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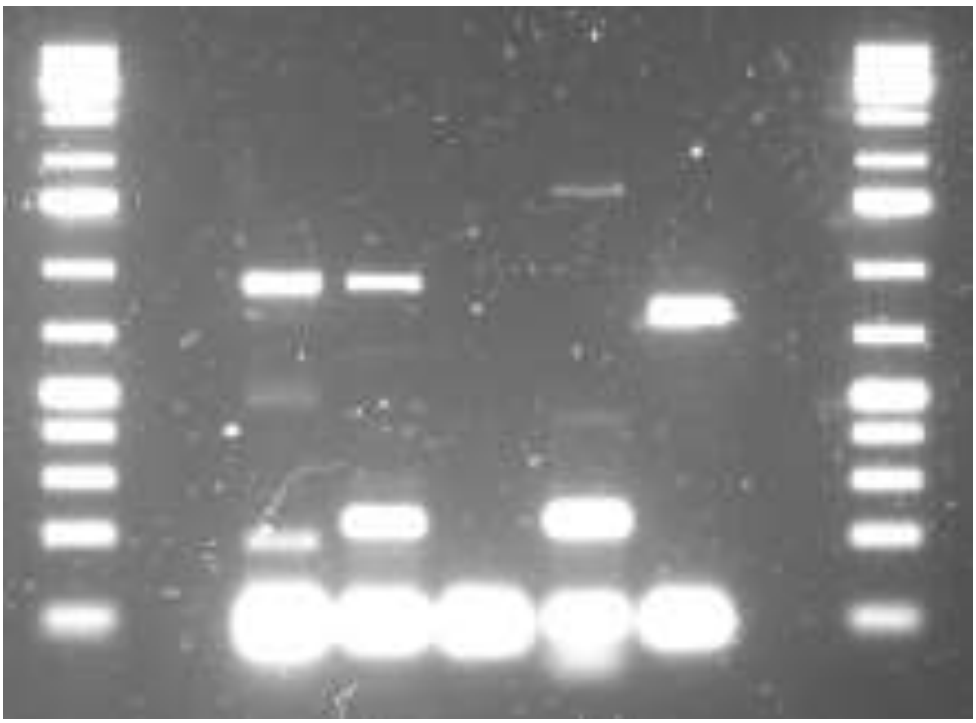
Searching for an increase of the protein content in potato (*Solanum tuberosum* L.)



MSc. Thesis Report

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M.Sc. Thesis

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Abbreviations

AMT	Ammonium Transporter
ANOVA	Analysis of Variance
B1	Batch 1
B2	Batch 1
bp	Base pair
C2H4	Ethylene
cDNA	Complementary Deoxyribonucleic acid
Clc	Chloride channel
CT	Cycle threshold
DB	Data Base
dCT	Delta Cycle threshold
ddCT	Delta Delta Cycle threshold
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
ETP	Explant Transformation Procedure
ETR	Ethylene receptor
GWAS	Genome-Wide Association Study
His	Histidine
HKG	House Keeping Gene
LB	Lysogeny Broth medium
LHT	Lysine/histidine transporter
Lys	Lysine
N	Nitrogen
NH_4^+	Ammonium
NO_2^-	Nitrite
NO_3^-	Nitrate
NRT	Nitrate transporter
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
pDNA	Plasmid Deoxyribonucleic acid
pENTR	Entry clone for the Gateway® system
PFJ	Potato fruit juice
pH	Potential Hydrogen
QTLs	Quantitative trait loci
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SOC	Super Optimal Broth with Catabolite repression medium
SPAD	Soil Plant Analysis Development
SPC	Soluble Protein Content
TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
Tm	Melting Temperature
WT	Wild Type

Abstract

Nowadays, plant-derived proteins occupy an important position in bio-economy. Plant proteins can be used for human consumption, animal feed and as resources for industrial applications. Potato (*Solanum tuberosum*, Solanaceae) provides great quality proteins, which can be compared to animal-derived proteins. Potato proteins are localized and dissolved in the potato fruit juice (PFJ) but this crop possesses a low overall protein content when compared to other vegetable crops. Therefore, an increase in the potato soluble protein content (SPC) is required to strongly position its participation in the bio-economy sector. Being Nitrogen (N) the limiting factor for protein biosynthesis, our research will focus on four candidate genes which are involved in N utilization and have been identified in GWAS studies in potato and other crops for investigation of their function in potato in relation to protein composition. We performed molecular cloning and transformation of the ammonium transporter (*AMT*), lysine (Lys)/histidine (His) transporter (*LHT*) and ethylene receptor *(ETR*) genes, aiming at the production of transgenic potato plants. In addition, transgenic lines overexpressing the *NRT1.11* were phenotyped and analyzed to evaluate its effect on the protein composition and its function in potato. *AMT* was amplified from PCR however, it could not be introduced into the vector plasmid. *LHT* and *ETR* genes were not PCR amplified. Cloning and transformation of these three genes still remain to be completed. Analysis of transgenic plants overexpressing the *NRT1.11* revealed no increment in yield and SPC in tubers. However, plant material from this transgenic lines still remains to be evaluated.

