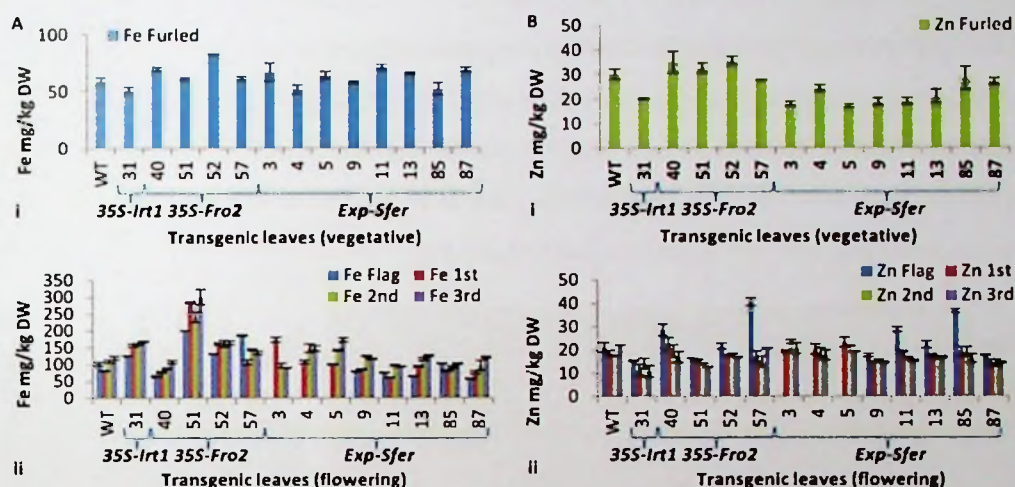
The percentage change in Zn content (Figure 4.7b iv) in the wild type leaves showed a reduction in Zn between the flowering and fruiting stages. This was also observed in lines 12, 42 and 39 (except in flag leaf). The other lines showed essentially no reduction in Zn content. Curiously, the flag leaf Zn content of lines 39 and 44 increased at the fruiting stage compared to flowering in contrast with the flag leaf change in other lines. This data suggested that Zn may also be transported within the plant during development.

#### 4.3.4.4 *Analysis of micronutrient content in Sukali Ndiizi leaves of lines transformed with 35S-IRT1, 35S-FRO2 and EXP-SFER at vegetative and flowering stage of development*

##### *Fe content*

In plants at the vegetative stage, the Fe content in the cultivar Sukali Ndiizi show that the furled leaf wild type (Figure 4.8 a i) was  $58.74 \pm 3.37$  mg/kg DW and that of the transgenic lines *35S-IRT1* was  $50.91 \pm 3.104$  (line 31) mg/kg DW, *35S-FRO2* ranged from  $61.01 \pm 0.85$  mg/kg DW (line 51) to  $83.15 \pm 0.64$  (line 57) mg/kg DW and *EXP1-SFER* ranged  $51.81 \pm 4.08$  mg/kg DW (line 4) to  $71.16 \pm 2.75$  mg/kg DW (line 11). In the flag leaf (Figure 4.8 a ii & iii), Fe content of transgenic lines was approximately 0.6 (line 40, 87), 1 (line 85), 1.2 (line 31) and 2 (line 51) fold higher than the respective wild type levels at flowering ( $101.47 \pm 5.15$  mg/kg DW). In the first leaf (Figure 4.8a ii), transgenic lines had 0.9 (lines 11, 40), 1.9 (line

31), 2.1 (line 3) to 3.5 (line 51) fold more Fe compared to WT level of  $81.09 \pm 0.12$  mg/kg DW at flowering. In the second leaf (Figure 4.8 a ii), transgenic lines had 0.8 (lines 11, 40), 1.4 (line 3, 31) to 2.2 (line 51) fold higher Fe content compared to wild type levels at flowering ( $109.20 \pm 4.05$  mg/kg DW) respectively. The third leaf Fe content of transgenic lines (Figure 4.8a ii) was approximately 1 (lines 3, 40), 1.4 (lines 5, 31) to 2.5 (line 51) fold higher than the wild type levels at flowering ( $120.09 \pm 5.22$  mg/kg DW) respectively.



**Figure 4.8:** Mineral content of transgenic banana leaves cultivar Sukali Ndiizi during plant development. A) Fe and B) Zn content at the i) vegetative and flowering stages of development. Samples included Wild type (WT), and transgenic lines 31 (35S-IRT1), 40 - 57 (35S-FRO2), 3 – 87 (EXP-Sfer).

#### Zn content

In plants at the vegetative stage, the Zn content in the cultivar Sukali Ndiizi show that the furlled leaf wild type Figure 4.8b i), Zn content of the wild type was  $30.13 \pm 2.19$  mg/kg DW; Transgenic lines transformed with *35S-IRT1* and *Exp1-Sfer* showed a reduction in Zn content compared to wild type with Zn ranging from  $16.80 \pm 0.74$  mg/kg DW (line 5) to  $28.16 \pm 4.66$  mg/kg DW (line 85). While transgenic line transformed with *35S-FRO2* had  $35.36 \pm 1.83$  mg/kg DW (line 52). In the flag leaf (Figure 4.8b ii), wild type tissue, Zn content was  $21.17 \pm 0.41$  mg/kg DW, transgenic lines had 0.8 (lines 31, 51, 87), 1.7 (line 85) to 1.9 (line 57) fold higher than wild type respectively at flowering. In the 1<sup>st</sup> leaf Zn content of  $18.72 \pm 1.29$  mg/kg DW in the wild type (Figure 4.8b ii), transgenic lines had 0.6 (line 31), 0.8 (lines 9, 51) and 1.2 (lines 5, 40) fold higher than the wild type respectively at flowering. In the 2<sup>nd</sup> leaf the wild type had Zn content of  $17.54 \pm 0.20$  mg/kg DW, while the transgenic lines had 0.8 (lines 31, 51, 87), 1.1 (line 40) to 1.3 (line 3) fold higher than the wild type respectively. In the 3<sup>rd</sup> leaf (Figure 4.8b ii) Zn content of the wild type was  $22.25 \pm 0.09$  mg/kg DW, while the

transgenic lines had 0.5 (lines 31, 51 and 87) to 0.9 (line 3) fold higher than the wild type respectively at flowering.

#### 4.3.5 Phenotypic characteristics of transgenic banana plants under field conditions

At the pre-flowering phase (vegetative stage), the phenotype of transgenic plants was rated according to four criteria (Table 4.5). During the post-flowering phase (flowering and fruiting stages), further phenotype analysis was performed (Table 4.6).

##### 4.3.5.1 Phenotypic expression during the pre-flowering phase

After planting, plantlets grew vigorously and gained normal height by the end of the fourth month and plants were assessed during the 5<sup>th</sup> month (Table 4.5). Transgenic line transformed with the *Sfer* transgene all grew normally, while some of the lines transformed with *35S-FRO2* and *35S-IRT1* were stunted (Figure 4.9).



Figure 4.9: Phenotype analysis of selected transgenic banana lines. *BT4-Sfer* line 01 (*Nakanyika*) represents a normal phenotype while B) and C) show *35S-IRT1* lines 24 and 101 (*Sukali Ndiizi*) that are examples of the stunted phenotype.

##### 4.3.5.2 Phenotypic expression during the post flowering phase

As soon as flowering occurred, a range of phenotypic parameters were recorded (Table 4.6). The mean corm size at maturity in cultivar *Sukali Ndiizi* wild type was  $78.25 \pm 6.50$  cm, while that of the transgenic lines transformed with *35S-IRT1* and *Exp1-Sfer* had reduced corm size. However, the *Nakanyika BT4-Sfer* lines had similar corm size to the control (68 cm). The *Sukali Ndiizi* transformed lines also tended to have smaller mean girth at maturity than the wild type girth ( $54.25 \pm 6.30$  cm) while that of the transgenic banana lines were  $45.00 \pm 11.53$  cm (*IRT1*),  $53.50 \pm 9.62$  cm (*FRO2*) and  $54.00 \pm 4.99$  cm (*Sfer*). Transgenic bananas transformed with *IRT1* showed a reduction in girth compared to other transgenes and control. Mean *Sukali Ndiizi* plant height at maturity of the control was  $293.0 \pm 18.76$  cm, while the transgenic banana lines were shorter than the control. In *Nakanyika* the wild type height was  $273.67 \pm 23.37$  cm, while the *BT4-Sfer* lines were taller was  $285.29 \pm 20.43$

cm. This shows transgenic banana lines transformed with *BT4-Sfer* grew taller than the wild types. The mean bunch weight of the wild type *Sukali Ndiizi* at maturity was  $10.87 \pm 0.65$  kg, which was larger than that of all the transgenic lines of this cultivar. However, the bunch weight of *Exp1-Sfer* lines was only  $8.40 \pm 1.33$  kg (*Exp1-Sfer*), suggesting a reduction in bunch weight in these lines. The *IRT1* and *FRO2* transgenic lines had comparable weights to the wild type. The mean number of days from flowering to bunch harvest in the *Sukali Ndiizi* control was  $133.75 \pm 19.96$  days. In comparison, the corresponding period for transgenic lines was  $142.89 \pm 18.6$  days (*35S-IRT1*),  $140.17 \pm 27.1$  days (*35S-FRO2*) and  $142.6 \pm 3.71$  days (*Exp1-Sfer*), which indicated that the transgenic lines matured more slowly than the wild type. In Nakinyika, the wild type time between flowering and bunch harvest was  $140.40 \pm 9.88$  days, compared to  $113.11 \pm 22.4$  days for the *BT4-Sfer* transgenic, suggesting faster maturity in the transgenic lines.

**Table 4.5: Field phenotypic expression of transgenic banana lines**  
Phenotypes (%)

| Transgene | P-1  | P-2* | P-3* | P-4* | Total No** |
|-----------|------|------|------|------|------------|
| 35S-FRO2  | 85.7 | 6.25 | 0.0  | 6.25 | 15         |
| 35S-IRT1  | 93.3 | 0.0  | 0.0  | 6.7  | 15         |
| BT4-Sfer  | 100  | 0.0  | 0.0  | 0.0  | 21         |
| Exp1-Sfer | 100  | 0.0  | 0.0  | 0.0  | 22         |

\*P-1: Normal phenotype; P-2: Yellow streaks; P-3: Purple pigmentation (petiole) and P-4: Stunted growth. \*\*Total number of plants assessed.

**Table 4.6: Post flowering phenotypic expression observed among transgenic banana**

| Gene      | Corm (cm)   | Girth (cm)  | Height (cm)  | TL*        | Clusters   | BWT (kg)*  | BH (cm)*   | BC (cm)*     | FH (days)*   |
|-----------|-------------|-------------|--------------|------------|------------|------------|------------|--------------|--------------|
| Wild SN   | 78.25±6.50  | 54.25±6.30  | 293.0±18.76  | 9.50±5.12  | 10.25±0.43 | 10.87±0.65 | 66.25±5.58 | 79.25±2.86   | 133.75±19.96 |
| 35S-IRT1  | 70.00±12.49 | 45.00±11.53 | 280.67±10.20 | 10.33±0.58 | 9.93±0.12  | 10.00±0.85 | 59.33±4.50 | 97.33±10.05  | 142.89±18.6  |
| 35S-FRO2  | 72.50±7.37  | 53.50±9.62  | 280.50±13.40 | 9.17±1.67  | 9.33±1.37  | 9.67±1.49  | 66.00±6.50 | 80.17±3.76   | 140.17±27.1  |
| Exp1-Sfer | 75.90±2.80  | 54.00±4.99  | 284.70±18.99 | 9.90±1.20  | 10.30±1.16 | 8.40±1.33  | 67.70±5.54 | 78.60±3.84   | 142.6±3.71   |
| Wild Nak  | 68.33±6.38  | 49.83±10.19 | 273.67±23.37 | 10.83±1.47 | 10.92±0.92 | 12.53±2.35 | 72.50±2.43 | 81.50±2.43   | 140.40±9.88  |
| BT4-Sfer  | 68.65±9.80  | 51.18±12.12 | 285.29±20.43 | 11.65±2.34 | 12.53±2.35 | 10.92±0.92 | 50.24±7.08 | 106.12±11.70 | 113.11±22.4  |

\*Abbreviated terms: TL(Functional leaves at flowering); BWT(Bunch weight); BH (Bunch height); BC(Bunch circumference); FH (Days from flowering to harvest)

## 4.4 DISCUSSION

### 4.4.1 Introduction

Biofortification of plants has potential to increase micronutrient density in the edible parts of the plant. In order to produce Fe biofortified banana, three transgenic approaches were investigated. First, enhancement of Fe storage in fruit pulp via expression of the Fe storage protein ferritin using both fruit specific and constitutive promoters and Secondly, enhancement of Fe translocation through overexpression of Fe reductase transporter (*IRT1*) and thirdly, enhancement of Fe reduction in the soil using ferric reductase transporters (*FRO2*) under the control of CaMV 35S constitutive promoter. As a result, Fe and Zn content from the baseline data in Chapter 3 showed that bananas did not have sufficient amounts of Fe and Zn to meet the nutritional requirements of humans. Therefore, biofortification using Fe enhancement and uptake genes would be a probable option. Various reports have stated that the use of storage enhancement and Fe uptake genes have potential to increase micronutrient densities in crops especially bananas. To date, however, there have been no reports of ferritin, *FRO2*, and *IRT1* overexpression in banana fruits using constitutive promoters. To investigate the effects of constitutive overexpression on Fe accumulation in transgenic bananas the *Sfer*, *FRO2* and *IRT1* genes were used with different promoters: BT4, Exp1 and CaMV 35S (35S).

### 4.4.2 Effect of transgenes on mineral accumulation in banana fruit transformed with the banana ferritin gene

The results suggest that Soybean ferritin overexpression has the potential to significantly increase Fe levels in banana fruit. This was shown in the fact that generally, most lines had moderately higher fruit Fe content of roughly 1.4 to 1.5 fold compared to WT, with the best line nearly 2 and 1.7 fold higher in *BT4-Sfer* (Figure 4.5) and *Exp1-Sfer* (Figure 4.6) lines, respectively. This was probably due to ferritin expression influencing the expression of other Fe homeostasis genes because ferritin is a known sink for Fe accumulation (Briat et al., 2010; Ravet et al., 2009a; Vasconcelos et al., 2003). In addition, these results are in line with previous studies in rice where Soybean ferritins have been used to obtain increases of 3 fold (Goto et al. (1999) and 3.7 fold (Vasconcelos et al. (2003) Fe content in rice seed compared to wild type. Furthermore, use of the ferritin gene from *Phaseolus vulgaris* obtained a 2 fold increment in Fe content in transgenic rice seed versus the wild type controls Lucca et al. (2001). Therefore, it is possible that increased ferritin protein levels due to ferritin overexpression improved the Fe storage capacity within the fruit. Consequently, this may result in high Fe content since one ferritin molecule can absorb up

to 4500 Fe atoms (Theil, 2000). The increased ferritin concentration may trigger increased inflow of Fe from other parts of the plant as the Fe absorption by ferritin, possibly inducing a localised Fe deficiency state. These findings indicate high levels of ferritin accumulation may contribute to improved Fe bioavailability in banana, as has been seen previously in rice (Lucca et al., 2001), since ferritin absorbs Fe (Theil and Briat, 2004) and serves as a bioavailable form (Lönnerdal, 2009; Murray-Kolb et al., 2002; Beard et al., 1996).

Interestingly, this study also suggested that ferritin overexpression was linked to a significant decrease in the accumulation of Zn in the fruit of transgenic lines compared to the wild type. Generally, the fruit Zn content across the *BT4-Sfer* and *Exp1-Sfer* lines was only about 0.7 fold relative to the wild type level (Figure 4.5). Interestingly, a few lines transformed with *BT4-Sfer* (line 9) and *Exp1-Sfer* (line 4 and 26) were observed with significantly high Zn content that was about 1.4 and 1.6 fold, respectively, compared to the wild type (Figure 4.6). This was not expected because previous studies in rice (Vasconcelos et al. (2003); (Goto et al., 1999) that used ferritin to increase the levels of both Fe and in transformed, without affecting the concentrations of other divalent metals. This indicated a positive correlation between Fe and Zn in rice grain (Vasconcelos et al., 2003). This may possibly be because phytosiderophores mobilize Zn as well as Fe (Zhang et al. (1991)). Interestingly, although ferritin is an Fe storage protein, it may be able to store other divalent metals such as Zn and Cu (Price and Joshi, 1982; Price and Joshi, 1983).

Previous studies suggested a positive correlation between Fe and Zn due to ferritin overexpression, yet generally, this study indicated an inverse relationship in the accumulation of these two elements. Although some lines with high Fe (lines 9 and 58) happened to have higher Zn than WT, other lines with higher Fe had Zn levels at or below the WT. Further, there was a significant overall reduction in Zn content in both transgenic lines irrespective of the promoter used. This therefore indicates a possible down regulation of Zn uptake resulting from enhanced Fe accumulation. An increase in Mn and Cu content (data not shown) was observed in addition to Fe, and a reduction in Zn in banana fruit pulp, which may indicate that ferritin up regulation, affects other divalent metals. This may also indicate that ferritins can accumulate other divalent cations at the expense of Zn in banana cultivars *Sukali Ndiizi* and *Nakinyika* under field conditions.

#### 4.4.3 Effect of transgenes on mineral accumulation in banana fruits transformed with *IRT1* and *FRO2* transgenes using the 35S promoter

There are no studies that have been conducted in banana plants to determine the effect of the constitutive expression of *FRO2* and *IRT1* genes in banana fruit tissue. This study assumed that constitutive overexpression of these transgenes could increase Fe and Zn contents in the banana fruit pulp. The results suggest that *FRO2* and *IRT1* constitutive overexpression did not boost Fe and Zn accumulation in the banana fruit pulp of *Sukali Ndiizi* except for line 40 (Figure 4.6) that had 1.9 times more Fe than the wild type. In addition, the Zn levels within the ferritins were lower than that of the wild type. It was thought that reduction of  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  enhances mineral uptake and translocation in different plant tissues. However, plants constitutively expressing both *IRT1* and *FRO2* genes may not enhance Fe uptake due to post-transcriptional regulation induced by high soil Fe levels (Connolly et al., 2002; Connolly et al., 2003). Probably, post-transcriptional regulation of *FRO2* may serve to protect plants against the accumulation of potentially toxic amounts of Fe (Connolly et al., 2003). This suggested that expression of *FRO2* in crops may yield lines with improved Fe efficiency as observed in line 40 that had high Fe content compared to the wild type.

In addition to Fe and Zn transportation *in planta*, *IRT1* appears to have a broad substrate specificity (Eide et al., 1996; Rogers et al., 2000), as it is known to transport Mn, Zn and probably even Cd and Co when expressed in plants (Buglio et al., 2002; Eckhardt et al., 2001), unlike *FRO2* (Connolly et al., 2003). The data obtained shows that in the banana fruit pulp constitutive expression of *IRT1* enhanced Mn but not Zn content (data not shown) compared to the wild type, while there was no significant difference in the amounts of other metals in lines transformed with 35S-*FRO2*. This is probably because overexpression of *FRO2* should not directly lead to accumulation of toxic amounts of Fe because it does not transport Fe itself but reduces it. The low Zn contents observed in line 31 transformed with 35S-*IRT1* (Figure 4.6) could be possibly due to post-transcriptional regulation of *IRT1* expression that prevents accumulation of divalent metals under conditions with high Fe levels.

#### 4.4.4 Effect of transgenes *BT4-Sfer* and *Exp1-Sfer* on mineral accumulation in banana leaf tissues

In addition to the fruit data, the Fe and Zn content of banana leaves were analysed using ICP-OES to determine the effects of *Sfer* overexpression on mineral accumulation in the different leaf positions at vegetative, flowering and fruit development stages of

development. The results suggest that Soybean ferritin overexpression has the potential to significantly increase Fe levels in banana leaf tissue. This was shown by the fact that generally, most *BT4-Sfer* (Figure 4.7) and *Exp1-Sfer* (Figure 4.8) lines had moderately higher leaf Fe content compared to wild type. This was probably due to promoter expression leakage although ferritin is a known sink for Fe accumulation (Briat et al., 2010; Ravet et al., 2009a; Vasconcelos et al., 2003). In addition, these results are in line with previous studies in vegetative leaf tissue of crops such as banana (Kumar et al. (2011), tobacco (Goto et al., 1998; Van Wuytswinkel et al., 1999), pineapple (Mhatre et al., 2011) and lettuce (Goto et al., 2000) where Soybean ferritins have been used to obtain increases in Fe and Zn. Notably, in banana leaf, Kumar et al. (2011) reported five and three fold enhancement in Fe and Zn content, respectively, in glasshouse conditions, which is similar to observations in other crops. However, under field conditions the Fe was enhanced and Zn content reduced. Probably under glasshouse controlled conditions had an effect on mineral uptake compared to field conditions.

The increases in Fe that were observed may be because ferritin overexpression may affect Fe homeostasis through mechanisms such as increased Fe storage capacity and affecting Fe regulatory mechanisms. Increases in ferritin protein content could significantly boost Fe storage capacity in transgenic lines as each additional ferritin molecule could potentially hold roughly 2600 to 4500 atoms (Korcz and Twardowski, 1992; Theil, 2000). Therefore, the Fe content of transgenic plants could be increased even in soils with high Fe levels. As a result, the increased Fe storage capacity due to increased ferritin protein levels may cause localized Fe deficiency in the tissues overexpressing *ferritin* genes. This could then affect the uptake and transport of elements such as Fe and Zn, which are mobilized by phytosiderophores (Zhang et al., 1991). Further, there is evidence that ferritin influences Fe content through the integrated pathways that govern Fe homeostasis (Van Wuytswinkel et al., 1999), which may also impact other minerals such as Zn, which share common regulatory mechanisms (Vasconcelos et al., 2003). This is possible because Fe influx and homeostasis in plants appears to be tightly matched to minimize iron toxicity at all points within the system (Grusak et al., 1999).

#### **4.4.5 Effect of transgenes *35S-IRT1* and *35S-FRO2* on mineral accumulation in banana leaf tissues**

##### **4.4.5.1 Effect of transgenes *35S-IRT1* on mineral accumulation in banana leaf tissues**

In this study leaf tissue of the dessert banana cultivar *Sukali Ndiizi* that was transformed with the *FRO2* and *IRT1* transgenes were analysed using ICP-OES to measure Fe and Zn

content. Both transgenes were controlled by the constitutive promoter CaMV 35S using Strategy I transgenes. However, leaf material was not obtainable at the fruiting stage, so no data was available.

The data indicates that constitutive overexpression of *35S-IRT1* in banana leaves enhances Fe uptake only at both vegetative and the flowering stage of development but results in reduction in Zn content at both the vegetative and flowering stages (Figure 4.8). The increase of Fe was expected but the reduction in Zn content was not expected because *IRT1* is a divalent metal transporter of both Fe and Zn. However, a possible explanation is that *IRT1* expression is normally induced by Fe deficiency and alkaline soil conditions (Eide et al., 1996; Korshunova et al., 1999; Rogers et al., 2000). This observation may be due to changes in mineral translocation as a result of altered plant metal homeostasis. However, it is important to note that only one *IRT1* line was available for analysis. In addition to high Fe content the banana lines showed signs of chlorosis although this was not expected. During Fe deficiency *IRT1* non-specifically transports Mn and Zn in the symplast, as well as non-essential but toxic cations such as Ni and Cd, increasing their concentrations in the plant during the Fe deficiency response (Eide et al., 1996; Korshunova et al., 1999; Rogers et al., 2000). Data shows that Ugandan soils are Fe sufficient (Chapter 3), therefore the chlorosis may not be attributed to Fe deficiency but overexpression of *IRT1* probably led to Mn and Ni accumulation at the expense of Zn in the leaf tissue of plants that did not flower.

This is probably attributed to the fact that other divalent elements replace Fe in some cellular processes under low-Fe conditions (Vert et al., 2002; Barberon et al., 2011). According to Varotto et al. (2002), constitutive expression of CaMV 35S promoter with *IRT1* transgene in *Arabidopsis thaliana* plants led to slight retardation in growth and a lighter pigmentation in the transgenic lines. Similar results were observed in the banana transgenic line in this study that reached both flowering and fruiting stage of development. In order for metal transport in plants to be optimal it requires tight spatial and temporal regulation of expression of the proteins involved (Eide et al., 1996; Vert et al., 2002), which may not be possible with the 35S promoter. The high metal accumulation in the line overexpressing *IRT1* may be due to higher root *IRT1* levels compared to wild type plants (Barberon et al., 2011).

#### **4.4.5.2 Effect of transgenes 35S-FRO2 on mineral accumulation in banana leaf tissues.**

The results obtained showed that transformation of banana lines with the constitutive promoter 35S using *FRO2* transgenes enhanced Fe uptake at both vegetative and flowering

stages of development (Figure 4.8) and enhanced Zn content in the first and second leaves but reduction in the furled, flag and third leaves. Probably constitutive overexpression of *FRO2* transgene in banana cultivar *Sukali Ndiizi* enhances Fe uptake but not Zn at different stages of leaf development. However, this was expected because *FRO2* transports Fe, but what was not known was the Fe and Zn distribution in the different leaf tissues at vegetative and flowering stage, therefore these are new findings in this study.

This may be attributed to the constitutive expression of the Fe reductase that might have enabled banana leaves to generate more ferrous Fe root absorption and subsequent utilization in the chlorophyll synthesis. Similar observation were reported by Pestana et al. (2012) in strawberry plants grown under high Fe conditions. Transgenic plants overexpressing *FRO2* showed better growth under low Fe condition compared with wild type plant (Connolly et al., 2003). Though chlorophyll and biomass content were not measured, the plants that were transformed with *35S-FRO2* had normal leaves like wild type control showing that *FRO2* does not affect the physiology of the leaf tissues.

In this study transgenic banana lines appeared shorter than the wild type control, with some lines exhibiting stunting. This indicates that constitutive expression of *FRO2* under normal conditions may lead to a decrease in plant productivity. Similar observations were reported in Soybean transgenic plants that showed a reduced biomass accumulation under non-iron stress conditions (Vasconcelos et al., 2006). Under Fe sufficient conditions constitutive expression of banana lines with *FRO2* transgenes probably compromises plant growth conditions. Nonetheless, elevating iron reductase activity enhanced Fe content in leaves and probably increased chlorophyll concentration, though it may impact negatively on banana fruit bunch size and plant growth. To improve human iron nutrition, it will not be sufficient merely to increase Fe uptake by plants but modified uptake and/or translocation is likely to be contributory factor. For example, in grains one key regulator of Fe and Zn concentrations is remobilization from leaves and translocation to grain (Zhao and McGrath, 2009).

#### 4.4.6 Conclusion

This study set out to determine the effect of over-expression of Fe uptake (*FRO2* and *IRT1*) and storage (*Sfer*) enhancement genes in the banana fruit pulp and leaves of the *Sukali Ndiizi* and Nakinyika cultivars under field conditions. Overall, these results show that *Sfer* overexpression produced notable increases in the Fe content of both leaf and fruit tissue. Further, though some improvement in Zn content was observed in leaf, there was a general

reduction in fruit Zn content. Similar results were observed with both the BT4 and Exp1 promoters. The use of the *IRT1* and *FRO2* genes lead to improved leaf and fruit Fe levels in some lines, but not to the extent seen in *Sfer* lines, coupled with a general decrease in the Zn content of both tissues. Therefore the best candidate so far is *Sfer* as it produced some lines that produced greater improvements in Fe content than *FRO2* and *IRT1*. Further *Sfer* lines had the best overall phenotypic characteristics, compared to both *IRT1* and *FRO2* transformants where some lines had stunting and leaf yellowing phenotypes.

However, repeating this study with increased numbers of lines, particularly the *FRO2* and *IRT1* genes, would give a more comprehensive picture of their potential as the number of lines analysed may be too low to identify promising lines. This may be attributed to a possible 'position effect' due to the location of gene insertion in multiple positions as seen in the multiple copy number analysis (Figure 4.4C and D). It may also be useful in future to obtain Fe and Zn content from other cultivars that were not included in this study. It may also be prudent to re-examine the selection of promoters for these genes that would target high expression of genes of interest in a more tissue-specific manner to get better increases in Fe and Zn content while reducing the potential for negative impacts on phenotype. For example, Qu et al. (2005) suggested that utilising strong promoters that target the specific tissue of interest could probably allow high Fe accumulation in the edible plant tissue. Interestingly, this study indicated that the genes that were examined may also affect the homeostasis of other divalent elements such as Mn, thus it may be worth expanding future experiments to investigate such effects. Such improvements to future studies would also have the benefit of providing further insight into the mechanisms of Fe accumulation in the different banana cultivars.

Previous studies have looked at transgene expression of ferritin genes in banana for Fe (Kumar et al. 2011). However, this is the first study, to my knowledge, to examine the effects of these genes in fruit tissue and across multiple leaves during development. Thus far *Sfer* shows the most potential for use in biofortification of banana using genetic modification. Although ferritin is a bioavailable form of Fe storage (Lönnerdal, 2009), the increases in Fe that I have observed so far are not sufficient to meet nutritional requirements.

# Chapter 5: Molecular and biochemical characterisation of transgenic bananas containing iron redistribution transgenes

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## 5.1 INTRODUCTION

Iron deficiency anaemia (IDA) affects over 50% of the world's population and if not tackled can cause reduced psychomotor and mental development in children, aborted pregnancies and compromised immunity (Nestelet al., 2006; Zimmermann and Hurrell, 2007). Globally, 90% of the population living Southeast Asia and Africa are impacted by IDA and often subsist on only major staple food crops like banana (Stephenson et al., 2000). In Uganda IDA is a critical public health problem, where the majority of children between 6 to 59 months old exhibit either moderate or severe IDA. In addition, key factors linked to the high prevalence of anaemia include: malaria, worm infestation, and inadequate dietary Fe intake (UBOS, 2011).

Biofortification of staples like banana through breeding or transgenic approach has potential to be one time investment for food fortification if the crop is adopted, and the nutrient is sufficiently bioavailable. Genetic improvement of crop or food nutrient content by breeding can be successful if there is sufficient genetic variation for the trait (Bouis et al., 2003). However, for some crops, there may be insufficient genetic variation to reach target nutrient levels via traditional breeding approaches (Mayer et al., 2008). This is the case in banana, the staple crop of most East Africans, including Ugandans. Banana is a strategy I crop that uses a reduction-based strategy for Fe uptake from the soil (Chapter 1.2.2). In previous work (Chapter 4), overexpression of *FRO2*, *IRT1* and *ferritin* genes obtained a 1.5 fold increase in fruit Fe content in transgenic lines compared to wild type, but with decreased Zn content. This approach, therefore, was unable to increase fruit Fe and Zn content to nutritionally significant levels, necessitating the exploration of alternative avenues.

Graminaceous plants use the strategy II chelation-based approach to facilitate Fe uptake (Chapter 1.2.3). Some graminaceous plants, such as rice, use both strategy I and Strategy II Fe uptake mechanisms (Ishimaru et al., 2006). Therefore, strategy II genes have been used

in this study as an alternative strategy to enhance Fe uptake to the different plant parts. In particular, the NAS genes from rice (*OsNAS1* and *OsNAS2*) were selected as they were previously used to significantly boost Fe and Zn in rice (Johnson et al., 2011). Recently, Trijatmiko et al. (2016) reported 7.5 and 2.5 fold increases in Fe and Zn content, respectively, in polished rice using a combination of *OsNAS2* and soy bean ferritin (*SferH-1*) transgenes. The rice *OsYSL2* transgene was included due to its role in Fe remobilization (Grotz and Gueriot, 2006), and the potential of the Fe assimilatory protein (*FEA1*) from *Chlamydomonas reinhardtii* was also explored because of its specificity in Fe accumulation and operation in high pH soils that limit Fe uptake (Narayanan et al., 2011).

The maize poly-ubiquitin promoter (Christensen et al., 1992; Christensen and Quail, 1996) was used to control transgene expression in this study because it has previously been used to demonstrate reporter gene expression in banana via particle bombardment (Becker et al., 2000; Arinaitwe et al., 2004) and *Agrobacterium*-mediated transformation (Khanna et al., 2007).

The specific objectives of this chapter were to:

1. Characterize transformed lines using PCR, RT-PCR and Southern hybridization
2. Biochemical analysis of banana fruit pulp from transgenic banana lines under field conditions
3. Biochemical analysis of banana leaves from transgenic banana lines under glasshouse conditions
4. Evaluate potential effects of transgenes on banana phenotypes developed under glasshouse conditions

## 5.2 MATERIALS AND METHODS

### 5.2.1 Production of transgenic plants

Transgenic Cavendish banana plants harbouring the iron assimilatory genes *FEA1*, *OsNAS1* and *OsNAS2* were generated at the CTCB as part of the research described in Chapter 2.4.1. These genes were incorporated into constructs cloned into a plant expression vector (pBMGF) under the control of the constitutive maize ubiquitin 1 promoter, Table 5.1 and Figure 5.1).

**Table 5.1:** Description of plant expression vectors used in this study

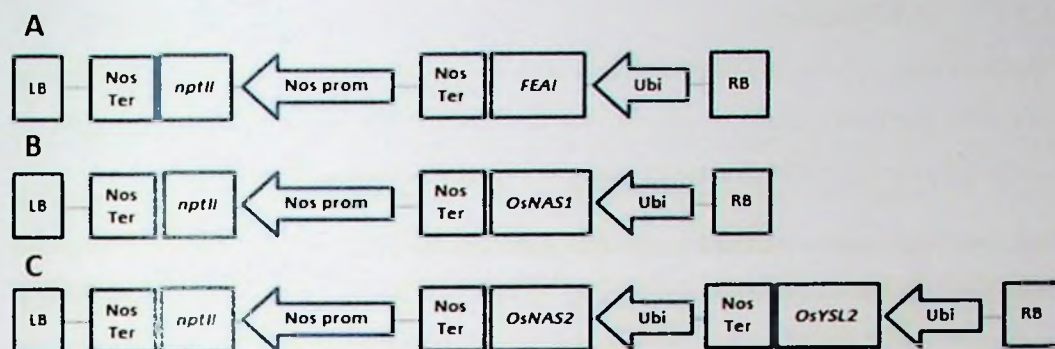
| Description                            | Transgene(s)          | Origin                           |
|----------------------------------------|-----------------------|----------------------------------|
| <i>pBMGF-DC-52-Ubi-FEA1</i>            | <i>FEA1</i>           | <i>Chlamydomonas reinhardtii</i> |
| <i>pBMGF-DC-53-Ubi-OsNAS1</i>          | <i>OsNAS1</i>         | <i>Oryza sativa</i>              |
| <i>pBMGF-DC-83-Ubi-OsNAS2+Ubi-YSL2</i> | <i>OsNAS2, OsYSL2</i> | <i>Oryza sativa</i>              |

### 5.2.2 Glass house acclimatization

Wild type and transgenic Cavendish banana plants (kindly provided by Phuong Hoang, CTCB) were acclimatized in the QCDF glasshouse for screening assays. Rooted plants were taken out of tissue culture jars and residual growth media was removed by washing under tap water. Individual plants were then transferred to 40 mm diameter plastic garden pots filled with Peat 80 plus<sup>®</sup> (Searles, Australia) commercial potting mix. Pots were placed in a container filled with tap water to one third of the height of the pot. The container was covered with clear plastic bag and placed in the glasshouse at 28°C, with a day/night cycle of 16 h/ 8 h for 2 weeks to allow plants to acclimatize. During the acclimation, the plants were sprayed with tap water twice per day. After 1 week, the bag was removed. After a period of 3 months the plants were fertilised with 5g of a water soluble fertilizer Aquasol at a concentration of (1 g/L).

### 5.2.3 Fe uptake screening assay

WT plant material (10 plants), and 20 plants (one plant per individual line) transformed with the plant expression vectors containing the *FEA1*, *OsNAS1*, *OsNAS2* and *OsYSL2* genes used in this study. The acclimatized plants (Chapter 5.2.2) were transplanted into 140 mm plastic pots containing Peat 80 plus<sup>®</sup> potting mix (Searles, Australia). The glasshouse experiments were conducted from October, 2013 to March, 2014 at the QCDF. Plants received regular water supply via drips drip irrigation to avoid water stress, and plants were monitored fortnightly for physiological changes for the 6 month duration of the experiment.



**Figure 5.1:** Schematic representation of binary expression vectors used for transformation of banana. The A) *FEA1*, B) *OsNAS1* and C) *OsNAS2* and *OsYSL2* transgenes were each flanked by the Ubi promoter and Nos terminator. Vectors also contained the *nptII* selection marker gene flanked by the Nos promoter and terminator. These genes were within the T-DNA region located between the right (RB) and left (LB) borders.

## 5.2.4 Molecular analysis of transgenic plants

### 5.2.4.1 Genomic DNA isolation and PCR analysis

Genomic DNA was isolated from leaves of putative transgenic and untransformed banana plants (Chapter 5.2.3) as described in Chapter 2.4.2. PCR of gDNA was carried in 20  $\mu$ L reactions as described (Chapter 2.5.1) using gene-specific primers (Table 5.2). Primers used for analysis of template quality and plasmid vector positive controls amplified *Actin*, and the corresponding gene, respectively. The negative and non-template controls were untransformed plants and water, respectively.

**Table 5.2:** List of primers used for PCR analysis of transgenic banana plants

| Primer name | Primer sequence (5' to 3')    | T <sub>m</sub> (°C) | Expected size (bp) |
|-------------|-------------------------------|---------------------|--------------------|
| F-Ubi       | GATTTTTTAGCCCTGCCTTC          | 50                  |                    |
| R-FEA1      | GTAGAGTAGATGGACAGAGCCTCG      | 63                  | 299                |
| F-Ubi       | GATTTTTTAGCCCTGCCTTC          | 59                  |                    |
| R-OsNAS1    | AGCAGAGCCTGATCAACTCTCC        | 53                  | 313                |
| F-Ubi       | GATTTTTTAGCCCTGCCTTC          | 54                  |                    |
| R-OsNAS2    | AGCGTAATGGGCCTCAAGATGG        | 58                  | 271                |
| F-Actin     | CTGGTGATGGTGTGAGCCAC          | 56                  |                    |
| R-Actin     | CATGAAATAGCTGCACAAATGGTATAAGG | 55                  | 640                |

### 5.2.5 RT-PCR analysis

RNA was used as template in RT-PCR as previously described (Chapter 2.5.1.2). The absence of DNA in the RNA extracts was confirmed by standard PCR (Chapter 2.5.1.1) using  $\beta$ -actin primers before RT-PCR (Chapter 2) with gene-specific primers (Table 5.3).

**Table 5.3:** List of primers used for RT-PCR analysis of transgenic banana plants

| Primer name      | Primer sequence (5' to 3') | T <sub>m</sub> (°C) | Expected size (bp) |
|------------------|----------------------------|---------------------|--------------------|
| F-FEA1           | ATGGCACTCGTGGATCTTCT       | 60                  | 154                |
| R-Nos            | ATGTGATAATCATCGCAAGACC     | 62.6                |                    |
| F-OsNAS1         | GGACCTCCATCTACCTGCTG       | 60                  | 163                |
| R-Nos            | ATGTGATAATCATCGCAAGACC     | 62.6                |                    |
| F-OsNAS2         | CAAGTGCTGCAAGATGGAAG       | 60                  | 155                |
| R-Nos            | ATGTGATAATCATCGCAAGACC     | 62.6                |                    |
| F- $\beta$ Actin | CAACAATACTTGGGAAAACGG      | 66.4                | 150                |
| R- $\beta$ Actin | TGGCTGACACTGACGACATTC      | 62.9                |                    |

### 5.2.6 Southern hybridisation

To confirm the stable integration of *FEA1*, *OsNAS1* and *OsNAS2* genes and assess copy number in transgenic banana plants, the plants that were PCR positive for the gene of interest and a randomly selected untransformed plant were analysed. The genomic DNA was isolated as per Chapter 2.4.2. The quality of the DNA was assessed by electrophoresis on a 0.8% agarose gel and quantified in Nanodrop 2000 before Southern hybridization as per Chapter 2.5.5. Hybridization probes were prepared by labelling a PCR product containing the *NPTII* gene, and products containing the respective transgenes (Chapter 2.5.5.1).

### 5.2.7 Banana leaf and fruit analysis

Banana leaf and fruit sample preparation was carried out as per Chapter 3.3.1. The normality and homogeneity of variance of the data were confirmed as per Chapter 4.3, before one-way ANOVA (XLSTAT, 2014). The Fisher LSD test was then used to determine the difference between means, expressed as mean  $\pm$  standard deviation.

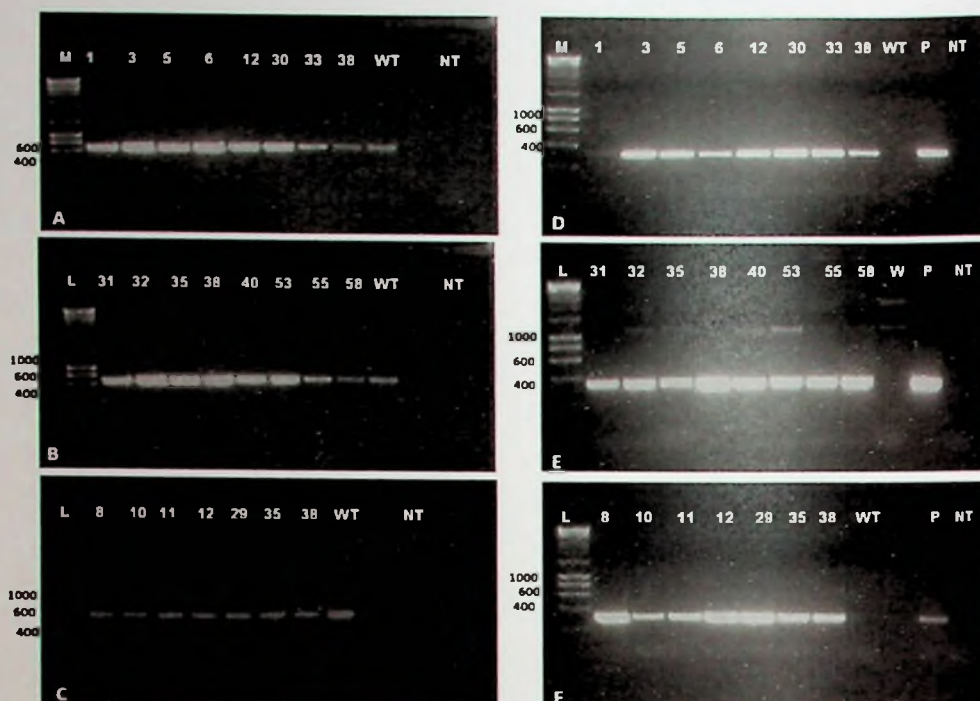
## 5.3 RESULTS

### 5.3.1 PCR screening of transgenic lines

After transformation, selection, regeneration, and acclimatisation, a total of 60 putative transgenic lines and 10 non-transgenic lines were available for analysis (Table 5.4). These plants were first characterized by PCR to confirm the integration of the transgene, followed by Southern hybridization analysis to establish transgene copy number integration in the genome and screened lines analysed for expression levels using RT-PCR. Total DNA was extracted from the leaves of putatively transformed banana leaf plantlets and was used in PCR with set of primers designed in the promoter and in the transgene (Figure 5.2; Table 5.4). Every extract was first assessed for quality by PCR using primers specific to the banana housekeeping gene actin.

**Table 5.4:** PCR screening results summary

| Transgene            | <i>Ubi-FEA1</i> | <i>Ubi-OsNAS1</i> | <i>Ubi-OsNAS2+YSL2</i> |
|----------------------|-----------------|-------------------|------------------------|
| Total lines screened | 20              | 20                | 20                     |
| Actin (+)            | 20              | 20                | 20                     |
| Transgene (+)        | 20              | 20                | 20                     |



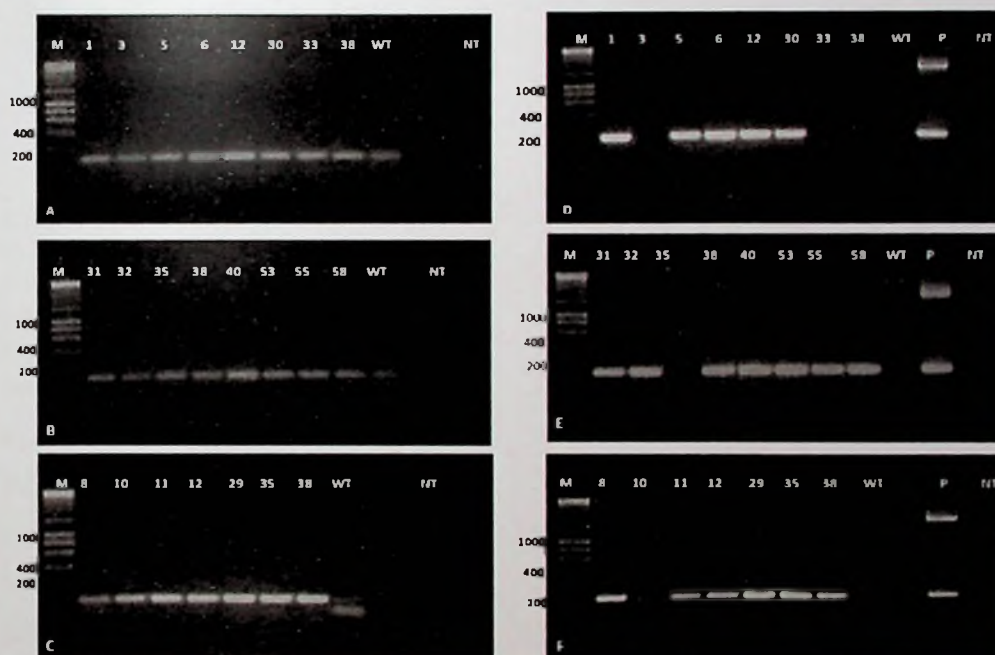
**Figure 5.2:** PCR screening of representative transgenic banana lines for the presence of each respective gene of interest. DNA integrity was assessed using primers to the banana *Actin* housekeeping gene in lines transformed with A) *Ubi-OsNAS1* (lines 1 – 38), B) *Ubi-OsNAS2+Ubi+OsYSL2* (lines 31 – 58) and C) *Ubi-FEA1* (lines 8 – 38). These D) *Ubi-OsNAS1* E) *Ubi-OsNAS2+Ubi+OsYSL2* and F) *Ubi-FEA1* lines were also tested for presence of the respective transgenes. The following lanes were also present: Hyper Ladder 1 (M), wild type (WT), plasmid DNA (P) and non-template control (NT).