Keywords: Molecular cloning, Nitrogen Transporter genes, SPC, *NRT1.11* gene, RT-PCR analysis

1 Introduction

1.1 Importance of potato proteins

Proteins can be used in a wide range of applications as human consumption, animal feed and as resources for industrial applications. Nowadays, plant-derived proteins occupy an important position in the bio-economy. Plant proteins can add value to bioenergy based value chains. Bio-refinery research focuses on the isolation of valuable protein derived compounds from diverse crops and plant residues. Crops with high yield and great quality proteins are of special interest in the bio-economy sector. Potato is the most important non-cereal food crop and is ranked fourth in terms of total global food production, cultivated in nearly 160 countries due to its high yield when compared with other food crops (Camire *et al*., 2009) and is estimated that more than a billion people include them on a daily basis (Chakraborty *et al*., 2010). Potatoes are a good source of energy containing a high amount of carbohydrates as well as of proteins, minerals, and vitamins (Navarre *et al*., 2016). Potatoes contain high-quality proteins with an important nutritional value that can be compared to wheat and soy proteins or animal-derived proteins as milk and egg proteins (Camire *et al*., 2009). Major classes of proteins have been described for potato. Patatin is the major storage protein in potato. This glycoprotein makes over 40% of the total soluble protein content in potato tubers. Patatin exhibit both lipid acyl hydrolase and acyltransferase activities. Other important potato tuber proteins are present as a diverse group of low molecular weight proteins and show protein digestion and protein inhibition activity. Proteins from potato tubers are localized and dissolved in the fruit juice, from where they can be recovered through acidic treatment (Delaplace *et al*., 2006). However, when proteins are extracted through this method, they lose important properties, such as water solubility. Therefore, extracted proteins can mostly be used for animal feed applications. Recently, the company Avebe has developed an alternative process. By using liquid chromatography approaches, the obtained potato proteins (Solanic®) maintain their natural biochemical properties making them suitable for human consumption. Soluble protein content in PFJ of tubers oscillates between 1% and 3% depending on the crop variety. However, when compared with other vegetable sources, potato is not typically considered as a noble protein source due to their low overall protein content (Camire *et al*., 2009). Therefore, an increase in the potato SPC is required to strongly position its participation in the bio-economy sector.

Biofortification is the process of increasing the content and/or bioavailability of essential nutrient in crops through agronomic and/or genetic pathways. Agronomical biofortification involves the use of fertilizer application, whereas genetic biofortification can be achieved by genetic engineering or classical breeding routes (de Valença *et al*., 2017). There are many strategies for enhancing the nutritional value of crops. From conventional breeding approaches to the development of transgenic varieties. While the former is very time-demanding, since it includes identification and introduction of useful traits into elite cultivars by several back-crossing events, making a transgenic plant is the most rapid way to introduce desirable traits into a given variety since the novel genetic information can be introduced directly into the plant genome (Le *et al*., 2016). Although genetic modification is the best approach, there are many ethical, political and socio-economic and legislative constrictions that impede the application of this strategy (Zhu *et al*., 2008 and Garcia-Casal *et al*., 2016). In order to increase the SPC on potato tubers, is important to identify and understand the complex process involved in protein production.

1.2 Importance of Nitrogen in plants

Potato yield and quality are affected by the variety used, the seed quality, plant environment, and crop management practices. However, the most limiting factor is the availability of mineral nutrients with N, at least quantitatively. Nitrogen assimilation presents a strong connection between the carbon metabolism and the photosynthesis. There is a positive correlation between the amount of N accumulated, plant growth rate and biomass production. (Gastal & Lemaire 2002). Nitrogen metabolism