The amplification of the *Actin* gene, the internal control for DNA quality, was observed for all plants, including non-transgenic controls (Figure 5.2). Distinct amplicons of the expected size (640 bp) showed that gDNA quality was good. PCR analysis of DNA extracted from lines transformed with *Ubi-FEA1*, *Ubi-OsNAS2+Ubi+YSL2* and *Ubi-OsNAS1* and (Figure 5.2) yielded the expected products of size 313 bp, 271 bp, and 299 bp respectively, consistent with those of the plasmid DNA positive control. This indicated that transformation of plants with each of the vectors was successful and that the PCR primers were specific to each gene of interest. This was further confirmed by the absence of transgene amplification in the wild type or non-template controls.

### 5.3.2 RT-PCR analysis

Semiquantitative RT-PCR analysis was carried out on the selected transgenic banana lines screened in Figure 5.2 using a gene specific primer and Nopaline synthase 3' regulatory sequence primers (Figure 5.3). RT-PCR expression analysis showed that in all cases tested, the amplification product obtained by RT-PCR analysis was identical to the one of the

positive PCR plasmid control. There were lines transformed with each of the transgene vectors (Table 5.1) where gene expression was detected. This is an indication that the genes were properly transcribed in the tested transformants. The RT-PCR reaction performed on the control PCR master mix yielded no product. Of equal importance, PCR analysis of DNase-treated RNA samples with actin-specific primers designed specifically for RT-PCR analysis indicated that the samples were not contaminated with DNA. The amplification products were 150 bp (*Actin*), 163 bp (*FEA1*), 155 bp (*OsNAS2*) and 154 bp (*OsNAS1*) fragments. Transgenic lines 3, 33 and 38 transformed with *Ubi-FEA1* transgenes and line 35 transformed with the *OsNAS2* and *OsYSL2* transgenes gave faint products, indicating weak transcription in those lines. Overall, RNA quality was sufficient to determine the presence of transgene expression by RT-PCR, given the strength of the bands observed.

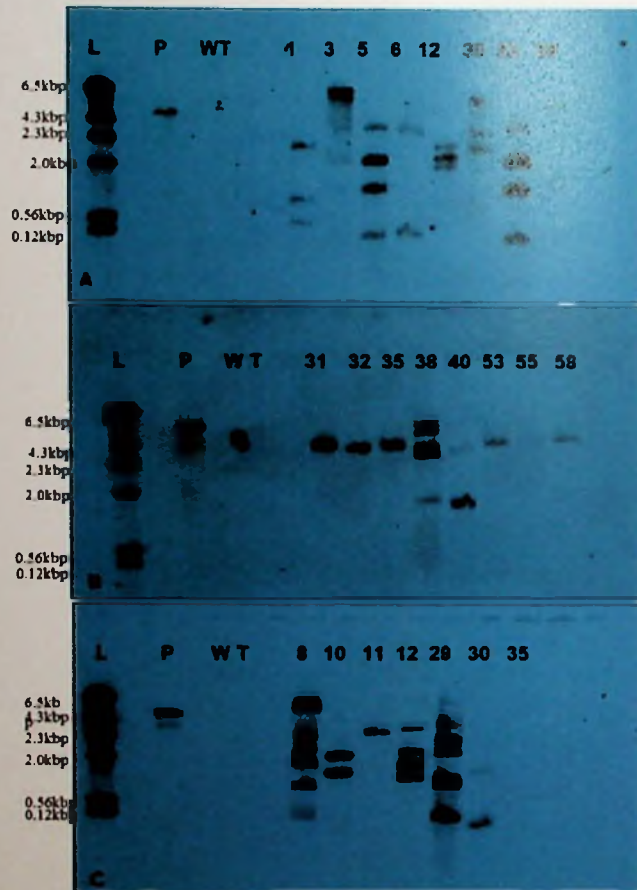


**Figure 5.3:** RT-PCR analysis of representative transgenic banana lines to determine whether the integrated transgenes are expressed. RNA integrity was assessed using primers to banana *Actin* housekeeping gene in lines transformed with **A**) *Ubi-OsNAS1* (lines 1 – 38), **B**) *Ubi-OsNAS2+Ubi+OsYSL2* (lines 31 – 58) and **D**) *Ubi-FEA1* (lines 8 – 38). **D**) *Ubi-OsNAS1*, **E**) *Ubi-OsNAS2+Ubi+OsYSL2* and **F**) *Ubi-FEA1* lines were also tested for expression of the respective transgenes. The following lanes were also present: Hyper Ladder 1 (M), wild type (WT), Genomic DNA (P) and non-template control (NT).

### 5.3.3 Southern analysis of transgenic lines transformed with *Ubi-FEA1*, *Ubi-OsNAS2+YSL2* and *Ubi-OsNAS1* transgenes

Integration patterns of the transgenes into the genome of banana lines expressing *Ubi-FEA1*, *Ubi-OsNAS2+Ubi-YSL2* and *Ubi-OsNAS1* were analysed by Southern blot analysis

(Figure 5.4). To detect the copy number, genomic DNA was digested with *EcoRI*, which cuts once within the T-DNA of the binary vector pCambia2300. Digests of respective genomic DNA samples were electrophoretically separated, transferred onto nylon membrane, and probed with 0.6 kb fragment from the different transgene coding regions. The mobility of the detected bands differed in most transgenic lines, indicating that these lines represent different events. The number of integrations varied between one and seven.



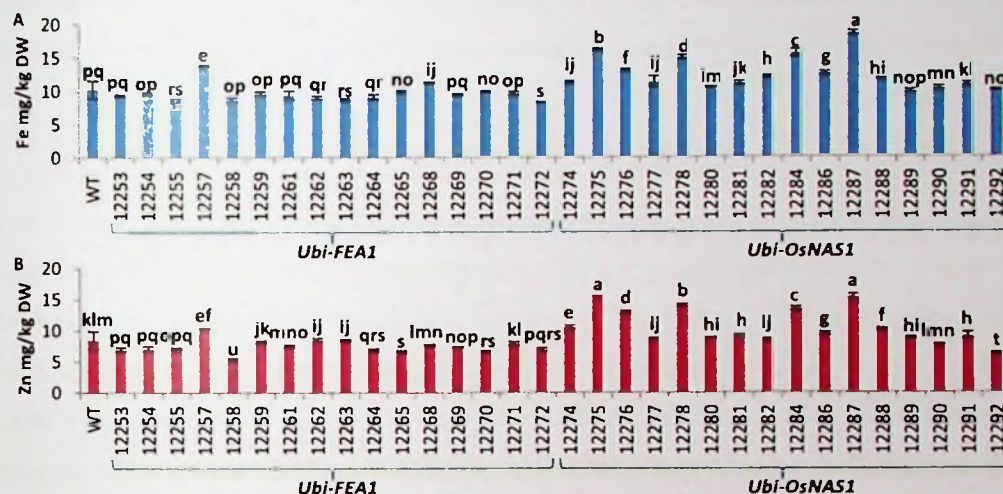
**Figure 5.4:** Southern blot analysis on selected banana lines transformed with different transgenes. Transgenic lines transformed with A) *Ubi-OsNAS1* (lines 1 – 38), B) *Ubi-OsNAS2+Ubi+OsYSL2* (lines 31 – 58) and C) *Ubi-FEA1* (lines 8 – 38) were screened for transgene integration. The following lanes were also present: Molecular Ladder (L), wild type (WT), and plasmid DNA (P). Unique cutter *EcoRI* restriction enzyme was used.

### 5.3.4 Biochemical analysis of transgenic banana lines

#### 5.3.4.1 Analysis of Fe and Zn content in fruit tissue

In this experiment the fruit of transgenic banana lines and non-transformed controls was analysed for Fe and Zn content because of their nutritional importance as biofortification targets. The data presented in Figure 5.5 was sourced from work done as part of the QUT

Bill and Melinda gates project field trial (provided by Dr. Bulukani Mlalazi) to supplement the glasshouse data because generating mature plants was beyond the scope of this thesis. In this experiment representative lines were selected for reporting the effect of *Ubi-FEA1* and *Ubi-OsNAS1* transgenes on Fe and Zn accumulation under field conditions.



**Figure 5.5:** Analysis of fruit mineral content in transgenic Cavendish banana lines. The levels of A) Fe and B) Zn were measured using ICP-OES in Cavendish plants transformed with *Ubi-FEA1* (lines 12253 – 12272) and *Ubi-OsNAS1* (lines 12274 – 12292) compared to the wild type (WT). Data is mean  $\pm$  SD (n=3). Means were separated using Fisher-LSD test. Samples with the same superscript letters represent groups that are not significantly different to each other at  $P \leq 0.05$ .

Figure 5.5 A) showed that the Fe content of the wild type was  $10.25 \pm 1.3$  mg/kg DW, Fe content of the transgenic lines transformed with *Ubi-FEA1* were less than that of the wild type except for lines 12257 that had significantly higher Fe at  $P \leq 0.05$ . Transgenic lines transformed with *Ubi-OsNAS1* had Fe content ranging 1.10 (lines 12274, 12277, 12282 and 12288), 1.27 (line 12276), 1.45 (lines 12278 and 12284), 1.57 (line 12275) and 1.81 (12287) fold higher than the wild type ( $10.25 \pm 1.30$  mg/kg DW). Transgenic lines transformed with *Ubi-OsNAS1* had accumulated more Fe than both *Ubi-FEA1* and wild type. The transgenic lines transformed with *Ubi-FEA1* (Figure 5.5B) had Zn content significantly less than the wild type ( $8.5 \pm 1.38$  mg/kg DW) at  $P \leq 0.05$  except for line 12257 that had 1.22 fold higher than that of the wild type. In addition, transgenic lines transformed with *Ubi-OsNAS1* had Zn content ranging from 1.12 (lines 12286 and 12291), 1.19 (line 12288), 1.25 (line 12274), 1.52 (lines 12276 and 12284), 1.65 (line 12278) and 1.82 (lines 12275 and 12288) compared to the wild type. This shows that probably transformation of Cavendish with *Ubi-OsNAS1* transgenes enhances Fe and Zn uptake in fruit pulp, while *Ubi-FEA1* has no apparent impact.

#### 5.3.4.2 *Analysis of mineral content in leaf tissue in glasshouse plants*

The mineral content of banana leaf tissue grown in the glasshouse was determined using ICP-OES to assess the effect of transgene on mineral uptake of Cavendish plants. Mineral elements Fe and Zn were assessed on the transgenic lines transformed with *Ubi-FEA1*, *Ubi-OsNAS1* and *Ubi-OsNAS2* and *Ubi-OsYSL2*, respectively. Results for Mn and Cu were also reported to give an insight into any broader impact of the transgenes of interest on plant mineral homeostasis.

The effect of transgene expression on Fe content accumulation in leaf tissue (Figure 5.6i) was analysed transgenic lines transformed with *Ubi-FEA1* had 1.04 (line 11), 1.10 (lines 8, 10, 12 and 35), 1.2 (line 29) and 1.3 (line 30) fold higher than the wild type ( $54.46 \pm 4.60$  mg/kg DW). In addition transgenic lines transformed with *Ubi-OsNAS1* had Fe content (Figure 5.6i) 1.13 (lines W6 and 38), 1.20 (line W-30), 1.41 (lines W-5 and 12) and 1.52 (lines W-3) fold higher than the wild type ( $54.46 \pm 4.60$  mg/kg DW). The data shows that Fe levels in transgenic lines transformed with both *Ubi-FEA1* and *Ubi-OsNAS1* transgenes were significantly higher than the wild type at  $P \leq 0.05$ . Overall, however, transgenic lines transformed with *Ubi-OsNAS1* accumulated more Fe. Transgenic lines transformed with *Ubi-FEA1* accumulated significantly less Zn (Figure 5.6ii) content at  $P \leq 0.05$  compared to the wild type ( $48.12 \pm 5.31$  mg/kg DW) except for line V-29 that accumulated 1.01 fold higher. In addition, transgenic lines transformed with *Ubi-OsNAS1* (Figure 5.6ii) had Zn content 3 fold (line W-5) and 1.21 (line W-12) less than the wild type ( $48.12 \pm 5.31$  mg/kg DW). Overall, this suggested that transformation of banana lines with the *Ubi-OsNAS1* (and possibly *Ubi-FEA1*) transgene enhances Fe accumulation in leaf tissue. However, neither transgene seemed to be able to improve Zn accumulation in leaf.

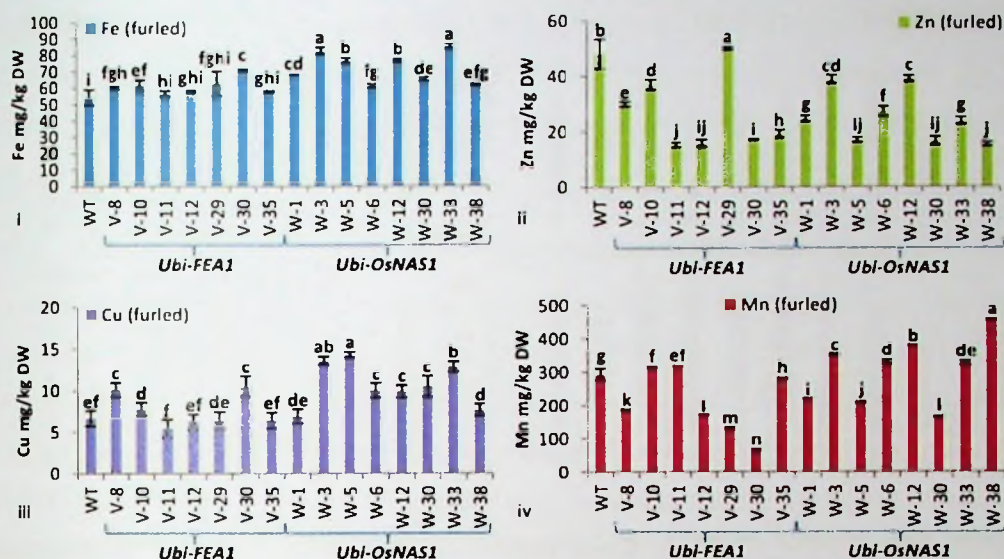
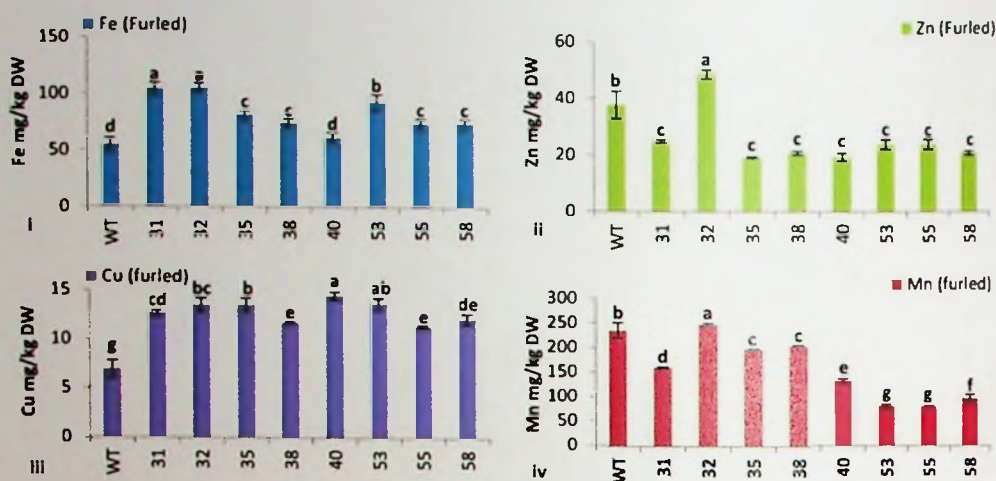


Figure 5.6: Mineral content of transgenic banana leaves transformed with Ubi-FEA1 and Ubi-OsNAS1 transgenes. The levels of i) Fe, ii) Zn, iii) Cu and iv) Mn were measured in transgenic plants transformed with *Ubi-FEA1* (lines V8–V35) and *Ubi-OsNAS1* (lines W1–W38) and in wild type (WT) plants. Data are mean  $\pm$  SD,  $n = 4$ . Means were separated using Fisher-LSD test. Samples with the same superscript letters represent groups that are not significantly different to each other at  $P \leq 0.05$ .

In addition to Fe and Zn constitutive expression of plants with transgenes is reported to accumulate other divalent elements. Thus, the effect of transgene expression on other divalent elements such as Cu and Mn content (Figure 5.6 iii and iv) accumulation in leaf tissue was analysed. Transgenic lines transformed with *Ubi-FEA1* had Cu content ranging 1.17 (line V-10) and 1.52 (lines V-8 and V-30) fold higher than the wild type ( $6.64 \pm 0.90$  mg/kg DW) respectively. In addition the Cu content of the transgenic lines transformed with *Ubi-OsNAS1* (Figure 5.6iii) ranged 1.05 (line W-1), 1.15 (line W-38), 1.51 (lines W-6, 12 and 30), 1.92 (line W-33), 2.04 (line 3) and 2.15 (line W-5) fold higher than wild type ( $6.64 \pm 0.90$  mg/kg DW) respectively. The Mn content of the transgenic lines transformed with *Ubi-FEA1* (Figure 5.6iv) was significantly less than the wild type except for lines V-10 and V-11 that had 1.1 fold higher than wild type ( $292.58 \pm 17.95$  mg/kg DW), respectively. In addition, transgenic lines transformed with *Ubi-OsNAS1* (Figure 5.6iv) had Mn content 1.11 (lines W-6 and W-33), 1.20 (line W-3), 1.3 (line W-12) and 1.56 (line W-38) fold higher than wild type ( $292.58 \pm 17.95$  mg/kg DW), respectively. The data shows that probably *OsNAS1* (but not *FEA1*) transgene transports Mn in banana lines in Cavendish. Similar results were observed with Cu and Mn accumulation to those seen for Fe and Zn, where *Ubi-OsNAS1* and *Ubi-FEA1* transformants seem to have improved Cu content, with reduction in Mn content, seen mainly in *Ubi-FEA1* lines.



**Figure 5.7:** Mineral content of transgenic banana leaves transformed with *Ubi-OsNAS2+Ubi-YSL2* transgenes. The levels of i) Fe, ii) Zn, iii) Cu and iv) Mn were measured in transgenic plants transformed with *Ubi-OsNAS2+Ubi-YSL2* (lines 31-58) and in wild type (WT) plants. Data are mean  $\pm$  SD,  $n = 4$ . Means were separated using Fisher-LSD test. Samples with the same superscript letters represent groups that are not significantly different to each other at  $P \leq 0.05$ .

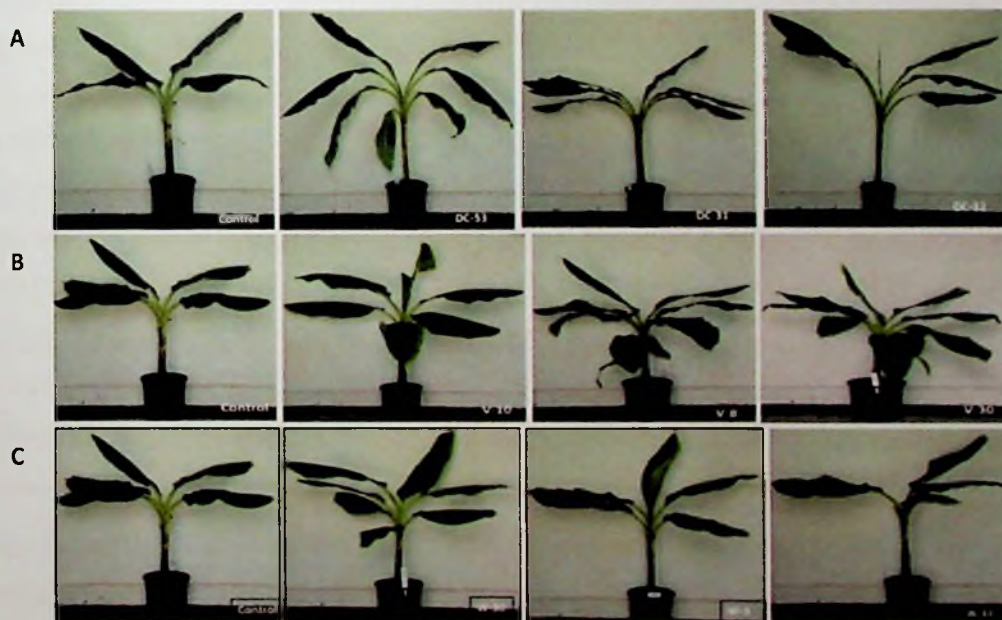
The transgenic lines transformed with *Ubi-OsNAS2+Ubi-OsYSL2* (Figure 5.7) had Fe content 1.12 (line 40), 1.36 (lines 35, 38, 55 and 58), 1.69 (line 53) and 1.91 (lines 31 and 32) fold higher than wild type ( $54.86 \pm 5.96$  mg/kg DW). The data shows that transgenic lines had significantly higher Fe than the wild type at  $P \leq 0.05$ . However, these transgenic lines accumulated significantly less Zn content (Figure 5.7ii) than the wild type ( $37.65 \pm 4.83$  mg/kg DW) at  $P \leq 0.05$  except for line 32 (1.29 fold higher). However, the majority of transgenic lines had less than half the Zn content of the wild type. The results indicate that transformation of banana cultivar Cavendish using *Ubi-OsNAS2+Ubi-OsYSL2* enhances Fe transportation in banana leaves but not Zn translocation in banana leaves cultivar Cavendish.

In addition to Fe and Zn, constitutive overexpression of plants with *Ubi-OsNAS2+Ubi-OsYSL2* transgenesis reported to accumulate other divalent elements. Thus, the effect of transgene expression on other divalent elements such as Cu and Mn content (Figure 5.7 iii and iv) accumulation in leaf tissue was analysed. The transgenic lines transformed using *Ubi-OsNAS2+Ubi-OsYSL2* had Cu content 1.64 (lines 38 and 55), 1.75 (line 58), 1.82 (line 31), 1.93 (lines 32 and 35) and 2.08 (line 40) fold higher than the wild type ( $6.96 \pm 0.84$  mg/kg DW). Transgenic lines accumulated significantly higher Cu than the wild type at  $P \leq 0.05$ . However, in these lines the Mn content of the transgenic lines transformed using *Ubi-OsNAS2+Ubi-OsYSL2* was significantly lower than that of the wild type ( $235.75 \pm 15.63$

mg/kg DW) at  $P \leq 0.05$ , except for transgenic line 32 that had 1.05 fold higher than wild type. Therefore, the data shows that *Ubi-OsNAS2+Ubi-OsYSL2* transgenes enhance Fe and Cu levels, but not Zn and Mn translocation in the vegetative banana leaf tissue during development under glasshouse conditions.

### 5.3.5 Effects of transgenes on phenotypic characteristics of bananas

In this study the effects of transgene expression on the phenotypic appearance of Cavendish plants transformed with *Ubi-FEA1*, *Ubi-OsNAS1* and *Ubi-OsNAS2+Ubi-YSL2* (Figure 5.8) were assessed. Transformation of bananas with *Ubi-FEA1* and *Ubi-OsNAS2+Ubi-OsYSL2* but not *Ubi-OsNAS1*, transgenes has physiological effects on the banana lines under similar growth conditions. Such physiological effects included stunting in *Ubi-FEA1* lines and elongation of the stem and petiole, as well as narrow leaf morphology in *Ubi-OsNAS2+Ubi-OsYSL2* as compared to the wild type.



**Figure 5.8:** Effect of transgenes on the banana phenotype. Wild type (Control) plants were compared transgenic lines transformed with A) *Ubi-OsNAS2+Ubi-YSL2* (DC), B) *Ubi-FEA1*(V), and C) *Ubi-OsNAS1*(W).

## 5.4 DISCUSSION

Biofortification of plants to enhance mineral uptake could probably alleviate micronutrient disorders such as Fe and Zn deficiency in Sub-Saharan African populations reliant on staple diets such as banana. In this study the effect of constitutive expression of the *FEA1*, *OsNAS1*, *OsNAS2* and *OsYSL2* transgenes was investigated in banana based on previous research in other staple crops such as rice (Johnson et al., 2011) and cassava (Ihemere et al., 2012). The aim of this work was achieved by identifying promising lines transformed with: *FEA1*; *OsNAS1*; and the combination of *OsNAS2* and *OsYSL2* through molecular characterisation. In addition, the mineral composition of promising lines was investigated via ICP-OES in order to assess the impact of these gens in both leaf and fruit tissue.

### 5.4.1 Effect of *FEA1* and *OsNAS1* transgenes on Fe and Zn accumulation in banana fruit

The levels of both Fe and Zn in banana fruit transformed with *FEA1* were generally similar to those of the wild type, with the exception of one line (12257), which had 1.4 and 1.3 fold more Fe and Zn, respectively. Previous work in *Arabidopsis* and *Cassava* suggested that the *FEA1* protein selectively increased Fe up to 3 fold, with no apparent impact on the levels of Zn or other elements (Ihemere et al., 2012; Narayanan, 2011). However, less Fe accumulation was observed than expected. One reason for this may be that despite using a constitutive promoter to drive *FEA1* expression, the mode of action of *FEA1* as an ion transporter may mean that it functions only in root, which is consistent with previous work (Ihemere et al., 2012; Narayanan, 2011). *FEA1* was previously shown to also work in Fe-limiting conditions in *Arabidopsis* (Narayanan, 2011), raising the possibility that any enhancement in Fe accumulation may only be observed under Fe-limiting conditions, given that even the wild type plants were otherwise capable of assimilating Fe in the optimal soil conditions used in this study.

The results show that in lines transformed with *OsNAS1*, both Fe and Zn content was generally enhanced, up to 1.8 fold, compared to the wild type, with a clear positive correlation between Fe and Zn improvement. This is consistent with previous work with *OsNAS* in rice, resulting in up to 3.5 and 2.7 fold increases in Fe and Zn content, respectively, with elevated NA levels in transgenic lines (Johnson et al., 2011). In addition, use of the barley *HvNAS1* gene in rice led to 1.5 fold increase of Fe in seeds (Usuda et al., 2009). The increased Fe and Zn content in fruit was likely due to increased NA levels, which are thought to regulate both Fe and Zn concentrations in rice grain, and are probably a major limiting factor in wild type rice plants (Johnson et al., 2011). In addition, NA forms

stable complexes with Fe (Lee et al., 2012) and is a transition metal chelator reported to facilitate intra- and intercellular transport of essential trace metal cations, including  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$  in plants (von Wirén et al., 1999). Interestingly, NA is believed to also improve Fe and Zn content and bioavailability in cereal grains (Lee et al., 2009; Zheng et al., 2010; Clemens et al., 2013), suggesting that this may also be the case in banana. Among the three rice NAS genes, the expression of *OsNAS1* and *OsNAS2* is reported to be similar, being strongly up-regulated by Fe deficiency (Masuda et al., 2009; Lee et al., 2009). However, *OsNAS2* overexpression produced the best results in rice (Johnson et al., 2011). Therefore, although this study did not assess the impact of *OsNAS2* expression in banana fruit, it is possible that this gene may yield a similar, if not greater enhancement of Fe-uptake in the fruit pulp. Further, this study shows that constitutive over-expression of *OsNAS1* genes can enhance Fe and Zn in the banana fruit pulp under field conditions with optimal soils.

#### 5.4.2 Effect of transgenes on mineral accumulation in banana leaf under glasshouse conditions

Generally, in glasshouse plants transformed with *Ubi-FEA1*, slight increases were observed in Fe and Cu content of up to 1.3 and 1.6 fold, respectively, with more improvement seen in Cu. However, quite dramatic reductions in Zn and Mn content were observed, particularly in the case of Zn. This was in contrast to previous work that suggested that the *FEA1* gene had a root-specific impact that was restricted to Fe (Ihemere et al., 2012; Narayanan, 2011). As *FEA1* appears to be preferentially active in root (Ihemere et al., 2012; Narayanan, 2011) it may be possible that *FEA1* enhances Fe uptake more in root tissue than in other plant parts. This therefore, suggests that *FEA1* transgene may perform better in more Fe-deficient soils. The levels of Cu and Mn were similar to those of Fe and Zn, respectively. Interestingly, the trends of Mn and Zn content in banana leaf were similar to these in transgenic *Arabidopsis* lines transformed with *FEA1*, which exhibited decreased Zn and Mn content in leaf relative to wild type plants, despite comparable Fe levels (Narayanan, 2011). This may indicate that overexpression of *FEA1* genes in banana may be associated with altered expression of multiple genes involved in Fe homeostasis in a variety of tissues similar to observations in previous work (Ihemere et al. (2012)), which may have broad effects on the accumulation of multiple metals. Interestingly, banana lines transformed with *Ubi-FEA1* were generally stunted compared to the wild type lines, similar to the effects observed in some *IRT1* and *FRO2* lines in Chapter 4. Since all three genes are involved in Fe uptake from the soil, it is possible that high expression of these genes may result in excessive accumulation of Fe in

roots that may cause negative impacts. Overall, the results suggest that expression of *FEA1* in banana may not be ideal strategy to increase Fe uptake in soils with abundant Fe.

Overall, in the leaf of glasshouse banana plants transformed with Ubi-*OsNAS1*, slight increases were observed in Fe and Cu as some lines has significantly higher element levels than WT, much greater improvement in Cu (up to 2 fold). A significant reduction in Mn content was also observed in some lines, and more generally in Zn levels. The same trend was also seen in banana lines transformed with both *OsNAS2* and *OsYSL2*, but these plants had lines with the highest Fe (almost 2 fold). The reduction of Mn, and particularly Zn, in banana leaf was not expected as previously, overexpression of *AtNAS1* in tobacco lead to higher Fe, Zn and Mn content in leaf (Douchkov et al., 2005; Takahashi et al., 2003), while *HvNAS1* overexpression enhanced Fe and Zn levels (Takahashi et al., 2003). The relationship between Fe and Zn content in *OsNAS1* transformants is similar to that of the inverse relationship previously observed in Chapter 4, giving further indication of common regulatory mechanisms for homeostasis of different metals (Vasconcelos et al., 2003). This also suggests that these mechanisms may operate differently in leaf and fruit, where both Fe and Zn content increased as expected. Such mechanisms would probably be influenced by increased NA concentration, itself attributed to higher *OsNAS* gene expression (Johnson et al., 2011). This is plausible as NA is through to be involved in leaf mineral homeostasis (Kim et al., 2005; Clemens et al., 2013).

The slight general improvement of leaf Fe and Cu content seen in banana lines also expressing *OsYSL2* may be due to better transport of these metals between plant tissues. *OsYSL2* is thought to be a plasma membrane transporter of Fe(II)-NA and Mn(II)-NA complexes, suggesting an involvement in long-distance metal transport (Ishimaru et al., 2010). Thus, the results in banana leaf of the elements that were observed further supports the hypothesis of altered metal homeostasis as NA is reported to chelate many metals, including Zn and Mn (Masuda et al., 2012). In rice, *OsYSL2* gene expression is thought is reported to be up regulated by Fe deficiency in phloem, and in developing seeds (Koike et al., 2004). In light of these results, this suggests that constitutive overexpression of both *OsNAS2*, which outperformed *OsNAS1* in rice (Johnson et al., 2011), and *OsYSL2* generates a synergistic effect that may be able to enhance the long-distance transport of Fe and other elements, such as Cu, into banana leaves. This may possibly improve mineral content in fruit as well.

The phenotypic effects observed in the *OsNAS2* lines may be due to the addition of *OsYSL2*. This is possible given that *OsNAS1* and *OsNAS2* enzymes have the same function, and that *OsNAS1* plants grew normally under the control of the same promoter in identical conditions. However, Ishimaru et al. (2010) reported that overexpression of *OsYSL2* in rice under Fe-sufficient conditions, showed slight changes in Fe and Mn concentrations, and no difference in the growth compared to wild type plants. However, in this study constitutive overexpression of *OsYSL2* led to aberrant phenotypes in transgenic lines. Therefore, it is difficult to predict the complex mechanism controlling the expression of *OsYSL2* under these conditions without extensive experiments. In addition, gene expression of *OsYSL2* is induced by Fe deficiency in plants (Koike et al., 2004; Inoue et al., 2008; Lee et al., 2009) but in this study the soils were Fe sufficient this shows that other factors may also be contributing to the phenotype observed. A shortage of NA is reported to cause disorders in the internal Fe transport, which leads to abnormal phenotypes suggesting that NA is indispensable for appropriate Fe translocation in plants (Ishimaru et al., 2010). However, since this work did not analyse the quantity of NA in the transgenic lines transformed with *OsNAS2+Ubi-OSYSL2* it is impossible to link the aberrant phenotype observed in Figure 5.8C with NA deficiency.

#### 5.4.3 Conclusion

In this study the focus was to examine the potential usefulness of the Fe assimilation *FEA1* gene as well as the *OsNAS1*, *OsNAS2* and *OsYSL2* genes involved in Fe mobilization for the biofortification of Fe and Zn in banana. This study demonstrated that constitutive overexpression of *OsNAS1* and *OsNAS2* genes can enhance Fe and Zn content in banana fruit pulp. However, some reduction in Zn was observed in leaf despite elevated Fe levels, indicating tissue- and element-specific plant mineral homeostasis in banana. Generally, this improvement in Fe and Zn was achieved without phenotypic side effects and was a great improvement from previous work (Chapter 4). It was unclear from the results whether *OsNAS2* or *OsYSL2* expression was responsible for the elongated stem and leaf phenotypes observed, but the normal *OsNAS1* phenotype points to a possible effect of *OsYSL2*. In contrast, constitutive *FEA1* transformants showed virtually no improvement in Fe and Zn in either leaf or fruit tissue in combination with larger reductions in leaf Mn and Zn content, compared to *OsNAS* transformants, and negative growth impacts in glasshouse plants. Therefore, the data indicated that constitutive *FEA1* expression was not a viable banana biofortification strategy.

From the results in this chapter, it is hypothesised that elevated NA levels may be responsible for the effect of *OsNAS* genes on leaf and fruit mineral content based on examination of the literature. However, analysis of NA content was beyond the scope of this study. In future this could shed light on the mechanisms behind the results observed in banana lines transformed with *OsNAS* genes. In addition, NA is believed to be an antihypertensive substance in humans, and rice lines with enhanced NA concentration may be useful for the functional food industry (Usuda et al., 2009). Analysis of the *Ubi-OsNAS2+Ubi-OsYSL2* lines under field conditions in future would help to assess the impact of these transgenes on fruit mineral content and on mature plant phenotype. In addition other studies on the individual constitutive expression of *OsNAS2* and *OsYSL2* in banana plants are essential to determine their respective effects on micronutrient accumulation in both fruit and leaf tissues. This would also allow direct comparison of the potential of *OsNAS1* and *OsNAS2* in banana. Further work is also needed to obtain more nutritionally significant increases in Fe and Zn content in banana fruit. For example, the combination of *ferritin* and *OsNAS* genes in banana may prove more effective (Johnson et al., 2011). However, these results show that using the *OsNAS* genes represent a significant step towards the goal of banana biofortification.

## Chapter 6: General discussion

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Dietary Fe deficiency is a major nutritional problem especially among women and children in developing countries like Uganda, where iron supplementation, food fortification and dietary diversification are not feasible. Fruits and vegetables are an important source of essential minerals (Ekholm et al., 2007; Milton, 2003; Tahvonen, 1993), which play a vital role in the proper development and good health of the human body (Wall, 2006). Banana is one of the most important staple foods for a large part of the East African community. Therefore, varieties containing large amounts of bioavailable Fe would have potential to ameliorate endemic Fe deficiency in this region. Breeding strategies to enhance nutrients in staple crops have been devised through conventional and genetic manipulation. However, in the case of banana conventional breeding does not seem an ideal option because no naturally high Fe bananas have yet been identified. Therefore, improving Fe content by genetic engineering is an important sustainable approach for solving Fe deficiency-related problems as key genes can be sourced from unrelated plants. However, breeding of high Fe content varieties from banana is time consuming and resource intensive (Qu et al., 2005; Goto and Yoshihara, 2001), making it important to pursue only the most promising strategies for further development.

This study aimed to understand the different interacting processes governing Fe translocation from the environment to the different plant tissues as well as the effect of different transgenes on micronutrient uptake, especially on Fe and Zn. The data obtained shows that in Uganda there are no banana genotypes with elevated Fe and Zn content in the fruit pulp. In banana leaves, Fe levels remained stable while Zn content decreased during plant development. In addition, Ugandan soils have Fe content (101–260 ppm) and pH (5–7) properties suitable for optimal banana growth, while environmental conditions have shown to play a key role in banana micronutrient uptake from the soil. Furthermore, biochemical analysis data of fruit pulp from transgenic lines showed that transformation of banana with *Sfer* and *OsNAS* genes enhanced Fe, while *IRT1* and *FRO2* genes did not. Interestingly, these genes all enhanced Fe but not Zn in the leaf tissues. Overall, *OsNAS1* lead to enhanced Fe and Zn in the fruit pulp and all transgenic lines grew normally, while some lines of other transgenes exhibited aberrant phenotypes.

Previously in Uganda, there was no comprehensive analysis of the micronutrient content of a broad range of banana cultivars. Interestingly, this study's finding that the average fruit Fe content of a range of cultivars was approximately 10 mg/kg DW of Fe was high compared to earlier reports. For example, Fungo et al. (2010) reported that cooking cultivar *Nakitembe* had Fe content between 4.4 to 5.5 mg/kg DW, while this was between 7.3 to 16.2 mg/kg DW in this study (Chapter 3). This difference may be attributed to the method of sample dissolution chosen for analysis, which according to Rodushkin et al. (1999), influences completeness and reproducibility of recovery of a given element. The variability in Fe and Zn content was observed among the different banana cultivars in this study and this has also been reported by other authors (Davey et al., 2009; Forster et al., 2002a; Forster et al., 2002b; Leterme et al., 2006; Hardisson et al., 2001a; Wall, 2006). This variability has been attributed to various environmental factors such as soil or climatic conditions that affect Fe content more than differences between cultivars (Forster et al., 2002a). Specifically, cultivation conditions such as soil fertility, soil pH, soil type, weather, water supply climate and season variation and maturity level (Tahvonen, 1993; Shewfelt, 1990) are known to affect the mineral content of vegetables and fruits reported for different regions of the world (Hardisson et al., 2001a). This variation is thought to have nutritional implications as long as the mineral bioavailability is not limiting (Leterme et al., 2006). Data obtained in this study indicate that bananas mineral content is influenced by the above mentioned factors, given that cultivar segregation by AHC clustering (Chapter 3) was more influenced by geographic origin than by cultivar genetic characteristics.

Soil is the main source of nutrients for plants therefore, the presence of nutrients in the soil is a primary indicator of their availability, and plant mineral micronutrient density variation is closely linked to soil factors (Briat and Lobréaux, 1997). However, the total concentration of nutrients is not strongly linked to their abundance but to soil variables that influence varied sorption and desorption of nutrients (Lombi et al., 2001; Kabata-Pendias, 2000). This work demonstrated that Ugandan soils have sufficient Fe to support banana plant growth, indicating that soil Fe was not a limiting factor for proper banana Fe metabolism in this region. Plants require approximately  $10^{-8}$  M total soluble Fe, but in alkaline soils this is no more than  $10^{-10}$  M (Briat et al., 1995). Thus, without active mechanisms for extracting Fe from the soil, most plants would therefore exhibit Fe-deficiency symptoms, such as leaf interveinal chlorosis (Briat et al., 2007). The soil pH affects mineral solubility, and thus the availability of various nutrients and chemical species to plant roots (Turner, 1980), and is therefore a good predictor of plant nutrient deficiency or toxicity (McKenzie et al. 2004).

According to (Turner, 1997), soil pH ranging from 4.7 – 8.0 has been reported to give good yields in bananas, which prefer neutral to moderately acidic conditions (Martin-Prével, 1989). The bioavailability of Fe has been reported to be highly dependent on the physiochemical properties of a given soil, pH, including solid phase composition, chemical speciation of Fe in solution and chelation and reduction processes (Lindsay, 1995). Interestingly, the PCA analysis in this study suggested that altitude of the banana growing district was negatively correlated with fruit Fe and Zn content in the second principle component. This might be because altitude may be an indication of the geographic and climatic characteristics of the sample's origin. Therefore, the altitude variable might incorporate the influence of rainfall patterns and temperature that have a profound effect on plant health and productivity (Leterme et al., 2006; Frossard et al., 2000). There appears to be three broad aspects of plant Fe metabolism that can be enhanced to enable biofortification of this mineral in important crops. These are: uptake from the soil by the roots, redistribution to other parts of the plant and storage in the desired destination organs. This study has examined approaches to enhance each of these three processes in order to enhance Fe accumulation in fruit, the edible portion of the banana plant. Biofortification of Fe is tricky as Fe levels within the plant are highly regulated as it is highly toxic due to its reactivity (Arosio et al., 2009; Grusak et al., 1999). This study examined the impact of enhancing each component of Fe metabolism. Fe uptake was enhanced using the *FRO2*, *IRT1* and *FEA1* genes to increase the influx of usable Fe into the plant. Redistribution of this mineral was improved using the *NAS2* and *YSL2* genes from rice to remobilise Fe from leaf into the fruit, as previous results (Chapter 3) indicate that the former tissue has significantly higher Fe content. Finally, Fe storage capacity in banana fruit was increased using the *Sfer* gene as each ferritin molecule can store thousands of Fe atoms (Theil, 2000) in a bioavailable form (Lönnerdal, 2009; Murray-Kolb et al., 2002; Beard et al., 1996). The most promising approaches were redistribution using *NAS* genes, followed by storage via *Sfer*, which gave plants with significant increases in fruit Fe content and normal phenotype. Notably, other approaches typically seemed to enhance fruit Fe content at the expense of Zn, another important micronutrient, while *NAS1* lines also have elevated Zn in this tissue. However, all transgenes enhance Fe uptake in the leaf tissues. Consequently, these results indicate that there is a complex mineral regulatory framework that simultaneously balances the levels of different minerals in different organs.

The varying levels of Fe and Zn in fruit compared to wild type plants may be linked to altered mineral homeostasis induced by the activity of the selected transgenes. Several

studies have shown that Fe uptake genes transport Zn in addition to Fe and other divalent elements (Eide et al., 1996; Korshunova et al., 1999; Vert et al., 2002). For example, the IRT1 protein is known to have a broad substrate range (Eide et al., 1996; Rogers et al., 2000) which may include Mn, Zn, Cd and Co (Bughio et al., 2002; Eckhardt et al., 2001). In addition, ferritin appears to be able to store other divalent metals such as Zn and Cu (Price and Joshi, 1982; Price and Joshi, 1983) as well as Fe under certain conditions. Increased reductase activity from *FRO2* overexpression may also have a significant impact on mineral homeostasis, despite being highly Fe-specific (Connolly et al., 2003), by increasing the amount of  $\text{Fe}^{2+}$  available to be absorbed via endogenous Fe transporters such as IRT1. Further, excessive accumulation of specific minerals such as Fe would likely impact the function of other components of mineral homeostasis with broad substrate specificity, such as the NRAMP protein family that can transport various metals including  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Al}^{3+}$  (Thomine et al., 2000; Nevo and Nelson, 2006; Xia et al., 2010). Therefore the accumulation of higher Fe levels may reduce the ability of the plant to manage the metabolism of other metals such as Zn, which have overlapping regulatory pathways (Vasconcelos et al., 2003), possibly explaining the apparent inverse correlation between fruit Fe and Zn content observed in this study. Interestingly, leaf Zn accumulation was shown to be negatively correlated with external supply of Fe (Wirth et al., 2009).

Plants that assimilate large amounts of free Fe are probably at risk of Fe-mediated oxidative stress because excess Fe amounts can catalyse the generation of reactive oxygen species via Fenton reaction chemistry (Arosio et al., 2009). This thesis shows that constitutive overexpression of the bananas with Fe uptake genes did not effectively enhance Fe and Zn uptake in the fruit pulp. However, increased Fe accumulation was observed in leaf tissue and aberrant phenotypes like stunting were observed in some of the transgenic lines. Similar observations were reported by (Vasconcelos et al., 2006), where constitutive *FRO2* expression under non-Fe stress conditions led to a decrease in plant productivity as reflected by reduced biomass accumulation in the transgenic events under non-Fe stress conditions. In addition, expression of *IRT1* and *FRO2* is complicated by their induction by local Fe in the surrounding soils (Vert et al., 2003). This is usually observed in high alkaline or low Fe soil conditions where Fe solubility is problematic (Robinson et al., 1999). In addition, according to Connolly et al. (2003), Zn levels also influences *FRO2* and *IRT1* transcript abundance. Thus, this work seems to confirm that plants must manage Fe influx and homeostasis carefully to minimize iron toxicity at all points within the system (Grusak et al., 1999). Therefore, it is important not only to increase Fe uptake ability, but also

enhance the storage capacity of iron in a non-toxic and bioavailable form (Qu et al., 2005). This may also explain why the storage and remobilisation approaches using *Sfer* and *NAS* genes, respectively, were more successful approaches. These strategies may have been able to improve Fe accumulation with a smaller impact on mineral homeostasis, possibly by interacting with Fe to sequester it in an un reactive state.

Ferritin molecules store excess Fe in the cytoplasm for release on demand (Theil and Hase, 1993) and appear to be involved in prevention of oxidative stress normally due to excess Fe (Goto and Yoshihara, 2001). Research showed that increasing Fe storage capacity effectively stimulated the uptake and sequestration of additional Fe into cells (Van Wuytswinkel et al., 1999), possibly explaining why ferritin can enhance Fe with no side effect. Interestingly, leaf appears to be a much better Fe sink in bananas than the fruit pulp in both wild type and transgenic lines, as much higher accumulation of Fe was observed. This is consistent with observations in other ferritin transformed plants (Goto et al., 1999; Lucca et al., 2001; Vasconcelos et al., 2003) where Fe content increased more in vegetative tissue than in the seed. This may be due to the fact that leaves are a major sink for plant Fe storage, with 80% of Fe localised in chloroplasts (Theil and Briat, 2004), and Fe metabolism changes during leaf development as ferritin levels increase in developing leaves (Briat and Lobréaux, 1997). This makes sense as leaves are typically more metabolically active than fruit, constantly managing photosynthesis and other complex metabolic processes. This also explains the overall trend during leaf development where Fe levels increased between the flowering and fruiting stages of both wild type and transgenic lines. The reduction of leaf Zn accumulation often observed during banana development may be due to the increasing Fe accumulation (Wirth et al., 2009), or the transport of Zn to other parts of the plant during this phase.