starts by the plant uptake of N from the soil. Nitrogen can be present in the soil in organic (as simple amino acids) and/or in inorganic form (such as nitrate (NO_3^-), nitrite (NO_2^-) and ammonium-ions (NH_4^+)) (Sohlenkamp *et al*., 2002). N forms are taken up by root cells through the activity of plasma membrane transport proteins (Perchlik *et al*., 2014). Plants have developed multiple transport systems for the acquisition of the different N forms available in the soil, and for their translocation from the apoplast root epidermal and cortical cells into the symplastic transport flow for its distribution within the plant organism (Sohlenkamp *et al*., 2002 and Hirner *et al*., 2006). For the most growing plant in agricultural soils is believed that the primary sources of N are the inorganic forms (Sohlenkamp *et al*., 2002). The preference for nitrate and ammonium over amino acids is not fully understood and might depend on the plant species (Perchlik *et al*., 2014). When ammonium and nitrate are available in similar concentrations, ammonium is generally taken up more rapidly than nitrate. This preference might be explained by the fact that plants directly assimilate ammonium into glutamine, in plastids. Meanwhile, for nitrate assimilation, nitrate needs first to be converted to nitrite in the cytoplasm by the nitrate reductase. Following, nitrite is translocated to the chloroplast for reduction to ammonia by the nitrite reductase (Ren et *al*., 2018). Therefore, plants must spend extra energy on reducing nitrate to ammonium before it can be incorporated into organic compounds (Howitt and Udvardib, 2000 and Sohlenkamp *et al*., 2002). N forms transportation and translocation are not fully understood in the potato cultivar. Consequently, in order to increase the SPC in potato tubers is first necessary to understand the participation of the multiple nitrogen transporters in this process.

1.3 Genes of interest

In this project, we focus our study on target genes which can significantly contribute to increasing the total protein content in potato tubers. These genes have been found within quantitative trait loci (QTLs) identified in GWAS studies in potato and other crops. Here, we will focus on 4 candidate genes: a) nitrate transporter b) ammonium transporter, c) lysine /histidine transporter and d) ethylene receptor.

1.3.1 Nitrate Transporters

Nitrate is the major source of N in higher plants (Bai *et al*., 2013). Nitrate is absorbed by plants through nitrate transporters (Bai *et al*., 2013). NO_3^- uptake, from the soil, is mediated by high-affinity and low-affinity nitrate uptake systems depending on the available concentration (low <1 mM or high >1 mM). Nitrate transporters have been comprehensively characterized in *Arabidopsis thaliana* (AtNRT). Dechorgnat and colleagues (2011) identified in *Arabidopsis* the presence of three NRT-families composed by NRT1/PTR (nitrate/peptide transporters), NRT2, and NRT3 members. *Arabidopsis* genome encodes at least 67 nitrate transporters, including 53 *NRT1* genes, 7 *NRT2* genes and 7 chloride channel (*Clc*) genes (Forde, 2000 and Dechorgnat *et al*., 2011). AtNRT1.1 shows an influence in both low and high-affinity NO_3^- transport regulating the nitrate uptake (Muños *et al*., 2004). It has been found that AtNRT1.1 also develops functions as NO_3^- sensor regulating the expression of NO_3^- related genes in plants (Ho *et al*., 2009). Other members of the AtNRT1 family as, AtNRT1.5, AtNRT1.8, and AtNRT1.9 are involved in regulating the nitrate translocation from root to shoot (Dechorgnat *et al*., 2011). *AtNRT1.4* is expressed in mid-rips and petioles intervening in the nitrate allocation to leaves (Dechorgnat *et al*., 2011). Hsu and Tsay (2013) research in *Arabidopsis*, reveal how NRT1.11 and NRT1.12 facilitate the redistribution of the stored N from mature leaves to youngest tissues. Nitrate transporters 1.11 and 1.12 are involved in loading xylem nitrate into the phloem for redistribution into sink tissues (Hsu *et al*, 2013).

M'hamdi and colleagues (2016) tested the gene expression of *NRT1.2*, *NRT1.5* and *NRT2.1 Arabidopsis* homologs in different potato cultivars. *StNRT1.2, StNRT1.5,* and *StNRT2.1* genes were expressed in leaves and tubers suggesting a role on the mobilization of nitrate from source to sink in potato. Accumulation of nitrate was credited to the expression levels of NRT genes. In potato, an increase in the transport of nitrate likely influence the biomass of the plants (M'hamdi *et al*., 2016). However, the increase of the protein content in tubers still needs to be evaluated. *Arabidopsis* NRTs genes present no significant phylogenetic divergence from *NRTs* potato species. This indicates that the function of *NRTs*

genes in potato could be determined according to the sequence homology to functionally characterized *Arabidopsis NRT* genes (M'hamdi *et al*., 2016). Following this idea, potato gene *PGSC0003DMG400015591* shows a high sequence homology to *AtNRT1.11*. With the purpose of facilitating the understanding of this report henceforth, the potato gene *PGSC0003DMG400015591* will be referred as *NRT1.11* gene.