NA, a chelator of Fe and other heavy metals, plays a key role in iron uptake, phloem transport and cytoplasmic distribution and ensures Fe solubility in the weakly alkaline environment of the cytoplasm (von Wirén et al., 1999; Hell and Stephan, 2003; Takahashi, 2003). As a transition metal-chelator, NA is reported to facilitate the intra- and intercellular transport of essential trace metal cations, including  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$ , in plants (von Wirén et al., 1999; Curie et al., 2009; Pich et al., 1994; Higuchi et al., 2001). The approach used in this study was similar to those used in previous research where enhanced Fe and Zn uptake in other plants was reported in rice (Johnson et al., 2011; Masuda et al., 2009; Zheng et al., 2010), tobacco and Arabidopsis (Kim et al., 2005) using constitutive overexpression of *OsNAS* genes. It is possible that the overexpression of *NAS* genes in banana resulted in

increased metal translocation in transgenic plants to the fruit pulp from sources such as leaf and root tissue, likely enabled by higher concentrations of NA in the plant. Although, NA content was not determined in this study, increased concentrations of this metabolite were observed in rice lines with increased grain Fe and Zn content was due to overexpression of NAS genes (Johnson et al., 2011). Furthermore, because of the positive effects of NA on Fe uptake and accumulation in cereals roots and seeds (Douchkov et al., 2005; Cheng et al., 2007), it is postulated from this study that elevating NA in the edible portions of banana fruit pulp might improve iron bioavailability to humans by chelating Fe to form a soluble NA-ferrous complex. Interestingly, the leaves of rice transformed with *OsNAS1* showed enhanced Fe and Zn content (Zhenget al., 2010), while this study reported enhanced Fe but not Zn content. This is possibly due to differences in how these minerals are regulated in rice compared to banana.

The results of this study has demonstrated that work to improve Fe and Zn accumulation in banana needs to be done while minimising disruption of banana mineral homeostasis. This could be achieved by improving control of the expression of the transgenes used in this study. One way to enhance transgene expression would be to assess the genes used in this study in combination with different promoters. For example, constitutive over-expression using Fe uptake transgenes does not, however, amount to enhanced metal uptake in plant tissues (Connolly et al., 2002; Connolly et al., 2003). Similar findings have been found in this study in the banana fruit pulp where the level of Fe was not significantly different from the control. In addition, several reports have shown enhanced Fe content in transgenic rice using soy ferritin under different promoters (Goto et al., 1999; Lucca et al., 2001; Vasconcelos et al., 2003), although Fe accumulation does not always parallel the ferritin levels (Ravet et al., 2009b). This study used constitutive overexpression of NAS genes to increase Fe levels in fruit, however, altered homeostasis of Zn, Mn and Cu was observed in leaf. Also, the precursor for NA biosynthesis is a methionine derivative (Kobayashi et al. (2005)). This means that care must to be taken not to disrupt the metabolism of this important amino acid, since using a strong constitutive promoter leads to a significant increase of NA content in transgenic plants (Johnson et al., 2011). In addition, further research should investigate the possibility of combining multiple genes, such as NAS and ferritin, and the use of genes involved in other Fe storage and transport mechanisms such as vacuolar Fe transporters. These combinations of genes could be investigated with appropriate promoters to optimise Fe accumulation in target organs. For example, moderate constitutive expression of a NAS gene in combination with a ferritin regulated by

a fruit-specific promoter could enhance Fe content in fruit by simultaneously improving Fe mobilisation throughout the plant for storage in the target tissue at an appropriate stage of development. Future research should also be done to quantify the levels of ferritins and NAS proteins in both the banana pulp as well as the leaf tissues since these transgenic lines gave promising results. In addition quantitative real-time PCR should be conducted on the *OsNAS1* plants to elucidate whether expression level played a significant role in Fe enhancement in both fruit pulp and vegetative leaf tissues.

In conclusion, this study has shown that biofortification could enhance Fe and Zn content in banana fruits since data obtained in baseline study showed that there are no wild genotypes with enhanced Fe and Zn content. In addition, this work examined the potential utility of various genes involved in Fe metabolism to enhance the accumulation of this mineral in banana fruit. Previously, these mechanisms have been reported in other plants, however, this thesis shows that manipulation of bananas with Fe assimilation proteins from other plants like soybean and rice is feasible. As far as it could be determined, this study represents the first time that this has been demonstrated in banana fruit. Furthermore, this shows that biofortified bananas can be generated with the potential to provide a source of bioavailable Fe in fruit through the use of ferritin or NAS genes, as well as an anti-hypertensive food where NAS genes are used. Thus far, the improvements observed in this study are not yet sufficient to meet the dietary Fe requirements of populations at risk of IDA. However, this study has identified potential strategies by which further improvements can be made. Importantly, the knowledge gained from this project could be useful to inform further Fe biofortification research in banana aimed at alleviating IDA in developing countries whose staple diet is banana.

## Bibliography

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- ABRAMS, S. A., GRIFFIN, I. J., HAWTHORNE, K. M., LIANG, L., GUNN, S. K., DARLINGTON, G. & ELLIS, K. J. 2005. A combination of prebiotic short- and long-chain inulin-type fructans enhances calcium absorption and bone mineralization in young adolescents. *The American journal of clinical nutrition*, 82, 471-476.
- ABRAMS, S. A., HAWTHORNE, K. M., ALIU, O., HICKS, P. D., CHEN, Z. & GRIFFIN, I. J. 2007. An inulin-type fructan enhances calcium absorption primarily via an effect on colonic absorption in humans. *The Journal of nutrition*, 137, 2208-2212.
- ACHAM, H., KIKAFUNDA, J., TYLLESKAR, T. & MALDE, M. 2012. Nutritional and health status primary schoolchildren in rural Uganda. *African Journal of Food, Agriculture, Nutrition and Development*, 12, 5862-5880.
- AGTE, V., TARWADI, K., MENGALE, S. & CHIPLONKAR, S. 2000. Potential of traditionally cooked green leafy vegetables as natural sources for supplementation of eight micronutrients in vegetarian diets. *Journal of Food Composition and Analysis*, 13, 885-891.
- AKED, J. & KYAMUHANGIRE, W. 1996. Postharvest aspects of highland bananas in Uganda. *Tropical Science*, 36.
- AQUIL, B., JAN, A. T., SARIN, N. B. & HAQ, Q. M. R. 2012. MICROPROPAGATION AND GENETIC TRANSFORMATION OF BANANA FOR CROP IMPROVEMENT AND SUSTAINABLE AGRICULTURE. *Journal of Crop Science*, 3, 64-77.
- AREMU, C. & UDOESSIEN, E. 1990. Chemical estimation of some inorganic elements in selected tropical fruits and vegetables. *Food Chemistry*, 37, 229-234.
- ARINAITWE, G., REMY, S., STROSSE, H., SWENNEN, R., SÁGI, L. & JAIN, S. Agrobacterium- and particle bombardment-mediated transformation of a wide range of banana cultivars. Banana improvement: cellular, molecular biology, and induced mutations. Proceedings of a meeting held in Leuven, Belgium, 24-28 September 2001., 2004. Science Publishers, Inc., 351-357.
- AROSIO, P., INGRASSIA, R. & CAVADINI, P. 2009. Ferritins: a family of molecules for iron storage, antioxidation and more. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1790, 589-599.
- BAKRY, F., CARREEL, F., JENNY, C. & HARRY, J.-P. 2009. Genetic improvement of banana. *Breeding plantation tree crops: tropical species*. Springer.
- BARBERON, M., ZELAZNY, E., ROBERT, S., CONÉJÉRO, G., CURIE, C., FRIML, J. & VERT, G. 2011. Monoubiquitin-dependent endocytosis of the iron-regulated transporter 1 (IRT1) transporter controls iron uptake in plants. *Proceedings of the National Academy of Sciences*, 108, E450-E458.
- BARNESA 2008. Banana and Plantain production. Available online. <http://www.asareca.org>.
- BASHIR, K., INOUE, H., NAGASAKA, S., TAKAHASHI, M., NAKANISHI, H., MORI, S. & NISHIZAWA, N. K. 2006. Cloning and characterization of deoxymugineic acid synthase genes from graminaceous plants. *Journal of Biological Chemistry*, 281, 32395-32402.

- BASHIR, K., ISHIMARU, Y. & NISHIZAWA, N. K. 2010. Iron uptake and loading into rice grains. *Rice*, 3, 122-130.
- BASHIR, K., ISHIMARU, Y., SHIMO, H., NAGASAKA, S., FUJIMOTO, M., TAKANASHI, H., TSUTSUMI, N., AN, G., NAKANISHI, H. & NISHIZAWA, N. K. 2011. The rice mitochondrial iron transporter is essential for plant growth. *Nature communications*, 2, 322.
- BEARD, J. L., BURTON, J. W. & THEIL, E. C. 1996. Purified ferritin and soybean meal can be sources of iron for treating iron deficiency in rats. *The Journal of nutrition*, 126, 154-160.
- BECKER, D., DUGDALE, B., SMITH, M., HARDING, R. & DALE, J. 2000. Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cvGrand Nain'via microprojectile bombardment. *Plant Cell Rep*, 19, 229-234.
- BERECZKY, Z., WANG, H.-Y., SCHUBERT, V., GANAL, M. & BAUER, P. 2003. Differential regulation of nramp and irt metal transporter genes in wild type and iron uptake mutants of tomato. *Journal of Biological Chemistry*, 278, 24697-24704.
- BHARGAVA, A., BOUIS, H. E. & SCRIMSHAW, N. S. 2001. Dietary intakes and socioeconomic factors are associated with the hemoglobin concentration of Bangladeshi women. *The Journal of nutrition*, 131, 758-764.
- BHULLAR, N. K. & GRUISSEM, W. 2013. Nutritional enhancement of rice for human health: The contribution of biotechnology. *Biotechnology Advances*, 31, 50 - 57.
- BLACK, R. E., ALLEN, L. H., BHUTTA, Z. A., CAULFIELD, L. E., DE ONIS, M., EZZATI, M., MATHERS, C. & RIVERA, J. 2008. Maternal and child undernutrition: global and regional exposures and health consequences. *The lancet*, 371, 243-260.
- BOARDMAN, R. & MCGUIRE, D. 1990. The role of zinc in forestry. I. Zinc in forest environments, ecosystems and tree nutrition. *Forest Ecology and Management*, 37, 167-205.
- BOSQUE-PÉREZ, N. A., MAY, G. D. & ARNTZEN, C. J. Applicability of an Agrobacterium-based system for the transformation of *Musa* species with diverse genomic constitution and ploidy level. I International Conference on Banana and Plantain for Africa 540, 1996. 193-201.
- BOUIS, H. E. 1999. Economics of enhanced micronutrient density in food staples. *Field Crops Research*, 60, 165-173.
- BOUIS, H. E. 2003. Micronutrient fortification of plants through plant breeding: can it improve nutrition in man at low cost? *Proceedings of the Nutrition Society*, 62, 403-412.
- BOUIS, H. E., CHASSY, B. M. & OCHANDA, J. O. 2003. 2. Genetically modified food crops and their contribution to human nutrition and food quality. *Trends in Food Science & Technology*, 14, 191-209.
- BOUIS, H. E., HOTZ, C., MCCLAFFERTY, B., MEENAKSHI, J. & PFEIFFER, W. H. 2011. Biofortification: a new tool to reduce micronutrient malnutrition. *Food & Nutrition Bulletin*, 32, 31S-40S.
- BOUIS, H. E. & WELCH, R. M. 2010. Biofortification—A sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. *Crop Science*, 50, S-20-S-32.
- BOUYOUCOS, G. J. 1962. Hydrometer method improved for making particle size analyses of soils. *Agronomy Journal*, 54, 464-465.

- BRIAT, J.-F., CURIE, C. & GAYMARD, F. 2007. Iron utilization and metabolism in plants. *Current opinion in plant biology*, 10, 276-282.
- BRIAT, J.-F., DUC, C., RAVET, K. & GAYMARD, F. 2010. Ferritins and iron storage in plants. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1800, 806-814.
- BRIAT, J.-F. & LOBRÉAUX, S. 1997. Iron transport and storage in plants. *Trends in Plant Science*, 2, 187-193.
- BRIAT, J. F., FOBIS-LOISY, I., GRIGNON, N., LOBRÉAUX, S., PASCAL, N., SAVINO, G., THOIRON, S., WIRÉN, N. & WUYTSWINKEL, O. 1995. Cellular and molecular aspects of iron metabolism in plants. *Biology of the Cell*, 84, 69-81.
- BROCK, J. H., HALLIDAY, J., PIPPARD, M. & POWELL, L. 1994. *Iron metabolism in health and disease*, WB Saunders.
- BRONICK, C. J. & LAL, R. 2005. Soil structure and management: a review. *Geoderma*, 124, 3-22.
- BRUCE, R. C. & RAYMENT, G. 1982. *Analytical methods and interpretations used by the Agricultural Chemistry Branch for soil and land use surveys*, Queensland Department of Primary Industries.
- BUGHIO, N., YAMAGUCHI, H., NISHIZAWA, N. K., NAKANISHI, H. & MORI, S. 2002. Cloning an iron-regulated metal transporter from rice. *Journal of Experimental Botany*, 53, 1677-1682.
- CAILLIATTE, R., SCHIKORA, A., BRIAT, J.-F., MARI, S. & CURIE, C. 2010. High-affinity manganese uptake by the metal transporter NRAMP1 is essential for Arabidopsis growth in low manganese conditions. *The Plant Cell Online*, 22, 904-917.
- CARSWELL, G. 2003. Food crops as cash crops: the case of colonial Kigezi, Uganda. *Journal of Agrarian Change*, 3, 521-551.
- CAULFIELD, L. E., RICHARD, S. A., RIVERA, J. A., MUSGROVE, P., BLACK, R. E., JAMISON, D., BREMAN, J., MEASHAM, A., ALLEYNE, G. & CLAESON, M. 2006. Stunting, wasting, and micronutrient deficiency disorders. *Disease control priorities in developing countries*, 551-567.
- CHANDLER, S. 1995. The nutritional value of bananas. *Bananas and plantains*. Springer.
- CHENG, L., WANG, F., SHOU, H., HUANG, F., ZHENG, L., HE, F., LI, J., ZHAO, F.-J., UENO, D. & MA, J. F. 2007. Mutation in nicotianamine aminotransferase stimulated the Fe (II) acquisition system and led to iron accumulation in rice. *Plant Physiology*, 145, 1647-1657.
- CHERNEY, J. & ROBINSON, D. 1983. A comparison of plant digestion methods for identifying soil contamination of plant tissue by Ti analysis. *Agronomy Journal*, 75, 145-147.
- CHRISTENSEN, A. H. & QUAIL, P. H. 1996. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Research*, 5, 213-218.
- CHRISTENSEN, A. H., SHARROCK, R. A. & QUAIL, P. H. 1992. Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Molecular Biology*, 18, 675-689.
- CHRISTOU, P. 1992. Genetic transformation of crop plants using microprojectile bombardment. *The Plant Journal*, 2, 275-281.

- CLEMENS, S., DEINLEIN, U., AHMADI, H., HÖRETH, S. & URAGUCHI, S. 2013. Nicotianamine is a major player in plant Zn homeostasis. *Biometals*, 26, 623-632.
- COLANGELO, E. P. & GUERINOT, M. L. 2004. The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. *The Plant Cell Online*, 16, 3400-3412.
- COLANGELO, E. P. & GUERINOT, M. L. 2006. Put the metal to the petal: metal uptake and transport throughout plants. *Current Opinion in Plant Biology*, 9, 322-330.
- CONNOLLY, E. L., CAMPBELL, N. H., GROTZ, N., PRICHARD, C. L. & GUERINOT, M. L. 2003. Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control. *Plant Physiology*, 133, 1102-1110.
- CONNOLLY, E. L., FETT, J. P. & GUERINOT, M. L. 2002. Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *The Plant Cell Online*, 14, 1347-1357.
- CONTE, S., CHU, H., RODRIGUEZ, D. C., PUNSHON, T., VASQUES, K., SALT, D. E. & WALKER, E. L. 2013. Arabidopsis thaliana Yellow Stripe1-Like4 and Yellow Stripe1-Like6 localize to internal cellular membranes and are involved in metal ion homeostasis. *Frontiers in Plant Science*, 4.
- CONTE, S. S. & WALKER, E. L. 2011. Transporters contributing to iron trafficking in plants. *Molecular plant*, 4, 464-476.
- COOK, L. L., MCGONIGLE, T. P. & INOUE, R. S. 2009. Titanium as an indicator of residual soil on arid-land plants. *Journal of environmental quality*, 38, 188-199.
- COSGROVE, D. J. 2000. New genes and new biological roles for expansins. *Current Opinion in Plant Biology*, 3, 73-78.
- CURIE, C., ALONSO, J., LE JEAN, M., ECKER, J. & BRIAT, J. 2000. Involvement of NRAMP1 from Arabidopsis thaliana in iron transport. *Biochem. J*, 347, 749-755.
- CURIE, C. & BRIAT, J.-F. 2003. Iron transport and signaling in plants. *Annual Review of Plant Biology*, 54, 183-206.
- CURIE, C., CASSIN, G., COUCH, D., DIVOL, F., HIGUCHI, K., LE JEAN, M., MISSON, J., SCHIKORA, A., CZERNIC, P. & MARI, S. 2009. Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. *Annals of botany*, 103, 1-11.
- CURIE, C., PANAVIENE, Z., LOULERGUE, C., DELLAPORTA, S. L., BRIAT, J.-F. & WALKER, E. L. 2001. Maize yellow stripe1 encodes a membrane protein directly involved in Fe (III) uptake. *Nature*, 409, 346-349.
- DAVEY, M. W., STALS, E., NGOH-NEWILAH, G., TOMEKPE, K., LUSTY, C., MARKHAM, R., SWENNEN, R. & KEULEMANS, J. 2007. Sampling strategies and variability in fruit pulp micronutrient contents of West and Central African bananas and plantains (Musa species). *Journal of agricultural and food chemistry*, 55, 2633-2644.
- DAVEY, M. W., VAN DEN BERGH, I., MARKHAM, R., SWENNEN, R. & KEULEMANS, J. 2009. Genetic variability in Musa fruit provitamin A carotenoids, lutein and mineral micronutrient contents. *Food Chemistry*, 115, 806-813.
- DELLAPORTA, S. L., WOOD, J. & HICKS, J. B. 1983. A plant DNA miniprep: version II. *Plant molecular biology reporter*, 1, 19-21.

- DIDONATO, R. J., ROBERTS, L. A., SANDERSON, T., EISLEY, R. B. & WALKER, E. L. 2004. Arabidopsis Yellow Stripe-Like2 (YSL2): a metal-regulated gene encoding a plasma membrane transporter of nicotianamine-metal complexes. *The Plant Journal*, 39, 403-414.
- DIVOL, F., COUCH, D., CONÉJÉRO, G., ROSCHZTTARDTZ, H., MARI, S. & CURIE, C. 2013. The Arabidopsis YELLOW STRIPE LIKE4 and 6 transporters control iron release from the chloroplast. *The Plant Cell Online*, 25, 1040-1055.
- DOUCHKOV, D., GRYCZKA, C., STEPHAN, U., HELL, R. & BÄUMLEIN, H. 2005. Ectopic expression of nicotianamine synthase genes results in improved iron accumulation and increased nickel tolerance in transgenic tobacco. *Plant, Cell & Environment*, 28, 365-374.
- DUGDALE, B., BECKER, D., BEETHAM, P., HARDING, R. & DALE, J. 2000. Promoters derived from banana bunchy top virus DNA-1 to- 5 direct vascular-associated expression in transgenic banana (*Musa* spp.). *Plant Cell Rep*, 19, 810-814.
- DUGDALE, B., BECKER, D. K., HARDING, R. M. & DALE, J. L. 2001. Intron-mediated enhancement of the banana bunchy top virus DNA-6 promoter in banana (*Musa* spp.) embryogenic cells and plants. *Plant Cell Rep*, 20, 220-226.
- DURRETT, T. P., GASSMANN, W. & ROGERS, E. E. 2007. The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiology*, 144, 197-205.
- ECKHARDT, U., MARQUES, A. M. & BUCKHOUT, T. J. 2001. Two iron-regulated cation transporters from tomato complement metal uptake-deficient yeast mutants. *Plant molecular biology*, 45, 437-448.
- EIDE, D., BRODERIUS, M., FETT, J. & GUERINOT, M. L. 1996. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proceedings of the National Academy of Sciences*, 93, 5624-5628.
- EKHOLM, P., REINIVUO, H., MATTILA, P., PAKKALA, H., KOPONEN, J., HAPPONEN, A., HELLSTRÖM, J. & OVASKAINEN, M.-L. 2007. Changes in the mineral and trace element contents of cereals, fruits and vegetables in Finland. *Journal of Food Composition and Analysis*, 20, 487-495.
- FAOSTAT 2001. Available online. <http://www.faostat.fao.org>.
- FAOSTAT 2004. Available online. <http://www.faostat.fao.org>.
- FAOSTAT 2010. <http://www.faostat.fao.org>.
- FORBES, J. R. & GROS, P. 2001. Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. *Trends in microbiology*, 9, 397-403.
- FORSTER, M. P., RODRÍGUEZ, E. R., MARTÍN, J. D. & ROMERO, C. D. 2002a. Statistical differentiation of bananas according to their mineral composition. *Journal of agricultural and food chemistry*, 50, 6130-6135.
- FORSTER, M. P., RODRÍGUEZ RODRÍGUEZ, E. & DÍAZ ROMERO, C. 2002b. Differential characteristics in the chemical composition of bananas from Tenerife (Canary Islands) and Ecuador. *Journal of agricultural and food chemistry*, 50, 7586-7592.
- FRISON, E. & SHARROCK, S. The economic, social and nutritional importance of banana in the world. Bananas and food security. International symposium, Douala, Cameroon, 1998. 10-14.

- FROSSARD, E., BUCHER, M., MÄCHLER, F., MOZAFAR, A. & HURRELL, R. 2000. Potential for increasing the content and bioavailability of Fe, Zn and Ca in plants for human nutrition. *Journal of the Science of Food and Agriculture*, 80, 861-879.
- FUNGO, R., KIKAFUNDA, J. & PILLAY, M. 2010.  $\beta$ -carotene, iron and zinc content in Papua New Guinea and East African highland bananas. *African Journal of Food, Agriculture, Nutrition and Development*, 10.
- GANAPATHI, T., HIGGS, N., BALINT-KURTI, P., ARNTZEN, C., MAY, G. & VAN ECK, J. 2001. Agrobacterium-mediated transformation of embryogenic cell suspensions of the banana cultivar Rasthali (AAB). *Plant Cell Reports*, 20, 157-162.
- GENSI, R., KYAMUHANGIRE, W. & CARASCO, J. Traditional production and characteristics of banana juice in Uganda. African Crop Science Conference Proceedings, 1994. 356-359.
- GIBERT, O., DUFOUR, D., GIRALDO, A. S., SÁNCHEZ, T., REYNES, M., PAIN, J.-P., GONZÁLEZ, A., FERNÁNDEZ, A. & DÍAZ, A. 2009. Differentiation between cooking bananas and dessert bananas. 1. Morphological and compositional characterization of cultivated Colombian Musaceae (*Musa* sp.) in relation to consumer preferences. *Journal of agricultural and food chemistry*, 57, 7857-7869.
- GÓMEZ-GALERA, S., ROJAS, E., SUDHAKAR, D., ZHU, C., PELACHO, A. M., CAPELL, T. & CHRISTOU, P. 2010. Critical evaluation of strategies for mineral fortification of staple food crops. *Transgenic research*, 19, 165-180.
- GOSWAMI, B. & BORTHAKUR, A. 1996. Chemical and biochemical aspects of developing culinary banana (< i> Musa</i> ABB)'< i> Kachka</i>'. *Food Chemistry*, 55, 169-172.
- GOTO, F. & YOSHIHARA, T. 2001. Improvement of micronutrient contents by genetic engineering—development of high iron content crops. *Plant Biotechnology*, 18, 7-15.
- GOTO, F., YOSHIHARA, T. & SAIKI, H. 1998. Iron accumulation in tobacco plants expressing soyabean ferritin gene. *Transgenic research*, 7, 173-180.
- GOTO, F., YOSHIHARA, T. & SAIKI, H. 2000. Iron accumulation and enhanced growth in transgenic lettuce plants expressing the iron-binding protein ferritin. *Theoretical and Applied Genetics*, 100, 658-664.
- GOTO, F., YOSHIHARA, T., SHIGEMOTO, N., TOKI, S. & TAKAIWA, F. 1999. Iron fortification of rice seed by the soybean ferritin gene. *Nature biotechnology*, 17, 282-286.
- GRAHAM, R. D., WELCH, R. M. & BOUIS, H. E. 2001. Addressing micronutrient malnutrition through enhancing the nutritional quality of staple foods: principles, perspectives and knowledge gaps. *Advances in Agronomy*, 70, 77-142.
- GREEN, L. S. & ROGERS, E. E. 2004. FRD3 controls iron localization in Arabidopsis. *Plant Physiology*, 136, 2523-2531.
- GROTZ, N. & GUERINOT, M. L. 2002. Limiting nutrients: an old problem with new solutions? *Current opinion in plant biology*, 5, 158-163.
- GROTZ, N. & GUERINOT, M. L. 2006. Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1763, 595-608.

- GRUSAK, M. A. 1994. Iron transport to developing ovules of *Pisum sativum* (L. Seed import characteristics and phloem iron-loading capacity of source regions). *Plant Physiology*, 104, 649-655.
- GRUSAK, M. A. 1999. Improving The Nutrient Composition OF Plants To Enhance Human Nutrition and Health. *Annual Review in Plant Physiology and Plant Molecular Biology* 50, 133-161.
- GRUSAK, M. A., PEARSON, J. & MARENTES, E. 1999. The physiology of micronutrient homeostasis in field crops. *Field crops research*, 60, 41-56.
- GUERINOT, J. J. A. M. L. 2009. Homing in on iron homeostasis in plants. *Trends in plant science*, 14, 280-285.
- GUERINOT, M. L. 2000. The ZIP family of metal transporters. *Biochimica et Biophysica Acta*, 1465, 190-198.
- GUERINOT, M. L. 2007a. It's elementary: Enhancing Fe<sup>3+</sup> reduction improves rice yields. *Proceedings of the National Academy of Sciences*, 104, 7311-7312.
- GUERINOT, S. A. K. A. M. L. 2007b. Mining iron: Iron uptake and transport in plants. *FEBS Letters*, 581, 2273 - 2280.
- HARDISSON, A., RUBIO, C., BAEZ, A., MARTIN, M., ALVAREZ, R. & DIAZ, E. 2001a. Mineral composition of the banana (< i> Musa acuminata</i>) from the island of Tenerife. *Food Chemistry*, 73, 153-161.
- HARDISSON, A., RUBIO, C., BAEZ, A., MARTIN, M. M. & ALVAREZ, R. 2001b. Mineral composition of the papaya (*Carica papaya* variety sunrise) from Tenerife island. *European Food Research and Technology*, 212, 175-181.
- HARRISON, P. M. & AROSIO, P. 1996. The ferritins: molecular properties, iron storage function and cellular regulation. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1275, 161-203.
- HELL, R. & STEPHAN, U. W. 2003. Iron uptake, trafficking and homeostasis in plants. *Planta*, 216, 541-551.
- HENRIQUES, R., JÁSIK, J., KLEIN, M., MARTINOIA, E., FELLER, U., SCHELL, J., PAIS, M. S. & KONCZ, C. 2002. Knock-out of Arabidopsis metal transporter gene IRT1 results in iron deficiency accompanied by cell differentiation defects. *Plant molecular biology*, 50, 587-597.
- HENTZE, M. W., MUCKENTHALER, M. U. & ANDREWS, N. C. 2004. Balancing acts: molecular control of mammalian iron metabolism. *Cell*, 117, 285-297.
- HIEI, Y., OHTA, S., KOMARI, T. & KUMASHIRO, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *The Plant Journal*, 6, 271-282.
- HIGUCHI, K., KANAZAWA, K., NISHIZAWA, N.-K., CHINO, M. & MORI, S. 1994. Purification and characterization of nicotianamine synthase from Fe-deficient barley roots. *Plant and Soil*, 165, 173-179.
- HIGUCHI, K., WATANABE, S., TAKAHASHI, M., KAWASAKI, S., NAKANISHI, H., NISHIZAWA, N. K. & MORI, S. 2001. Nicotianamine synthase gene expression differs in barley and rice under Fe-deficient conditions. *The Plant Journal*, 25, 159-167.
- HILTY, F. M., ARNOLD, M., HILBE, M., TELEKI, A., KNIJNENBURG, J. T., EHRENSPERGER, F., HURRELL, R. F., PRATSINIS, S. E., LANGHANS, W. & ZIMMERMANN, M. B. 2010. Iron from nanocompounds containing

- iron and zinc is highly bioavailable in rats without tissue accumulation. *Nature nanotechnology*, 5, 374-380.
- HOLDSWORTH, M. J., BIRD, C., RAY, J., SCHUCH, W. & GRIERSON, D. 1987. Structure and expression of an ethylene-related mRNA from tomato. *Nucleic Acids Research*, 15, 731-739.
- HOSSAIN, S. M. & MOHIUDDIN, A. 2013. Study on Biofortification of Rice by Targeted Genetic Engineering. *International Journal of Agricultural Research, Innovation and Technology*, 2, 25-35.
- HUE, N., UCHIDA, R. & HO, M. 2000. Sampling and analysis of soils and plant tissues: How to take representative samples, how the samples are tested.
- HUNT, J. M. 2002. Reversing productivity losses from iron deficiency: the economic case. *The Journal of nutrition*, 132, 794S-801S.
- HURRELL, R. & EGLI, I. 2010. Iron bioavailability and dietary reference values. *The American journal of clinical nutrition*, 91, 1461S-1467S.
- IHEMERE, U. E., NARAYANAN, N. N. & SAYRE, R. T. 2012. Iron biofortification and homeostasis in transgenic cassava roots expressing the algal iron assimilatory gene, FEAL. *Frontiers in plant science*, 3.
- INOUE, H., TAKAHASHI, M., KOBAYASHI, T., SUZUKI, M., NAKANISHI, H., MORI, S. & NISHIZAWA, N. K. 2008. Identification and localisation of the rice nicotianamine aminotransferase gene OsNAAT1 expression suggests the site of phyto siderophore synthesis in rice. *Plant molecular biology*, 66, 193-203.
- ISHIMARU, Y., MASUDA, H., BASHIR, K., INOUE, H., TSUKAMOTO, T., TAKAHASHI, M., NAKANISHI, H., AOKI, N., HIROSE, T. & OHSUGI, R. 2010. Rice metal-nicotianamine transporter, OsYSL2, is required for the long-distance transport of iron and manganese. *The Plant Journal*, 62, 379-390.
- ISHIMARU, Y., SUZUKI, M., TSUKAMOTO, T., SUZUKI, K., NAKAZONO, M., KOBAYASHI, T., WADA, Y., WATANABE, S., MATSUHASHI, S. & TAKAHASHI, M. 2006. Rice plants take up iron as an Fe<sup>3+</sup>-phyto siderophore and as Fe<sup>2+</sup>. *The Plant Journal*, 45, 335-346.
- IVANOV, R., BRUMBAROVA, T. & BAUER, P. 2012. Fitting into the harsh reality: regulation of iron-deficiency responses in dicotyledonous plants. *Molecular plant*, 5, 27-42.
- JEAN, M. L., SCHIKORA, A., MARI, S., BRIAT, J. F. & CURIE, C. 2005. A loss-of-function mutation in AtYSL1 reveals its role in iron and nicotianamine seed loading. *The Plant Journal*, 44, 769-782.
- JEONG, J. & CONNOLLY, E. L. 2009. Iron uptake mechanisms in plants: Functions of the FRO family of ferric reductases. *Plant Science*, 176, 709-714.
- JOHNSON, A. A., KYRIACOU, B., CALLAHAN, D. L., CARRUTHERS, L., STANGOULIS, J., LOMBI, E. & TESTER, M. 2011. Constitutive overexpression of the OsNAS gene family reveals single-gene strategies for effective iron-and zinc-biofortification of rice endosperm. *PLoS ONE*, 6, e24476.
- KABATA-PENDIAS, A. 2000. *Trace elements in soils and plants*, CRC press.
- KAISER, B. N., MOREAU, S., CASTELLI, J., THOMSON, R., LAMBERT, A., BOGLIOLO, S., PUPPO, A. & DAY, D. A. 2003. The soybean NRAMP homologue, GmDMT1, is a symbiotic divalent metal transporter capable of ferrous iron transport. *The Plant Journal*, 35, 295-304.

- KANAZAWA, K., HIGUCHI, K., NISHIZAWA, N.-K., FUSHIYA, S., CHINO, M. & MORI, S. 1994. Nicotianamine aminotransferase activities are correlated to the phytosiderophore secretions under Fe-deficient conditions in Gramineae. *Journal of Experimental Botany*, 45, 1903-1906.
- KARUGABA, A. & KIMARU, G. 1999. *Banana production in Uganda: An essential food and cash crop*, Relma.
- KENNEDY, G., NANTEL, G. & SHETTY, P. 2003. The scourge of "hidden hunger": global dimensions of micronutrient deficiencies. *Food Nutrition and Agriculture*, 8-16.
- KHANNA, H., BECKER, D., KLEIDON, J. & DALE, J. 2004. Centrifugation assisted *Agrobacterium tumefaciens*-mediated transformation (CAAT) of embryogenic cell suspensions of banana (*Musa* spp. Cavendish AAA and Lady finger AAB). *Molecular Breeding*, 14, 239-252.
- KHANNA, H. K., PAUL, J.-Y., HARDING, R. M., DICKMAN, M. B. & DALE, J. L. 2007. Inhibition of *Agrobacterium*-induced cell death by antiapoptotic gene expression leads to very high transformation efficiency of banana. *Molecular Plant-Microbe Interactions*, 20, 1048-1054.
- KHUSH, G. S., LEE, S., CHO, J.-I. & JEON, J.-S. 2012. Biofortification of crops for reducing malnutrition. *Plant biotechnology reports*, 6, 195-202.
- KIKAFUNDA, J. K., LUKWAGO, F. B. & TURYASHEMERERWA, F. 2009. Anaemia and associated factors among under-fives and their mothers in Bushenyi district, Western Uganda. *Public health nutrition*, 12, 2302.
- KIM, S., TAKAHASHI, M., HIGUCHI, K., TSUNODA, K., NAKANISHI, H., YOSHIMURA, E., MORI, S. & NISHIZAWA, N. K. 2005. Increased nicotianamine biosynthesis confers enhanced tolerance of high levels of metals, in particular nickel, to plants. *Plant and cell physiology*, 46, 1809-1818.
- KIM, S. A., PUNSHON, T., LANZIROTTI, A., LI, L., ALONSO, J. M., ECKER, J. R., KAPLAN, J. & GUERINOT, M. L. 2006. Localization of iron in *Arabidopsis* seed requires the vacuolar membrane transporter VIT1. *Science*, 314, 1295-1298.
- KLATTE, M., SCHULER, M., WIRTZ, M., FINK-STRAUBE, C., HELL, R. & BAUER, P. 2009. The analysis of *Arabidopsis* nicotianamine synthase mutants reveals functions for nicotianamine in seed iron loading and iron deficiency responses. *Plant Physiology*, 150, 257-271.
- KOBAYASHI, T., NAKANISHI, H., TAKAHASHI, M., KAWASAKI, S., NISHIZAWA, N.-K. & MORI, S. 2001. In vivo evidence that *Ids3* from *Hordeum vulgare* encodes a dioxygenase that converts 2'-deoxymugineic acid to mugineic acid in transgenic rice. *Planta*, 212, 864-871.
- KOBAYASHI, T. & NISHIZAWA, N. K. 2012. Iron uptake, translocation, and regulation in higher plants. *Annual review of plant biology*, 63, 131-152.
- KOBAYASHI, T., SUZUKI, M., INOUE, H., ITAI, R. N., TAKAHASHI, M., NAKANISHI, H., MORI, S. & NISHIZAWA, N. K. 2005. Expression of iron-acquisition-related genes in iron-deficient rice is co-ordinately induced by partially conserved iron-deficiency-responsive elements. *Journal of Experimental Botany*, 56, 1305-1316.
- KOIKE, S., INOUE, H., MIZUNO, D., TAKAHASHI, M., NAKANISHI, H., MORI, S. & NISHIZAWA, N. K. 2004. OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. *The Plant Journal*, 39, 415-424.

- KORCZ, A. & TWARDOWSKI, T. 1992. Effect of selected heavy metal ions on the in vitro translation system of wheat germ-protective function of plant ferritin. *Acta Physiologiae Plantarum (Poland)*.
- KORSHUNOVA, Y. O., EIDE, D., CLARK, W. G., GUERINOT, M. L. & PAKRASI, H. B. 1999. The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. *Plant Molecular Biology*, 40, 37-44.
- KUMAR, G. B. S., SRINIVAS, L. & GANAPATHI, T. R. 2011. Iron fortification of banana by the expression of soybean ferritin. *Biological trace element research*, 142, 232-241.
- KUMARI, M., GUPTA, S., LAKSHMI, A. J. & PRAKASH, J. 2004. Iron bioavailability in green leafy vegetables cooked in different utensils. *Food Chemistry*, 86, 217-222.
- LACOEUILHE, J. & MARTIN-PREVEL, P. 1971. Culture sur milieu artificiel carences en N, P, S chez le bananier: analyse foliaire. *Fruits*.
- LAHAV, E. 1995. *Banana nutrition*, Springer.
- LANGENEGGER, W. & PLESSIS, S. D. 1977. The determination of the nutritional status of dwarf cavendish bananas in South Africa.
- LANQUAR, V., LELIÈVRE, F., BOLTE, S., HAMÈS, C., ALCON, C., NEUMANN, D., VANSUYT, G., CURIE, C., SCHRÖDER, A. & KRÄMER, U. 2005. Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. *The EMBO journal*, 24, 4041-4051.
- LEE, S., JEON, U. S., LEE, S. J., KIM, Y.-K., PERSSON, D. P., HUSTED, S., SCHJØRRING, J. K., KAKEI, Y., MASUDA, H. & NISHIZAWA, N. K. 2009. Iron fortification of rice seeds through activation of the nicotianamine synthase gene. *Proceedings of the National Academy of Sciences*, 106, 22014-22019.
- LEE, S., KIM, Y.-S., JEON, U. S., KIM, Y.-K., SCHJØRRING, J. K. & AN, G. 2012. Activation of Rice nicotianamine synthase 2 (OsNAS2) enhances iron availability for biofortification. *Molecules and Cells*, 33, 269-275.
- LEMANCEAU, P., BAUER, P., KRAEMER, S. & BRIAT, J.-F. 2009. Iron dynamics in the rhizosphere as a case study for analyzing interactions between soils, plants and microbes. *Plant and Soil*, 321, 513-535.
- LETERME, P., BULDGEN, A., ESTRADA, F. & LONDOÑO, A. M. 2006. Mineral content of tropical fruits and unconventional foods of the Andes and the rain forest of Colombia. *Food Chemistry*, 95, 644-652.
- LINDSAY, W. 1995. Chemical reactions in soils that affect iron availability to plants. A quantitative approach. *Iron nutrition in soils and plants*. Springer.
- LING, H.-Q., KOCH, G., BAUMLEIN, H. & GANAL, M. W. 1999. Map-based cloning of chloronerva, a gene involved in iron uptake of higher plants encoding nicotianamine synthase. *Proceedings of the National Academy of Sciences*, 96, 7098-7103.
- LOBREAUX, S. & BRIAT, J.-F. O. 1991. Ferritin accumulation and degradation in different organs of pea (*Pisum sativum*) during development. *Biochem. J*, 274, 601-606.
- LOMBI, E., ZHAO, F., DUNHAM, S. & MCGRATH, S. 2001. Phytoremediation of heavy metal-contaminated soils. *Journal of environmental quality*, 30, 1919-1926.

- LÖNNERDAL, B. 2009. Soybean ferritin: implications for iron status of vegetarians. *The American journal of clinical nutrition*, 89, 1680S-1685S.
- LÓPEZ, M. A. & MARTOS, F. C. 2004. Iron availability: An updated review. *International journal of food sciences and nutrition*, 55, 597-606.
- LUCCA, P., HURRELL, R. & POTRYKUS, I. 2001. Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains. *Theoretical and Applied Genetics*, 102, 392-397.
- LUQING ZHENG, Y. Y., LU WANG, FANG WANG, JAMES WHELAN, AND HUIXIA SHOU 2010. Identification of a novel iron regulated basic helix-loop-helix protein involved in Fe homeostasis in *Oryza sativa*. *Plant Biology* 10, 1 - 9.
- MA, J. F. & NOMOTO, K. 1993. Two related biosynthetic pathways of mugineic acids in gramineous plants. *Plant Physiology*, 102, 373-378.
- MA, J. F., TAKETA, S., CHANG, Y.-C., IWASHITA, T., MATSUMOTO, H., TAKEDA, K. & NOMOTO, K. 1999. Genes controlling hydroxylations of phytosiderophores are located on different chromosomes in barley (*Hordeum vulgare* L.). *Planta*, 207, 590-596.
- MARSCHNER, H. 1996. Mineral nutrition of higher plants. Wiley Online Library.
- MARSCHNER, H. & RÖMHELD, V. 1994. Strategies of plants for acquisition of iron. *Plant and Soil*, 165, 261-274.
- MARTIN-PRÉVEL, P. 1980. nutrition minerale du bananier dans le monde. 1. *Fruits*.
- MARTIN-PRÉVEL, P. Past, present and future of tropical fruit nutrition with special reference to the banana. International Symposium on the Culture of Subtropical and Tropical Fruits and Crops 275, 1989. 523-534.
- MARTIN-PRÉVEL, P., GAGNARD, J. & GAUTIER, P. 1987. *Plant analysis as a guide to the nutrient requirements of temperate and tropical crops*, Technique et Documentation-Lavoisier.
- MASUDA, H., ISHIMARU, Y., AUNG, M. S., KOBAYASHI, T., KAKEI, Y., TAKAHASHI, M., HIGUCHI, K., NAKANISHI, H. & NISHIZAWA, N. K. 2012. Iron biofortification in rice by the introduction of multiple genes involved in iron nutrition. *Scientific Reports*, 2.
- MASUDA, H., USUDA, K., KOBAYASHI, T., ISHIMARU, Y., KAKEI, Y., TAKAHASHI, M., HIGUCHI, K., NAKANISHI, H., MORI, S. & NISHIZAWA, K., NAOKO 2009. Overexpression of the Barley Nicotianamine Synthase Gene HvNAS1 Increases Iron and Zinc Concentrations in Rice Grains. *Rice* 2, 155 - 166.
- MAYER, J. E., PFEIFFER, W. H. & BEYER, P. 2008. Biofortified crops to alleviate micronutrient malnutrition. *Current Opinion in Plant Biology*, 11, 166-170.
- MAZIAH, M., SARIAH, M. & SREERAMANAN, S. 2007. Transgenic banana Rastali (AAB) with  $\beta$ -1, 3-glucanase gene for tolerance to Fusarium wilt race 1 disease via Agrobacterium-mediated transformation system. *Plant Pathology Journal*, 6.
- MCKIE, A. T., BARROW, D., LATUNDE-DADA, G. O., ROLFS, A., SAGER, G., MUDALY, E., MUDALY, M., RICHARDSON, C., BARLOW, D. & BOMFORD, A. 2001. An iron-regulated ferric reductase associated with the absorption of dietary iron. *Science*, 291, 1755-1759.
- MEDINA-SUÁREZ, R., MANNING, K., FLETCHER, J., AKED, J., BIRD, C. R. & SEYMOUR, G. B. 1997. Gene expression in the pulp of ripening bananas (two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis

- of in vitro translation products and cDNA cloning of 25 different ripening-related mRNAs). *Plant Physiology*, 115, 453-461.
- MHATRE, M., SRINIVAS, L. & GANAPATHI, T. R. 2011. Enhanced iron and zinc accumulation in genetically engineered pineapple plants using soybean ferritin gene. *Biological trace element research*, 144, 1219-1228.
- MILTON, K. 2003. Micronutrient intakes of wild primates: are humans different? *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 136, 47-59.
- MIZUNO, D., HIGUCHI, K., SAKAMOTO, T., NAKANISHI, H., MORI, S. & NISHIZAWA, N. K. 2003. Three nicotianamine synthase genes isolated from maize are differentially regulated by iron nutritional status. *Plant Physiology*, 132, 1989-1997.
- MIZUNO, T., USUI, K., HORIE, K., NOSAKA, S., MIZUNO, N. & OBATA, H. 2005. Cloning of three ZIP/Nramp transporter genes from a Ni hyperaccumulator plant *Thlaspi japonicum* and their Ni<sup>2+</sup>-transport abilities. *Plant Physiology and Biochemistry*, 43, 793-801.
- MOH 2002. Anemia Policy. Kampala, Uganda. *Ministry of Health*.
- MORTON, J. F. 1987. *Fruits of warm climates*, JF Morton.
- MURRAY-KOLB, L. E., TAKAIWA, F., GOTO, F., YOSHIHARA, T., THEIL, E. C. & BEARD, J. L. 2002. Transgenic rice is a source of iron for iron-depleted rats. *The Journal of nutrition*, 132, 957-960.
- MWADIME, R. K. 2001. HEALTH SECTOR ACTIONS TO IMPROVE NUTRITION: CHALLENGES AND OPPORTUNITIES IN SUB-SAHARAN AFRICA. *African Journal of Food, Agriculture, Nutrition and Development*, 1, 29-33.
- NAIR, K. M. & IYENGAR, V. 2009. Iron content, bioavailability & factors affecting iron status of Indians. *Indian J Med Res*, 130, 634-45.
- NAKANISHI, H., OKUMURA, N., UMEHARA, Y., NISHIZAWA, N.-K., CHINO, M. & MORI, S. 1993. Expression of a gene specific for iron deficiency (Ids3) in the roots of *Hordeum vulgare*. *Plant and cell physiology*, 34, 401-410.
- NAMANYA, P., MAGAMBO, S., MUTUMBA, G. & TUSHEMEREIRWE, W. 2004. Somatic embryogenesis from immature male inflorescences of East African highland banana CV 'Nakyetengu'. *African Crop Science Journal*, 12, 43-49.
- NARAYANAN, N. N., IHEMERE, U., CHIU, W. T., SIRITUNGA, D., RAJAMANI, S., SINGH, S., ODA, S. & SAYRE, R. T. 2011. The iron assimilatory protein, FEA1, from *Chlamydomonas reinhardtii* facilitates iron-specific metal uptake in yeast and plants. *Front. Plant Sci*, 2, 3389.
- NARAYANAN, N. N., UZOMA IHEMERE, WAITING CHIU, DIMUTH SIRITUNGA, SATHISH RAJAMANI, SAREENA SINGH, SAHARU ODA AND RICHARD.T.SAYRE 2011. The iron assimilatory protein, FEA1, from *Chlamydomonas reinhardtii* facilitates iron-specific metal uptake in yeast and plants. *Frontiers in Plant Science*, 2, 1-13.
- NESTEL, P., BOUIS, H. E., MEENAKSHI, J. & PFEIFFER, W. 2006. Biofortification of staple food crops. *The Journal of Nutrition*, 136, 1064-1067.
- NEVO, Y. & NELSON, N. 2006. The NRAMP family of metal-ion transporters. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1763, 609-620.