1.3.2 Ammonium Transporters

Ammonium is an important source of N vital for the growth of plants (Mayer *et al*., 2006). NH_4^+ is a central precursor of nucleic acids, proteins and other organic molecules (Ludewig *et al*., 2007). This chemical compound is up taken from the soil by plant roots via ammonium transporters and then distributed to vacuoles, chloroplast and mitochondria (Howitt and Udvardib, 2000). AMT are integral membrane proteins organized as homotrimers were each monomer possesses a central hydrophobic channel that assists in the translocation of ammonium ions from the sites of production to the sites of consumption (Khademi *et al*., 2004 and Andrade *et al*., 2005). Two different AMT-families are known in higher plants genome, AMT1 and AMT2. Both family members present a high ammonium affinity, but the AMT2 family shows incapacity of transporting methylammonium (ammonium analog) (Pantoja, 2012). In *Arabidopsis*, five different AMT1s (*AtAMT1*; 1–5) and one AMT2 have been identified (Ludewig *et al*., 2007). *AtAMT1*, expressed in roots, assist in the NH_4^+ uptake from soil (Yuan *et al*., 2007). AtAMT2 is involved in ammonium recycling and translocation from the apoplast of root cells throughout the plant via symplastic stream flow, especially after photorespiration (Sohlenkamp *et al*., 2002). In *Arabidopsis*, *AMT* transcriptional control is a major response to N or carbon availability regulating ammonium contents and concentration in different plant tissues. N deficiency upregulates transcription of 4 roots AMTs. On the contrary, AMTs expression and ammonium influx are down-regulated after re-supply to N-starved plants and these effects are negatively correlated with glutamine levels suggesting that glutamine may function as a feedback signal for ammonium after N re-supply (Ludewig *et al*., 2007).

Loqué and colleagues (2006) observed in *Arabidopsis*, that loss of either AMT1:1 or AMT1:3 led to a decrease in the high-affinity ammonium influx of approximately 30% and in AMT double knock out accessions a reduction of 70% in ammonium influx was observed, suggesting that there is an additive contribution of AMT1:1 or AMT1:3 on the overall ammonium uptake under N deficiency.

1.3.3 Lysine/Histidine Transporters

Amino acids are the predominant form of organic N. Amino acids are precursors of many essential molecules including proteins, nucleic acids, and phytohormones. Lysine and histidine are α-amino acids used for the biosynthesis of proteins. Both amino acids contain an α-amino group, an α-carboxylic acid group and in case of Lys a lysyl side chain meanwhile, His possess an imidazole side chain (Hirner *et al*., 2006). Organic forms of N are imported into the root and mesophyll cell by LHTs (Hirner *et al*., 2006). Lysine/histidine transporters are localized on the plasma membrane assisting in the transportation of a broad spectrum of amino acids from the cell wall space into the cell (Hirner *et al*., 2006). *Arabidopsis* genome contains a family of 10 LHTs (*AtLHT1-10*) members (Hirner *et al*., 2006 and Tegeder et al., 2012). *Arabidopsis* LHTs are divided in two different clusters: cluster 1 contains eight of ten AtLHTs (*AtLHT1, 2, 3, 5, 6, 8, 9, and 10*) meanwhile, cluster 2 contains *AtLHT4* and *AtLHT7* (Tegeder and Ward., 2012). Cluster 1 LHT transporters including AtLHT1, 2, 5, and 6 are expressed in both female and male floral tissues, suggesting that these transporters might play an essential role during sexual plant reproduction (Tegeder and Ward, 2012). Cluster 2 is also expressed in anther and pollen, suggesting a participation in plant reproduction. However, *AtLHT4* is also expressed in roots and stem suggesting additional functions (Tegeder and Ward, 2012).

Svennerstam and colleagues (2007) found that AtLHT1 mutants exhibited an impaired growth when N was supplied in organic forms, at the same time, lower amounts of amino acids were acquired by AtLTH1 mutants when compared to wild-type (WT) plants from solid growth media. On the other hand, overexpression of *AtLHT1* increments the capacity for amino acid uptake, and thus N use efficiency under limited inorganic N supply, accelerating plant growth (Hirner *et al*., 2006). These results suggest that AtLTH1 is involved in root amino acid uptake in plants. Similar studies show that AtLHT1 and AtLHT2 exhibit a preference for transporting neutral and acidic amino acids (Hirner *et al*., 2006; Svennerstam *et al*., 2007, and Tegeder *et al*., 2012). Most studies of lysine/histidine transporters have been performed in *Arabidopsis*, while few information is available in potato and its participation in SPC in potato tubers is still unclear. Therefore, functions and behavior of these transporters are not completely understood.

1.3.4 Ethylene Receptors

Ethylene (C_2H_4) is a gaseous plant hormone involved in growth and developmental regulation processes including seed germination, the growth of seedling, leaf/petal abscission, fruit ripening, organ senescence and pathogen responses (Shakeel *et al*., 2015). Ethylene is recognized and modulated by plants through ethylene receptors (ETR). In *Arabidopsis*, five ethylene receptors have been identified: ethylene response 1 (AtETR1), AtETR2, ethylene insensitive 4 (AtEIN4), ethylene response sensor 1 (AtERS1) and AtERS2. Based on phylogenetic analysis, ethylene receptors can be divided into two subfamilies. Subfamily 1 is composed by ETR1 and ERS1. Meanwhile, subfamily 2 is composed by ETR2, ERS2, and EIN4. A genetic analysis of these receptors exhibits a functional overlapping between both subfamilies. However, subfamily one plays the dominant role in ethylene signaling (Shakeel *et al*., 2015). Ethylene receptors possess an overall similar modular structure. In both ETR subfamilies three conserved transmembrane domains, a GAF domain, and signal output motifs in the C-terminal half are localized. The transmembrane domain contains the ethylene binding site, the GAF domain is involved in protein-protein interactions among the receptors, and the signal output domains of all five receptors contain a kinase-like domain. Kinase domains among these subfamilies differ, subfamily 1 shows a functional histidine-kinase domain, whereas subfamily 2 was reported to have a serine/threonine kinase domain (Shakeel *et al*., 2015). Ethylene receptors, in combination with the constitutive response 1 (CTR1) Raf-like kinase, negatively regulates ethylene signal transduction (Shakeel *et al*., 2015). Membrane-associated CTR1 levels vary in response to ethylene in a post-transcriptional manner that correlates with ethylene-mediated changes in levels of the ethylene receptors ERS1, ERS2, EIN4, and ETR2. CTR1 interaction with ETR1 protects ETR1 from ethylene-induced turnover. A model where two opposing factors controls the ethylene receptor/CTR1 levels is supported by kinetic and dose-response analysis (Shakeel *et al*., 2015). In potato, ETR genes are expressed in tubers, stolons, stems, leaves and flower parts such as petals, sepals, petioles, and stamina.

In *Arabidopsis*, a mutation in the transmembrane domain causes ethylene insensitivity, since the receptor is unable to bind ethylene. Therefore, no ethylene recognition responses are observed even in presence of this gas hormone (Gallie, 2015). *Arabidopsis* and tomato mutants, presenting an ETR loss-of-function, display either an increased sensitivity to ethylene or a loss of recognition to ethylene response (Gallie, 2015). Ethylene functions and plant response is yet not completely understood. In order to understand more about this plant hormone, Wuriyanghan and colleagues (2009) studied in rice the plant responses to overexpression and the silencing, by RNA interference (RNAi), of *ERT2*. Rice plants overexpressing *ERT2* exhibit a decrease in ethylene sensitivity and showed phenotypes with delayed floral transition and suppression in sugar translocation from stems to grains, leading to a reduced seed rate and weight. In contrast, silenced plants in *ETR2* showed an increased ethylene sensitivity, early flowering, a decrease in starch accumulation and an increase in sugar translocation, leading to higher seed weights. Lastly, analogous studies in ethylene receptors had revealed that a loss in ETR1 or/and EIN4 leads to accelerated germination; loss of function ETR2 delays germination meanwhile a loss of

either ERS1 or ERS2 present no measurable effect in germination (Wilson *et al*., 2014). Gallie (2015) research demonstrated that loss of function of subfamily 1 receptors cannot be rescued by the ectopic expression of subfamily 2.

2 Objectives of the research

This project aims to increase the final SPC in potato tubers (*Solanum tuberosum* L.) by cloning the *AMT*, *LHT* and *ETR* genes and overexpress them in potato plants via *Agrobacterium tumefaciens* transient assays (ATTA). In addition, phenotype and analyze transformed potato plants overexpressing the *NTR1.11* gene in comparison to WT potatoes, in terms of SPC.

3 Material and methods

3.1 Plant material

Plant material used in this project included potato wild-type cultivar Kardal and transgenic lines overexpressing the *NRT1.11* gene. Young leaves, tubers, and stems samples were kept frozen at -80°C before being grinded. Only plant material extracted from wild-type Kardal was used for cloning of the *AMT*, *LHT*, and *ETR* genes.

Two different batches of transformed plants deferring in dates placed in the greenhouse (Batch 1 and 2) were used for phenotyping and analysis of data. Batch one was integrated by eight different transformants and one wild-type (WT1) for control. Meanwhile, Batch two was formed by five different transformants and one wild-type (WT2) control. Each transformed line or wild-type was intended to have 8 different clones, however, in some lines, one or more plants development was compromised. Batch one and three were placed in the greenhouse (Batch one 28 weeks after the explant transformation procedure (ETP) and Batch two after 31 weeks after ETP). Half-harvest of each batch was performed after 10 weeks in the greenhouse, and the final harvest was completed after 19 weeks in the greenhouse. The table in Appendix 9.5.1 shows the number of clones for each line.