- NOWAKUNDA, K., RUBAIHAYO, P. R., AMENY, M. & TUSHEMERIRWE, W. 2000. Consumer acceptability of introduced bananas in Uganda. *InfoMusa*, 9, 22-25.
- ODUOL, P. A. & ALUMA, J. 1990. The banana (*Musa* spp.)—Coffee robusta: traditional agroforestry system of Uganda. *Agroforestry systems*, 11, 213-226.
- OOMEN, R. J., WU, J., LELIÈVRE, F., BLANCHET, S., RICHAUD, P., BARBIER-BRYGOO, H., AARTS, M. G. & THOMINE, S. 2009. Functional characterization of NRAMP3 and NRAMP4 from the metal hyperaccumulator *Thlaspi caerulescens*. *New Phytologist*, 181, 637-650.
- ORTIZ, R., FERRIS, R. & VUYLSTEKE, D. 1995. Banana and plantain breeding. *Bananas and Plantains*. Springer.
- PALMER, C. M. & GUERINOT, M. L. 2009. Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Nature chemical biology*, 5, 333-340.
- PÉREZ HERNÁNDEZ, J. B., REMY, S., GALÁN SAUCO, V., SWENNEN, R. & SÁGI, L. 1999. Chemotactic Movement and Attachment of *Agrobacterium tumefaciens* to Banana Cells and Tissues. *Journal of plant physiology*, 155, 245-250.
- PESTANA, M., GAMA, F., SAAVEDRA, T., DE VARENNES, A. & CORREIA, P. 2012. The root ferric-chelate reductase of *Ceratonia siliqua* (L.) and *Poncirus trifoliata* (L.) Raf. responds differently to a low level of iron. *Scientia Horticulturae*, 135, 65-67.
- PICH, A., SCHOLZ, G. & STEPHAN, U. W. 1994. Iron-dependent changes of heavy metals, nicotianamine, and citrate in different plant organs and in the xylem exudate of two tomato genotypes. Nicotianamine as possible copper translocator. *Plant and Soil*, 165, 189-196.
- PLOETZ, R., THOMAS, J. & SLABAUGH, W. 2003. Diseases of banana and plantain. *Diseases of tropical fruit crops*, 73-134.
- POLETTI, S., GRUISSEM, W. & SAUTTER, C. 2004. The nutritional fortification of cereals. *Current Opinion in Biotechnology*, 15, 162-165.
- POLETTI, S. & SAUTTER, C. 2005. Biofortification of the crops with micronutrients using plant breeding and/or transgenic strategies. *Minerva Biotechnologica*, 17, 1-11.
- PRICE, D. & JOSHI, J. G. 1982. Ferritin: a zinc detoxicant and a zinc ion donor. *Proceedings of the National Academy of Sciences*, 79, 3116-3119.
- PRICE, D. J. & JOSHI, J. 1983. Ferritin. Binding of beryllium and other divalent metal ions. *Journal of Biological Chemistry*, 258, 10873-10880.
- QU, L. Q., YOSHIHARA, T., OYAMA, A., GOTO, F. & TAKAIWA, F. 2005. Iron accumulation does not parallel the high expression level of ferritin in transgenic rice seeds. *Planta*, 222, 225-233.
- RAVET, K., TOURAINE, B., BOUCHEREZ, J., BRIAT, J. F., GAYMARD, F. & CELLIER, F. 2009a. Ferritins control interaction between iron homeostasis and oxidative stress in Arabidopsis. *The Plant Journal*, 57, 400-412.
- RAVET, K., TOURAINE, B., KIM, S. A., CELLIER, F., THOMINE, S., GUERINOT, M. L., BRIAT, J.-F. & GAYMARD, F. 2009b. Post-translational regulation of AtFER2 ferritin in response to intracellular iron trafficking during fruit development in Arabidopsis. *Molecular plant*, 2, 1095-1106.
- RENGEL, Z., BATTEN, G. & CROWLEY, D. D. 1999. Agronomic approaches for improving the micronutrient density in edible portions of field crops. *Field crops research*, 60, 27-40.

- ROBINSON, J. C. & SAÚCO, V. G. 2010. *Bananas and plantains*, Cabi
- ROBINSON, N. J., PROCTER, C. M., CONNOLLY, E. L. & GUERINOT, M. L. 1999. A ferric-chelate reductase for iron uptake from soils. *Nature*, 397, 694-697.
- RODUSHKIN, I., RUTH, T. & HUHTASAARI, Å. 1999. Comparison of two digestion methods for elemental determinations in plant material by ICP techniques. *Analytica Chimica Acta*, 378, 191-200.
- ROGERS, E. E., EIDE, D. J. & GUERINOT, M. L. 2000. Altered selectivity in an Arabidopsis metal transporter. *Proceedings of the National Academy of Sciences*, 97, 12356-12360.
- SADIK, K., MUKASA, S., ARINAITWE, G., RUBAIHAYO, P., KIGGUNDU, A., GIBSON, P. & SSEBULIBA, J. Enhancing Callus induction and embryogenic cell suspension development in East African highland banana. Second RUFORUM Biennial Regional Conference on "Building capacity for food security in Africa", Entebbe, Uganda, 20-24 September 2010., 2010. RUFORUM, 405-408.
- SAGI, L., MAY, G. D., REMY, S. & SWENNEN, R. 1998. Recent developments in biotechnological research on bananas (*Musa* spp.). *Biotechnology and Genetic Engineering Reviews*, 15, 313-328.
- SAGI, L., PANIS, B., REMY, S., SCHOOF, H., DE SMET, K., SWENNEN, R. & CAMMUE, B. 1995. Genetic transformation of banana and plantain (*Musa* spp.) via particle bombardment. *Biotechnology*, 13, 481-485.
- SAGI, L., REMY, S. & SWENNEN, R. 1997. Genetic transformation for the improvement of bananas—a critical assessment. *Networking banana and plantain: INIBAP Annual Report*, 33-36.
- SAHRAWAT, K. 2005. Iron toxicity in wetland rice and the role of other nutrients. *Journal of Plant Nutrition*, 27, 1471-1504.
- SALTZMAN, A., BIROL, E., BOUIS, H. E., BOY, E., DE MOURA, F. F., ISLAM, Y. & PFEIFFER, W. H. 2013. Biofortification: progress toward a more nourishing future. *Global Food Security*.
- SAMBROOK, J., RUSSELL, D. W. & RUSSELL, D. W. 2001. *Molecular cloning: a laboratory manual (3-volume set)*, Cold spring harbor laboratory press Cold Spring Harbor, New York.
- SCHAAF, G., LUDEWIG, U., ERENOGLU, B. E., MORI, S., KITAHARA, T. & VON WIRÉN, N. 2004. ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. *Journal of Biological Chemistry*, 279, 9091-9096.
- SCHAGERLÖF, U., WILSON, G., HEBERT, H., AL-KARADAGHI, S. & HÄGERHÄLL, C. 2006. Transmembrane topology of FRO2, a ferric chelate reductase from Arabidopsis thaliana. *Plant molecular biology*, 62, 215-221.
- SCHULER, M. & BAUER, P. 2012. Strategies for Iron Biofortification of Crop Plants. *Food Quality*.
- SHAMAH-LEVY, T., VILLALPANDO, S., RIVERA, J. A., MEJÍA-RODRÍGUEZ, F., CAMACHO-CISNEROS, M. & MONTERRUBIO, E. A. 2003. Anemia in Mexican women: A public health problem. *salud pública de méxico*, 45, 499-507.
- SHEWFELT, R. L. 1990. Sources of variation in the nutrient content of agricultural commodities from the farm to the consumer. *Journal of food quality*, 13, 37-54.

- SHIBATA, D. & LIU, Y.-G. 2000. Technical Focus-Agrobacterium-mediated plant transformation with large DNA fragments. *Trends in Plant Science*, 5, 354-357.
- SHUMAN, L. M. 1998. Micronutrient fertilizers. *Journal of crop production*, 1, 165-195.
- SOCHA, A. L. & GUERINOT, M. L. 2014. Mn-euvering manganese: the role of transporter gene family members in manganese uptake and mobilization in plants. *Plant Nutrition*, 5, 106.
- SOUTHERN, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of molecular biology*, 98, 503-517.
- SPEROTTO, R. A., RICACHENEVSKY, F. K., WALDOW, V. D. A. & FETT, J. P. 2012. Iron biofortification in rice: It's a long way to the top. *Plant Science*, 190, 24-39.
- STEIN, A. J. 2010. Global impacts of human mineral malnutrition. *Plant and soil*, 335, 133-154.
- STEPHENSON, L. S., LATHAM, M. C. & OTTESEN, E. 2000. Malnutrition and parasitic helminth infections. *Parasitology*, 121, S23-S38.
- STOVER, R. H. & SIMMONDS, N. W. 1987. *Bananas*, Longman Scientific & Technical.
- SVITASHEV, S. K., PAWLOWSKI, W. P., MAKAREVITCH, I., PLANK, D. W. & SOMERS, D. A. 2002. Complex transgene locus structures implicate multiple mechanisms for plant transgene rearrangement. *The Plant Journal*, 32, 433-445.
- TAHVONEN, R. 1993. Contents of selected elements in some fruits, berries, and vegetables on the finnish market in 1987-1989. *Journal of Food Composition and Analysis*, 6, 75-86.
- TAKAGI, S. I., NOMOTO, K. & TAKEMOTO, T. 1984. Physiological aspect of mugineic acid, a possible phytosiderophore of graminaceous plants. *Journal of Plant Nutrition*, 7, 469-477.
- TAKAHASHI, M. 2003. Overcoming Fe deficiency by a transgenic approach in rice. *Plant Cell, Tissue and Organ Culture* 72, 211 - 220.
- TAKAHASHI, M., TERADA, Y., NAKAI, I., NAKANISHI, H., YOSHIMURA, E., MORI, S. & NISHIZAWA, N. K. 2003. Role of nicotianamine in the intracellular delivery of metals and plant reproductive development. *The Plant Cell Online*, 15, 1263-1280.
- TAKIZAWA, R., NISHIZAWA, N. K., NAKANISHI, H. & MORI, S. 1996. Effect of iron deficiency on S-adenosylmethionine synthetase in barley roots. *Journal of Plant Nutrition*, 19, 1189-1200.
- TERRY, N. & ABADÍA, J. 1986. Function of iron in chloroplasts. *Journal of Plant Nutrition*, 9, 609-646.
- THEIL, E. & HASE, T. 1993. Plant and microbial ferritins.
- THEIL, E. C. & BRIAT, J.-F. 2004. *Plant ferritin and non-heme iron nutrition in humans*, International Food Policy Research Institute (IFPRI).
- THEIL, J. W. A. E. C. 2000. Identification and Characterization of the Iron Regulatory Element in the Ferritin Gene of a Plant (Soybean). *THE JOURNAL OF BIOLOGICAL CHEMISTRY*, 275, 17488-17493.
- THOMINE, S., LELIÈVRE, F., DEBARBIEUX, E., SCHROEDER, J. I. & BARBIER-BRYGOO, H. 2003. AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *The Plant Journal*, 34, 685-695.

- THOMINE, S., WANG, R., WARD, J. M., CRAWFORD, N. M. & SCHROEDER, J. I. 2000. Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to Nramp genes. *Proceedings of the National Academy of Sciences*, 97, 4991-4996.
- TRIFONOVA, A., MADSEN, S. & OLESEN, A. 2001. Agrobacterium-mediated transgene delivery and integration into barley under a range of in vitro culture conditions. *Plant Science*, 161, 871-880.
- TRIJATMIKO, K. R., DUEÑAS, C., TSAKIRPALOGLOU, N., TORRIZO, L., ARINES, F. M., ADEVA, C., BALINDONG, J., OLIVA, N., SAPASAP, M. V. & BORRERO, J. 2016. Biofortified indica rice attains iron and zinc nutrition dietary targets in the field. *Scientific reports*, 6.
- TRIPATHI, L., TRIPATHI, J. & HUGHES, J. D. A. 2005. Agrobacterium-mediated transformation of plantain (*Musa* spp.) cultivar Agbagba. *African Journal of Biotechnology*, 4.
- TRIPATHI, L., TRIPATHI, J. N. & TUSHEMERREIRWE, W. K. 2008. Rapid and efficient production of transgenic East African Highland Banana (*Musa* spp.) using intercalary meristematic tissues. *African Journal of Biotechnology*, 7.
- TRIVEDI, P. K. & NATH, P. 2004. MaExp1, an ethylene-induced expansin from ripening banana fruit. *Plant Science*, 167, 1351-1358.
- TRUMBO, P., YATES, A. A., SCHLICKER, S. & POOS, M. 2001. Dietary reference intakes: vitamin A, vitamin K, arsenic, boron, chromium, copper, iodine, iron, manganese, molybdenum, nickel, silicon, vanadium, and zinc. *Journal of the American Dietetic Association*, 101, 294-301.
- TURNER, D. 1980. Some factors related to yield components of bananas, in relation to sampling to assess nutrient status. *Fruits*, 35.
- TURNER, D. Ecophysiology of bananas: the generation and functioning of the leaf canopy. I International Symposium on Banana in the Subtropics 490, 1997. 211-222.
- TURNER, D. & BARKUS, B. 1974. The effect of season, stage of plant growth and leaf position on nutrient concentration in banana leaves on a krasnozem in New South Wales. *Animal Production Science*, 14, 112-117.
- TURNER, D. W. 1984. Growth, yield and leaf nutrient composition of 30 banana varieties in subtropical New South Wales.
- TUSHEMERREIRWE, W., KARAMURA, D., SALI, H., BWAMIKI, D., KASHAJA, I., NANKINGA, C., BAGAMBA, F., KANGIRE, A. & SEBULIBA, R. 2001. Bananas (*Musa* spp.). *Agriculture in Uganda*, 11.
- TWYFORD, I. & WALMSLEY, D. 1974. The mineral composition of the Robusta banana plant II. The concentration of mineral constituents. *Plant and Soil*, 41, 459-470.
- UBOS 2006. Uganda Bureau of Statistics & ORC Macro UDHS (*Uganda Demographic and Health Survey*), Kampala, Uganda/Calverton, MD: . Uganda Bureau of Statistics Kampala, Uganda and ICF International Calverton, Maryland, USA.
- UBOS 2011. Uganda demographic and health survey 2010. UBOS, Kampala, Uganda and Calverton, Maryland, USA; Uganda Bureau of Statistics and Macro International Inc.
- UDHS 2011. Uganda Demographic and Health Survey. Uganda Bureau of Statistics Kampala, Uganda and ICF International Calverton, Maryland, USA.
- USUDA, K., WADA, Y., ISHIMARU, Y., KOBAYASHI, T., TAKAHASHI, M., NAKANISHI, H., NAGATO, Y., MORI, S. & NISHIZAWA, N. K. 2009.

- Genetically engineered rice containing larger amounts of nicotianamine to enhance the antihypertensive effect. *Plant biotechnology journal*, 7, 87-95.
- VALDERRAMA-CHÁIREZ, M. L., CRUZ-HERNÁNDEZ, A. & PAREDES-LÓPEZ, O. 2002. Isolation of functional RNA from cactus fruit. *Plant molecular biology reporter*, 20, 279-286.
- VAN WUYTSWINKEL, O., VANSUYT, G., GRIGNON, N., FOURCROY, P. & BRIAT, J. F. 1999. Iron homeostasis alteration in transgenic tobacco overexpressing ferritin. *The Plant Journal*, 17, 93-97.
- VANSUYT, G., MENCH, M. & BRIAT, J.-F. 2000. Soil-dependent variability of leaf iron accumulation in transgenic tobacco overexpressing ferritin. *Plant Physiology and Biochemistry*, 38, 499-506.
- VAROTTO, C., MAIWALD, D., PESARESI, P., JAHNS, P., SALAMINI, F. & LEISTER, D. 2002. The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in *Arabidopsis thaliana*. *The Plant Journal*, 31, 589-599.
- VASCONCELOS, M., DATTA, K., OLIVA, N., KHALEKUZZAMAN, M., TORRIZO, L., KRISHNAN, S., OLIVEIRA, M., GOTO, F. & DATTA, S. K. 2003. Enhanced iron and zinc accumulation in transgenic rice with the *Atferritin* gene. *Plant Science*, 164, 371-378.
- VASCONCELOS, M., ECKERT, H., ARAHANA, V., GRAEF, G., GRUSAK, M. A. & CLEMENTE, T. 2006. Molecular and phenotypic characterization of transgenic soybean expressing the *Arabidopsis* ferric chelate reductase gene, *FRO2*. *Planta*, 224, 1116-1128.
- VERT, G., BARBERON, M., ZELAZNY, E., SÉGUÉLA, M., BRIAT, J.-F. & CURIE, C. 2009. *Arabidopsis* IRT2 cooperates with the high-affinity iron uptake system to maintain iron homeostasis in root epidermal cells. *Planta*, 229, 1171-1179.
- VERT, G., GROTZ, N., DÉDALDÉCHAMP, F., GAYMARD, F., GUERINOT, M. L., BRIAT, J.-F. & CURIE, C. 2002. IRT1, an *Arabidopsis* transporter essential for iron uptake from the soil and for plant growth. *The Plant Cell Online*, 14, 1223-1233.
- VERT, G. A., BRIAT, J.-F. & CURIE, C. 2003. Dual regulation of the *Arabidopsis* high-affinity root iron uptake system by local and long-distance signals. *Plant Physiology*, 132, 796-804.
- VON WIRÉN, N., KLAIR, S., BANSAL, S., BRIAT, J.-F., KHODR, H., SHIOIRI, T., LEIGH, R. A. & HIDER, R. C. 1999. Nicotianamine chelates both FeIII and FeII. Implications for metal transport in plants. *Plant Physiology*, 119, 1107-1114.
- VON WIRÉN, N., MARSCHNER, H. & ROMHELD, V. 1996. Roots of iron-efficient maize also absorb phyto siderophore-chelated zinc. *Plant physiology*, 111, 1119-1125.
- VUYLSTEKE, D. Breeding bananas and plantains: from intractability to feasibility. I International Conference on Banana and Plantain for Africa 540, 1996. 149-156.
- WALL, M. M. 2006. Ascorbic acid, vitamin A, and mineral composition of banana (*Musa* sp.) and papaya (*Carica papaya*) cultivars grown in Hawaii. *Journal of Food Composition and Analysis*, 19, 434-445.
- WALMSLEY, D. & TWYFORD, I. 1976. The mineral composition of the robusta banana plant. *Plant and Soil*, 45, 595-611.

- WELCH, R. M. & GRAHAM, R. D. 2004. Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of Experimental Botany*, 55, 353-364.
- WELCH, R. M., NORVELL, W. A., SCHAEFER, S. C., SHAFF, J. E. & KOCHIAN, L. V. 1993. Induction of iron (III) and copper (II) reduction in pea (*Pisum sativum* L.) roots by Fe and Cu status: Does the root-cell plasmalemma Fe (III)-chelate reductase perform a general role in regulating cation uptake? *Planta*, 190, 555-561.
- WHEAL, M. S., FOWLES, T. O. & PALMER, L. T. 2011. A cost-effective acid digestion method using closed polypropylene tubes for inductively coupled plasma optical emission spectrometry (ICP-OES) analysis of plant essential elements. *Analytical Methods*, 3, 2854-2863.
- WHITE, P. J. & BROADLEY, M. R. 2005. Biofortifying crops with essential mineral elements. *Trends in plant science*, 10, 586-593.
- WHITE, P. J. & BROADLEY, M. R. 2009. Biofortification of crops with seven mineral elements often lacking in human diets—iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytologist*, 182, 49-84.
- WHO 2000. Nutrition for health and development: Turning the tide of malnutrition. *WHO/NDH.00.7*, <http://www.who.int/nut/publications.htm>.
- WIRTH, J., POLETTI, S., AESCHLIMANN, B., YAKANDAWALA, N., DROSSE, B., OSORIO, S., TOHGE, T., FERNIE, A. R., GÜNTHER, D. & GRUISSEM, W. 2009. Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotechnology Journal*, 7, 631-644.
- XIA, J., YAMAJI, N., KASAI, T. & MA, J. F. 2010. Plasma membrane-localized transporter for aluminum in rice. *Proceedings of the National Academy of Sciences*, 107, 18381-18385.
- XLSTAT 2014. <http://www.addinsoft.com>.
- YEN, M.-R., TSENG, Y.-H. & SAIER, M. 2001. Maize Yellow Stripe1, an iron-phytosiderophore uptake transporter, is a member of the oligopeptide transporter (OPT) family. *Microbiology*, 147, 2881-2883.
- YI, Y. & GUERINOT, M. L. 1996. Genetic evidence that induction of root Fe (III) chelate reductase activity is necessary for iron uptake under iron deficiency†. *The Plant Journal*, 10, 835-844.
- ZANCANI, M., PERESSON, C., BIROCCIO, A., FEDERICI, G., URBANI, A., MURGIA, I., SOAVE, C., MICALI, F., VIANELLO, A. & MACRÌ, F. 2004. Evidence for the presence of ferritin in plant mitochondria. *European Journal of Biochemistry*, 271, 3657-3664.
- ZHANG, F., ROMHELD, V. & MARSCHNER, H. 1991. Release of zinc mobilizing root exudates in different plant species as affected by zinc nutritional status. *Journal of Plant Nutrition*, 14, 675-686.
- ZHANG, Y., XU, Y.-H., YI, H.-Y. & GONG, J.-M. 2012a. Vacuolar membrane transporters OsVIT1 and OsVIT2 modulate iron translocation between flag leaves and seeds
- in rice. *The Plant Journal*, 1 -11.
- ZHANG, Y., XU, Y. H., YI, H. Y. & GONG, J. M. 2012b. Vacuolar membrane transporters OsVIT1 and OsVIT2 modulate iron translocation between flag leaves and seeds in rice. *The Plant Journal*, 72, 400-410.
- ZHAO, F.-J. & MCGRATH, S. P. 2009. Biofortification and phytoremediation. *Current opinion in plant biology*, 12, 373-380.

- ZHAO, F.-J. & SHEWRY, P. R. 2011. Recent developments in modifying crops and agronomic practice to improve human health. *Food Policy*, 36, S94-S101.
- ZHENG, L., CHENG, Z., AI, C., JIANG, X., BEI, X., ZHENG, Y., GLAHN, R. P., WELCH, R. M., MILLER, D. D. & LEI, X. G. 2010. Nicotianamine, a novel enhancer of rice iron bioavailability to humans. *PLoS One*, 5, e10190.
- ZHU, C., NAQVI, S., GOMEZ-GALERA, S., PELACHO, A. M., CAPELL, T. & CHRISTOU, P. 2007. Transgenic strategies for the nutritional enhancement of plants. *Trends in plant science*, 12, 548-555.
- ZIMMERMANN, M. B. & HURRELL, R. F. 2007. Nutritional iron deficiency. *The lancet*, 370, 511-520.