3.2 Culturing *E. coli* containing the *Arabidopsis* gene of interest

Before being able to perform Plasmid DNA (pDNA) extraction single colonies from *E. coli* had to be isolated. Petri dishes containing 20 mL of Lysogeny broth (LB) solid medium + 1/2000 of gentamicin (50 mg/ml) or 1/500 spectinomycin (100 mg/ml) depending on the origin of the bacteria were used. Bacteria were placed on agar plates in a streaking way for a single colony and then kept at 37°C overnight. Single colonies were picked with a toothpick from overnight Petri dishes and placed in tubes containing 3 mL LB liquid medium and gentamicin concentration of 1/2000 or 1/500 of spectinomycin. Four clones were used per construct and then kept at 37°C for shaking overnight. The culture of single bacteria colonies intends to increase the amount of plasmid for further pDNA isolation.

3.3 Plasmid DNA isolation

Plasmid DNA (pDNA) isolation was performed using the QIAprep® Spin Miniprep kit from QIAGEN. Following the manufacturer's protocol (Appendix 9.2.2). This QIAGEN kit recover a low quantity of plasmids since high amounts of plasmids are lost during the filtering steps nevertheless, a highly pure pDNA is recovered.

3.4 NanoDrop spectrophotometer

NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) is used for measuring concentration and purity of nucleic acid samples. For this step, 1.5 µl of the genetic sample was pipetted onto the end of a fiber optic cable (receiving fiber) and then closed with a second fiber optic cable (the source fiber). A PC based software provides the report of the concentration and purity of the different samples.

3.5 Medium

LB medium was used for culture *E. coli* from the different cloning steps. Two types of LB medium were used: the liquid and the solid one, both of them contain 20 g/L of LB medium, the difference lies on the use of 1.5% of agar from the final volume for the solid LB medium. All mediums were autoclaved before used. LB liquid medium was mainly used for propagating the selected single colonies in 10 mL tubes for further pDNA isolation and LB solid medium was used for culture bacteria after transformation in plates.

3.6 DNA extraction

DNA extraction was performed to confirm successful transformation events. An alkaline lysis DNA extraction method was performed on young leaves. DNA extraction was performed following the protocol presented in Appendix 9.1.1. After DNA extraction, samples were stored in the freezer at -20°C.

3.7 RNA extraction

RNA was extracted using the protocol presented in Appendix 9.1.2. Extracted RNA was then loaded onto the gel page to confirm the RNA quality. Amount of isolated RNA was quantified by NanoDrop analysis. Isolated RNA was stored at –80°C for long and at -20°C for short storage periods.

3.8 cDNA synthesis

Complementary DNA was utilized for cloning purposes and analysis of gene expression by RT-PCR. Before cDNA synthesis, a DNase treatment was used (Appendix 9.1.3). cDNA was synthesized using iScript™ cDNA synthese kit from Biorad following the protocol described in Appendix 9.1.4. Synthesized cDNA was confirmed by PCR.

3.9 Polymerase Chain Reaction (PCR)

3.9.1 Dream Taq polymerase

Use of polymerase chain reaction results in the amplification of a delimited sequence. For this technique sample mix of 25 µl were placed in 96 well plates. Each 25 µl sample mixture contained the following: 0.05 µl of Dream Taq polymerase, 2.5 µl of 10X deTaq buffer, 0.5 µl (100µM) forward primer, 0.5 µl (100µM) reverse primer, 0.5 µl of dNTPs (5 pmol/l), 1 µl DNA extraction or DNA sample, and 19.95 µl Milli-Q. Configuration of PCR steps were: Firstly denaturation at 94°C for 5 min, then 35 cycles of 94°C for 30 seconds for the denaturing stage, 55° for 30 seconds for the annealing stage and 72°C for 1 min for each kilobase of the expected product for the extending stage, followed by 72°c for 10 min and finally 10°C for unspecified time. The use of this polymerase was principally used for confirmation of transformed plants. Extending stage time depended directly on the size of the delimited sequence.

3.9.2 Phusion® polymerase

Phusion®® polymerase was only used for cloning purposes. A single sample mixture contained the following: 0.5 µl Phusion® polymerase, 10.0 µl 5X Phusion® buffer, 2.5 µl (100µM) forward primer and 2.5 µl (100µM) reverse primer; 2.0 µl of dNTPs (5 pmol/l), 2.0 µl of DNA/RNA extraction or DNA/RNA sample; and 30.5 µl of Milli-Q water. Configuration of PCR steps were: Firstly 98°C for 30 seconds, then a stage of 5 cycles(98°C for 10 seconds for the denaturing stage, 56°C during 30 seconds for the annealing stage and 72°C for 1 min for each kilobase of the expected product), followed by another stage of 28 cycles (98°C for 10 seconds for the denaturing stage, 58°C for 30 seconds for the annealing stage and 72°C for 1 min for each kilobase of the expected product, followed by 72°C for 10 min and finally 10°C for unspecified time.

3.10 Gel DNA recovery

The product from a PCR was loaded on a TAE 1.0% agarose gel. Using a sterile blade, the fragment of gel containing the amplified product with the expected length was carefully excised. The solid gel was then dissolved by heat and product purification was realized using Zymoclean ™ Gel DNA Recovery Kit following the manufacturer's protocol (Appendix 9.2.1).

3.11 Recombinant PCR

Two segments of the *AMT* gene containing an overlapping section were used as a template for the amplification of the complete gene fragment by recombinant PCR. All PCR assays followed the previously mentioned Phusion® protocol. Primers pair 80F-43R and 42F-83R (Table 1) were used for obtaining the two overlapping PCR products. Both PCR products were recovered from gel and purified. Equal concentration of products served as a template for a third PCR using only external primers 80F and 83R.

3.12 Sequencing

Sequencing of the DNA fragments was used for the purpose of proving the presence of the gene of interest in the isolated plasmid. For this step, 400-500 ng of pDNA is used, plus 2.5 µl of one primer (25 pmol) and fully filled with Milli-Q water for a final volume of 10 µl. Samples were sequenced by GATC BIOTECH, an external sequencing service.

3.13 Vector construction

The ORF was amplified by PCR using Phusion® Polymerase from the cDNA of *S. tuberosum* using specific primers (Table 1). PCR products were then purified using Zymoclean ™ Gel DNA Recovery Kit and quantified by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The purified PCR products were then cloned into the Gateway pENTR™ TOPO® vector following the protocol described in Appendix 9.3. Selection after cloning in the pENTR™ TOPO® vector was performed under LB solid medium plates containing of 1/1000 Kanamycin (50 mg/ml).

3.14 Soluble protein content analysis

SPC expressed in percentage exhibits the amount of protein in potato tubers at a certain time. The SPRINT Rapid Protein Analyzer (CEM Corporation, NC, U.S.A.) assay following the manufacturer´s protocol available in "http://cem.com/sprint/" was used to measure protein content in the potato fruit juice. For the measurement of the protein content for the half-way harvest one tuber, over 2 cm of diameter, from 3-4 plants per line (Appendix 9.5.2 and 9.5.4) was harvested and then pooled into a common sample. The common sample was then grinded and used for the SPRINT assay. On the other hand, the remaining tubers of each clone line were collected for the final harvest and then grinded individually for a single protein content assessment per line clone (Appendix 9.5.3 and 9.5.5).

3.15 Real-Time Polymerase Chain Reaction (RT-PCR) and CT analysis

Expression of the NRT gene in tubers was analyzed using Real-Time PCR assay. Samples were prepared using the iQ™ SYBR® Green Super Mix from BIO-RAD following protocol showed in Appendix 9.4. Cycle threshold (CT) values obtained were validated by the analysis of the melting curves using the software Bio-Rad CFX Manager from Bio-Rad. One replica was used for each cDNA sample and an average of Ct values from correspondent samples was employed. Delta Ct (dCT) values were obtained by the difference between the housekeeping gene (HKG) and the NRT Ct average values. The 2^{-ddCT} method was then utilized to analyze the relative changes in gene expression. Using this method, wild-type used as reference acquire a value of "1" and values under 1 represent a lower expression compared to the wild-type meanwhile, values over 1 represent a higher gene expression.

3.16Statistical analysis

All data were analyzed statistically by Student's test (t test) for each parameter separately using Microsoft Excel software. Principal component analysis (PCA) showing the correlations between transformant traits was performed by the utilization of the SPSS. All significance difference in traits among transformants vs WT was determined at a $p < 0.05$.

3.17 Primer design

Sequences from the different genes of interest were obtained from Solanaceae Genemonic Resource from the Michigan State University. Identification of the open reading frame (ORF) was performed by the software Vector NTI. Candidate primers were obtained by the software Vector NTI or the online platform Primer3 (version 4.0). Software Oligo 6 was used for testing the interaction between primers for dimerization analysis, hairpin formation and melting temperature (Tm). Primers were selected with a Tm above 60°C (65-68°C) for cloning purposes and below 60 °C (around 55°C) for RT-PCR assay; at least 20 base pair (bp) length and g/c content over 50%. All primers were synthesized by Biolegio BV Laboratories.

Table 1. List of relevant primers used during the project for amplification purposes.