# Appendices

## APPENDIX I: ANALYSIS OF POTENTIAL HEAVY METAL CONTAMINATION IN BANANA FRUIT SAMPLES ANALYSED BY ICP-OES IN CHAPTER 3

| Sample ID | Cultivar      | Transgene | Cd content<br>(mg/kg) | Pb content<br>(mg/kg) | Ti content<br>(mg/kg) |
|-----------|---------------|-----------|-----------------------|-----------------------|-----------------------|
| 1         | Mpologoma     | Wakiso    | 0.16±0.0              | -1.66±0.7             | 0.06±0.1              |
| 2         | Nakitembe     | Wakiso    | 0.08±0.0              | -1.78±0.8             | 0.59±0.0              |
| 3         | Mbwazirume    | Wakiso    | 0.03±0.0              | -1.52±0.3             | 0.61±0.1              |
| 4         | Kisansa       | Wakiso    | 0.02±0.0              | -3.42±1.6             | 0.23±0.7              |
| 5         | Gonja         | Wakiso    | 0.01±0.0              | -2.78±0.7             | 0.24±0.2              |
| 8         | Mpologoma     | Nakaseke  | 0.02±0.0              | 0.76±1.5              | 0.29±0.1              |
| 9         | Nakitembe     | Nakaseke  | 0.05±0.0              | -1.06±1.2             | 0.89±0.0              |
| 10        | Mbwazirume    | Nakaseke  | 0.02±0.1              | -0.08±1.3             | 0.09±0.2              |
| 11        | Kisansa       | Nakaseke  | 0.00±0.0              | -3.20±0.0             | 0.71±0.2              |
| 12        | Gonja         | Nakaseke  | 0.04±0.0              | -4.06±0.6             | 0.40±0.2              |
| 13        | Bogoya        | Nakaseke  | 0.24±0.1              | -2.40±0.2             | 0.11±0.0              |
| 14        | Sukali Ndiizi | Nakaseke  | 0.21±0.0              | -1.30±0.1             | 0.07±0.0              |
| 15        | Mpologoma     | Masaka    | 0.19±0.1              | -1.28±0.4             | 0.75±0.1              |
| 16        | Mbwazirume    | Masaka    | 0.08±0.0              | -1.92±0.3             | 0.59±0.2              |
| 17        | Kivuvu        | Masaka    | 0.24±0.2              | -1.80±0.3             | 0.11±0.1              |
| 18        | Gonja         | Masaka    | 0.02±0.0              | -3.89±1.3             | 0.32±0.2              |
| 19        | Mpologoma     | Mbarara   | 0.15±0.0              | 0.27±0.31             | 0.65±0.0              |
| 20        | Nakitembe     | Mbarara   | 0.01±0.1              | -2.19±0.6             | 0.13±0.1              |
| 21        | Mbwazirume    | Mbarara   | 0.05±0.1              | -1.90±0.5             | 0.69±0.4              |
| 22        | Kisansa       | Mbarara   | 0.03±0.1              | -2.53±1.5             | 0.22±0.4              |
| 23        | Gonja         | Mbarara   | 0.03±0.1              | -3.29±0.4             | 0.44±0.8              |
| 24        | Bogoya        | Mbarara   | 0.28±0.2              | -1.25±0.4             | 0.13±0.1              |
| 25        | Sukali Ndiizi | Mbarara   | 0.28±0.3              | -2.40±0.5             | 0.07±0.1              |
| 27        | M9            | Mbarara   | 0.16±0.0              | -1.42±0.7             | 0.17±0.2              |
| 28        | Kivuvu        | Mbarara   | 0.15±0.2              | -2.50±0.5             | 0.10±0.1              |
| 30        | Mpologoma     | Bushenyi  | 0.17±0.1              | -1.17±0.9             | 0.95±1.1              |
| 31        | Nakitembe     | Bushenyi  | 0.01±0.0              | -2.41±0.3             | 0.39±0.1              |
| 32        | Mbwazirume    | Bushenyi  | 0.03±0.0              | -1.61±0.4             | 0.41±0.2              |
| 33        | Kisansa       | Bushenyi  | 0.07±0.0              | -2.47±0.9             | 0.81±0.7              |
| 34        | Gonja         | Bushenyi  | 0.04±0.0              | -3.28±0.5             | 0.17±0.4              |
| 35        | Bogoya        | Bushenyi  | 0.31±0.0              | -2.34±0.1             | 0.10±0.0              |
| 36        | Sukali Ndiizi | Bushenyi  | 0.20±0.4              | -2.40±1.0             | 0.09±0.1              |
| 37        | M9            | Bushenyi  | 0.15±0.0              | 0.50±0.1              | 0.46±1.1              |
| 39        | Mbwazirume    | Kasese    | 0.01±0.0              | -1.35±0.9             | 0.05±0.2              |
| 41        | Bogoya        | Kasese    | 0.23±0.0              | -2.40±0.4             | 0.11±0.1              |
| 42        | Gonja         | Kasese    | 0.04±0.0              | -3.82±0.9             | 0.06±0.0              |
| 43        | M9            | Kasese    | 0.14±0.0              | -1.41±0.0             | 0.34±0.1              |
| 44        | Sukali Ndiizi | Kasese    | 0.23±0.0              | -1.45±0.0             | 0.20±0.2              |
| 45        | Mbwazirume    | Kabalore  | 0.02±0.0              | -2.59±1.0             | 0.20±0.1              |

## APPENDIX I: CONTINUED

| Sample ID | Cultivar      | Transgene  | Cd content<br>(mg/kg) | Pb content<br>(mg/kg) | Ti content<br>(mg/kg) |
|-----------|---------------|------------|-----------------------|-----------------------|-----------------------|
| 46        | Kisansa       | Kabalore   | 0.05±0.1              | -1.89±0.7             | 0.28±0.1              |
| 48        | Gonja         | Kabalore   | 0.21±0.0              | -1.24±0.7             | 0.09±0.1              |
| 49        | Kivuvu        | Kabalore   | 0.18±0.0              | 1.40±0.8              | 0.11±0.1              |
| 50        | Mpologoma     | Buginyanya | 0.12±0.0              | 0.32±0.9              | 0.57±0.1              |
| 51        | Nakitembe     | Buginyanya | 0.06±0.1              | -2.36±0.6             | 0.2±0.1               |
| 52        | Mbwazirume    | Buginyanya | 0.02±0.0              | -2.34±0.5             | 0.31±0.1              |
| 53        | Bogoya        | Buginyanya | 0.24±0.3              | -1.40±0.7             | 0.15±0.0              |
| 54        | SukaliNdiizi  | Buginyanya | 0.20±0.3              | -2.40±0.1             | 0.14±0.0              |
| 55        | M9            | Buginyanya | 0.11±0.3              | -0.32±1.6             | 0.27±0.1              |
| 56        | Mpologoma     | Sironko    | 0.20±0.5              | 0.81±2.5              | 0.66±0.0              |
| 57        | Nakitembe     | Sironko    | 0.08±0.0              | -1.57±0.9             | 0.33±0.0              |
| 58        | Mbwazirume    | Sironko    | 0.03±0.0              | -1.99±0.7             | 0.33±0.1              |
| 59        | Bogoya        | Sironko    | 0.26±0.0              | -2.46±0.8             | 0.09±0.1              |
| 60        | Kivuvu        | Sironko    | 0.16±0.0              | -1.28±0.9             | 0.11±0.1              |
| 61        | M9            | Sironko    | 0.06±0.0              | -0.68±0.1             | 0.45±0.2              |
| 62        | Mpologoma     | Mbale      | 0.15±0.08             | 0.39±0.9              | 0.78±0.0              |
| 63        | Nakitembe     | Mbale      | 0.09±0.06             | -1.87±0.5             | 0.54±0.1              |
| 64        | Mbwazirume    | Mbale      | 0.02±0.0              | -2.14±0.4             | 0.44±0.1              |
| 66        | Gonja         | Mbale      | 0.26±0.0              | -2.56±0.5             | 0.09±0.1              |
| 67        | Bogoya        | Mbale      | 0.33±0.1              | -2.45±0.5             | 0.06±0.0              |
| 68        | Sukali Ndiizi | Mbale      | 0.16±0.1              | -2.40±1.0             | 0.09±0.0              |
| 69        | Kivuvu        | Mbale      | 0.17±0.2              | -1.40±0.5             | 0.16±0.0              |
| 70        | Mpologoma     | Tororo     | 0.15±0.1              | 0.36±1.2              | 0.68±0.0              |
| 71        | Nakitembe     | Tororo     | 0.03±0.0              | -2.79±0.9             | 0.49±0.1              |
| 72        | Mbwazirume    | Tororo     | 0.06±0.0              | -1.13±0.8             | 0.08±0.1              |
| 73        | M9            | Tororo     | 0.08±0.0              | -0.67±0.9             | 0.80±0.3              |
| 74        | Mpologoma     | Serere     | 0.17±0.0              | -1.07±0.5             | 0.09±0.0              |
| 75        | Nakitembe     | Serere     | 0.02±0.0              | -2.93±0.4             | 0.59±0.1              |
| 76        | Mbwazirume    | Serere     | 0.09±0.1              | -2.12±1.0             | 0.61±0.0              |
| 77        | Kisansa       | Serere     | 0.06±0.1              | -3.20±0.6             | 0.45±0.3              |
| 78        | Bogoya        | Serere     | 0.30±0.0              | -3.20±0.1             | 0.05±0.0              |
| 79        | SukaliNdiizi  | Serere     | 0.27±0.1              | -2.07±0.5             | 0.10±0.5              |
| 80        | Kivuvu        | Serere     | 0.19±0.1              | -1.25±1.0             | 0.10±0.0              |
| 81        | M9            | Serere     | 0.04±0.0              | -1.80±0.6             | 0.56±0.1              |
| 82        | Mpologoma     | Lira       | 0.22±0.1              | 0.47±0.3              | 0.75±0.2              |
| 83        | Nakitembe     | Lira       | 0.04±0.1              | -3.12±0.1             | 0.04±0.1              |
| 84        | Mbwazirume    | Lira       | 0.05±0.0              | -2.29±1.3             | 0.54±0.1              |
| 85        | Kisansa       | Lira       | 0.02±0.0              | -2.87±1.9             | 0.28±0.0              |
| 86        | Bogoya        | Lira       | 0.22±0.0              | -3.12±0.0             | 0.14±0.0              |
| 87        | Gonja         | Lira       | 0.13±0.1              | -2.17±0.1             | 0.01±0.0              |
| 88        | SukaliNdiizi  | Lira       | 0.25±0.0              | -3.20±0.0             | 0.08±0.0              |
| 89        | Kivuvu        | Lira       | 0.22±0.1              | -1.27±0.0             | 0.12±0.1              |
| 90        | M9            | Lira       | 0.11±0.0              | -0.57±0.6             | 0.56±0.1              |

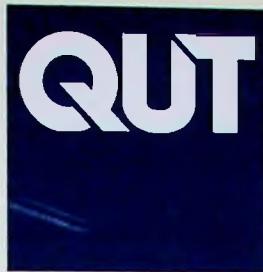
APPENDIX II: ANALYSIS OF POTENTIAL HEAVY METAL CONTAMINATION IN BANANA  
FRUIT SAMPLES ANALYSED BY ICP-OES IN CHAPTER 4 (FIGURE 4.5 AND 4.6)

| Sample ID | Cultivar      | Transgene       | Cd content<br>(mg/kg) | Pb content<br>(mg/kg) | Ti content<br>(mg/kg) |
|-----------|---------------|-----------------|-----------------------|-----------------------|-----------------------|
| 4         | Nakinyika     | <i>BT4-Sfer</i> | 0.17±0.5              | 0.05±0.0              | 0.89±0.0              |
| 5         | Nakinyika     | <i>BT4-Sfer</i> | 0.05±0.3              | 0.26±0.5              | 0.75±0.0              |
| 9         | Nakinyika     | <i>BT4-Sfer</i> | 0.08±0.1              | 0.15±0.3              | 0.93±0.1              |
| 12        | Nakinyika     | <i>BT4-Sfer</i> | 0.03±0.0              | 0.15±0.1              | 0.89±0.4              |
| 17        | Nakinyika     | <i>BT4-Sfer</i> | 0.06±0.0              | 0.12±0.1              | 0.87±0.8              |
| 22        | Nakinyika     | <i>BT4-Sfer</i> | 0.05±0.0              | 0.13±0.2              | 0.85±0.5              |
| 25        | Nakinyika     | <i>BT4-Sfer</i> | 0.03±0.0              | 0.12±0.2              | 0.76±0.1              |
| 29        | Nakinyika     | <i>BT4-Sfer</i> | 0.09±0.1              | 0.05±0.1              | 0.97±0.1              |
| 33        | Nakinyika     | <i>BT4-Sfer</i> | 0.01±0.2              | 0.28±0.3              | 0.98±0.1              |
| 42        | Nakinyika     | <i>BT4-Sfer</i> | 0.02±0.1              | 0.16±0.2              | 0.89±0.2              |
| 44        | Nakinyika     | <i>BT4-Sfer</i> | 0.02±0.0              | 0.25±0.0              | 0.69±0.0              |
| 58        | Nakinyika     | <i>BT4-Sfer</i> | 0.08±0.1              | 0.19±0.4              | 0.96±0.3              |
| 64        | Nakinyika     | <i>BT4-Sfer</i> | 0.06±0.0              | 0.05±0.1              | 0.87±0.5              |
| 67        | Nakinyika     | <i>BT4-Sfer</i> | 0.08±0.1              | 0.06±0.1              | 0.96±0.1              |
| WT        | Nakinyika     | Control         | 0.07±0.0              | 0.22±0.1              | 0.89±0.1              |
| 1         | Sukali Ndiizi | <i>Exp-Sfer</i> | -0.32±0.0             | 0.45±1.1              | -0.61±0.0             |
| 3         | Sukali Ndiizi | <i>Exp-Sfer</i> | -0.36±0.0             | 0.84±1.1              | -0.59±0.0             |
| 4         | Sukali Ndiizi | <i>Exp-Sfer</i> | -0.33±0.0             | 0.73±0.0              | -0.61±0.0             |
| 5         | Sukali Ndiizi | <i>Exp-Sfer</i> | -0.34±0.0             | 0.93±1.5              | -0.54±0.0             |
| 8         | Sukali Ndiizi | <i>Exp-Sfer</i> | 0.30±0.0              | 0.65±1.1              | -0.72±0.0             |
| 9         | Sukali Ndiizi | <i>Exp-Sfer</i> | -0.26±0.0             | 0.44±0.1              | -0.40±0.3             |
| 11        | Sukali Ndiizi | <i>Exp-Sfer</i> | -0.34±0.0             | 0.89±0.2              | -0.64±0.0             |
| 13        | Sukali Ndiizi | <i>Exp-Sfer</i> | 0.06±0.0              | 0.10±0.1              | -0.05±0.0             |
| 15        | Sukali Ndiizi | <i>Exp-Sfer</i> | 0.02±0.0              | 0.10±0.4              | -0.15±0.0             |
| 19        | Sukali Ndiizi | <i>Exp-Sfer</i> | 0.34±0.0              | 0.96±0.4              | 0.97±0.0              |
| 24        | Sukali Ndiizi | <i>Exp-Sfer</i> | 0.02±0.1              | 0.08±0.3              | 0.10±0.1              |
| 26        | Sukali Ndiizi | <i>Exp-Sfer</i> | -0.36±0.05            | 1.00±0.1              | -0.56±0.0             |
| 81        | Sukali Ndiizi | <i>Exp-Sfer</i> | -0.29±0.0             | 2.20±0.9              | -0.56±0.0             |
| 83        | Sukali Ndiizi | <i>Exp-Sfer</i> | -0.32±0.0             | 1.29±1.19             | -0.59±0.0             |
| 85        | Sukali Ndiizi | <i>Exp-Sfer</i> | -0.31±0.0             | 1.68±1.2              | -0.51±0.0             |
| 31        | Sukali Ndiizi | <i>35S-Irt1</i> | 0.17±0.1              | -1.21±0.3             | 0.06±0.1              |
| 40        | Sukali Ndiizi | <i>35S-Fro2</i> | 0.20±0.0              | -1.52±0.8             | 0.84±0.2              |
| 51        | Sukali Ndiizi | <i>35S-Fro2</i> | 0.17±0.0              | -2.33±0.4             | 0.09±0.4              |
| 52        | Sukali Ndiizi | <i>35S-Fro2</i> | 0.21±0.0              | -2.11±0.8             | 0.09±0.7              |
| 57        | Sukali Ndiizi | <i>35S-Fro2</i> | 0.27±0.0              | -0.94±0.4             | 0.10±0.2              |
| WT        | Sukali Ndiizi | Control         | -0.25±0.0             | 0.89±1.4              | -0.72±0.0             |

**APPENDIX III: ANALYSIS OF POTENTIAL HEAVY METAL CONTAMINATION IN  
BANANA FRUIT SAMPLES ANALYSED BY ICP-OES IN CHAPTER 5**

| Sample ID | Cultivar  | Transgene  | Cd content<br>(mg/kg) | Pb content<br>(mg/kg) | Ti content<br>(mg/kg) |
|-----------|-----------|------------|-----------------------|-----------------------|-----------------------|
| 12253     | Cavendish | Ubi-FEA1   | -0.8 ± 0.1            | -0.2 ± 0.4            | -0.3 ± 0              |
| 12254     | Cavendish | Ubi-FEA1   | -0.8 ± 0              | -0.2 ± 0.3            | -0.3 ± 0              |
| 12255     | Cavendish | Ubi-FEA1   | -0.8 ± 0.1            | -0.5 ± 0.2            | -0.3 ± 0              |
| 12257     | Cavendish | Ubi-FEA1   | -0.8 ± 0              | -0.6 ± 0.6            | -0.2 ± 0              |
| 12258     | Cavendish | Ubi-FEA1   | -0.7 ± 0              | -0.3 ± 0.1            | -0.2 ± 0              |
| 12259     | Cavendish | Ubi-FEA1   | -0.8 ± 0              | -0.8 ± 0.3            | -0.2 ± 0.1            |
| 12261     | Cavendish | Ubi-FEA1   | -0.8 ± 0              | -0.7 ± 0.2            | -0.2 ± 0              |
| 12262     | Cavendish | Ubi-FEA1   | -0.8 ± 0              | -0.6 ± 0.4            | -0.2 ± 0              |
| 12263     | Cavendish | Ubi-FEA1   | -0.8 ± 0              | -0.6 ± 0.5            | -0.2 ± 0              |
| 12264     | Cavendish | Ubi-FEA1   | -0.7 ± 0.1            | -0.5 ± 0.1            | -0.2 ± 0              |
| 12265     | Cavendish | Ubi-FEA1   | -0.7 ± 0.1            | -0.5 ± 0.3            | -0.2 ± 0              |
| 12266     | Cavendish | Ubi-FEA1   | 0 ± 0                 | 0.2 ± 0               | 0.3 ± 0.1             |
| 12267     | Cavendish | Ubi-FEA1   | 0 ± 0                 | 0 ± 0.2               | 0.3 ± 0.1             |
| 12268     | Cavendish | Ubi-FEA1   | -0.8 ± 0.1            | -0.6 ± 0.2            | -0.2 ± 0              |
| 12269     | Cavendish | Ubi-FEA1   | -0.8 ± 0.1            | -0.4 ± 0.5            | -0.2 ± 0              |
| 12270     | Cavendish | Ubi-FEA1   | -0.8 ± 0              | -0.4 ± 0.1            | -0.2 ± 0              |
| 12271     | Cavendish | Ubi-FEA1   | -0.7 ± 0              | -0.5 ± 0.4            | -0.2 ± 0              |
| 12272     | Cavendish | Ubi-FEA1   | -0.8 ± 0              | -0.4 ± 0.2            | -0.2 ± 0              |
| 12273     | Cavendish | Ubi-OsNAS1 | 0.2 ± 0.1             | 1.8 ± 0.4             | 0.3 ± 0               |
| 12274     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.8 ± 0.5            | -1.2 ± 0              |
| 12275     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.5 ± 0.2            | -1.1 ± 0              |
| 12276     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.5 ± 0.1            | -1.1 ± 0.1            |
| 12277     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.6 ± 0.2            | -1.1 ± 0              |
| 12278     | Cavendish | Ubi-OsNAS1 | -0.4 ± 0.1            | -0.5 ± 0.3            | -1.1 ± 0              |
| 12280     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.5 ± 0.4            | -1.1 ± 0              |
| 12281     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.7 ± 0.4            | -1.1 ± 0.1            |
| 12282     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.9 ± 0.3            | -1.1 ± 0.1            |
| 12283     | Cavendish | Ubi-OsNAS1 | -0.1 ± 0              | 0 ± 0.4               | 0.1 ± 0               |
| 12284     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.5 ± 0.4            | -1.1 ± 0.1            |
| 12285     | Cavendish | Ubi-OsNAS1 | -0.1 ± 0              | 0.2 ± 0.2             | 0.1 ± 0               |
| 12286     | Cavendish | Ubi-OsNAS1 | -0.4 ± 0              | -0.7 ± 0.3            | -1.1 ± 0              |
| 12287     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.6 ± 0.4            | -1.1 ± 0              |
| 12288     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.6 ± 0.6            | -1.2 ± 0              |
| 12289     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.3 ± 0.1            | -1.1 ± 0              |
| 12290     | Cavendish | Ubi-OsNAS1 | -0.4 ± 0              | -0.3 ± 0.1            | -1.1 ± 0.1            |
| 12291     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.7 ± 0.8            | -1 ± 0                |
| 12292     | Cavendish | Ubi-OsNAS1 | -0.5 ± 0              | -0.7 ± 0.1            | -1.1 ± 0              |
| WT        | Cavendish | Wild-type  | -0.7 ± 0.2            | -0.6 ± 0.1            | -0.5 ± 0.4            |

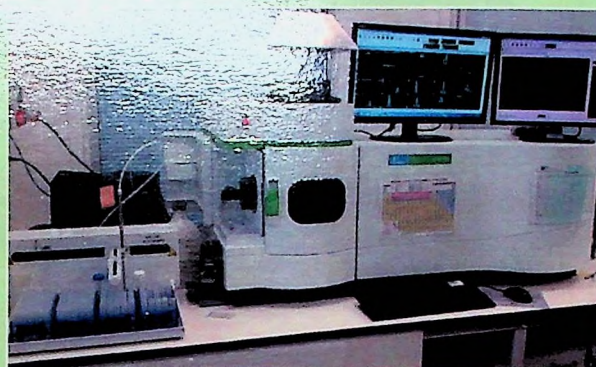




# Molecular and Biochemical characterisation of transgenic banana lines containing iron uptake and storage enhancing genes

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Bananas are an important staple crop to millions in developing countries, like Uganda, who suffer from iron and zinc deficiency. This study confirmed that Ugandan bananas are poor in these important nutrients by analysing ten banana varieties grown across the country, and demonstrated the potential to improve iron levels in staple bananas through biofortification using plant mineral metabolism genes. This research yields critical insights for future developments in the biofortification of banana to meet the nutrient requirements of vulnerable groups.

A full version of this thesis is available online at <http://eprints.qut.edu.au>