5' - 3' SEQUENCE[†]	CODE
cacccatcatctcaatcttgtgtgaagca	Pr050F[1]
cacctcttgtgtgaagcaaggaataatgg	Pr052F[1]
tgaggtgtgcaccttattattgttg	Pr053R[1]
cgggtttaatcctggatcattcgac	Pr054F[1]
cgtccaaacaaggttacaatcccc	Pr055R[1]
tcattacatcattctacttccccgtagcaga	Pr013R[2]
ccctgccttataaactccactcc	Pr041R[2]
caccgattaggaagcgttggtgctgaa	Pr065F[2]
caccgtgctgaaatggaaatttaatacag	Pr067F[2]
ttgcgagtagtggagggagtaggga	Pr069F[2]
cctgctttgcttgttgcagagctcg	Pr070R[2]
cacttagggcgagtcaagcaaggaa	Pr071F[2]
gaaatattataacccctataagcac	Pr072R[2]
caactatagcatgggtgggttgtg	Pr042F[3]
gtactctggaaggcttttcaggtg	Pr043R[3]
caccatttgcaaacatggtttcatcttct	Pr080F[3]
aatgtattcttcatgaatagaactcatag	Pr081R[3]
caccatggtttcatcttctcctccac	Pr082F[3]
tcatgaatagaactcataggaggatgaa	Pr083R[3]
attaggattgttccaagcaggtg	Pr023F[4]
tcaggatcttcaatcatacaggctct	Pr024R[4]

[†]5′ - 3′ sequences are all shown in forward strand. [+] Indicates for what purpose the primers were used during the project. F=Forward primer; R=Reverse primer. [1]*AMT* gene primers. [2]*ETR* gene primers. [3]*LHT* gene primers. [4]*NRT* gene primers used in RT-PCR.

4 Results

Experiments with 3 candidate genes were performed with the intention of amplifying and cloning them into the Gateway® cloning system to introduce them into *Agrobacterium tumefaciens*. Results for the Lysine/histidine transporter, Ethylene receptor and Ammonium transporter are presented.

4.1 Lysine/Histidine transporter

Lysine/histidine transporter transcript sequence according to Spud DB presents a length of 1527 bp. An Open Reading Frame of 1314 bp with a start codon in position 47 and a stop codon in 1360 was identified. PCR using the four different combinations of external primers (Figure 1) yielded no bands after ethidium bromide staining and electrophoresis. PCR using all possible combination of external and internal primers yielded 4 bands with expected product length (figure 1). Products A and B showed in figure 1 were used as a template for a recombinant PCR. No band after recombinant PCR was observed. Consequently, following steps within the cloning strategy were not performed.

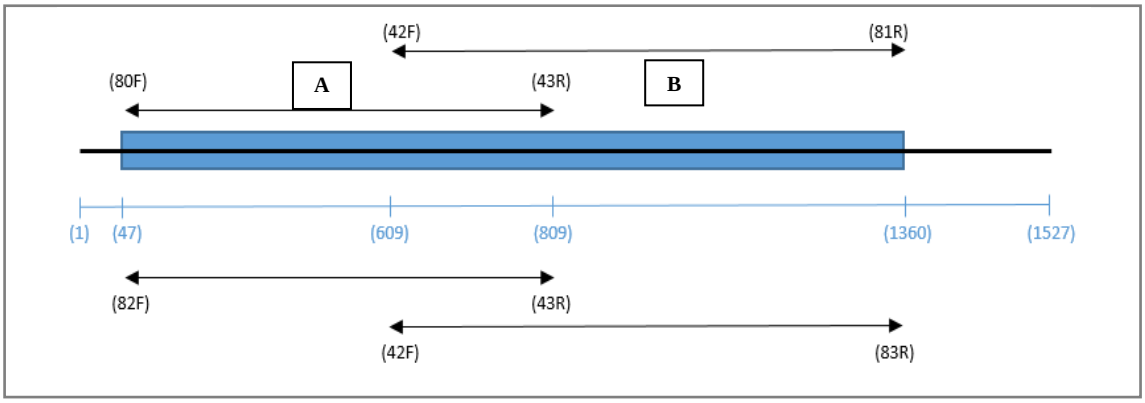


Figure 1. Representation of the LHT gene. The wide dark central line represents the complete sequence obtained from Spud DB database. Blue square indicates the ORF. Forward primer = F; reverse primer = R. Thin black lines between F and R primers represent the amplified gene fragment.

4.2 Ethylene receptor

Ethylene receptor sequence obtained from Spud DB for potato exhibit a gene fragment of 3778 bp. An ORF of 2333 bp with a start codon in position 530 and a stop codon in 2863 was identified. PCR using all four possible combinations with external primers yielded no band after electrophoresis. PCR using all external and internal primers was performed to test each primer and obtained amplified sequences are shown in figure 2. Unfortunately, the search for the complete gene or two products with an overlapping region was interrupted after that several attempts failed. Consequently, following steps within the cloning strategy were not performed.

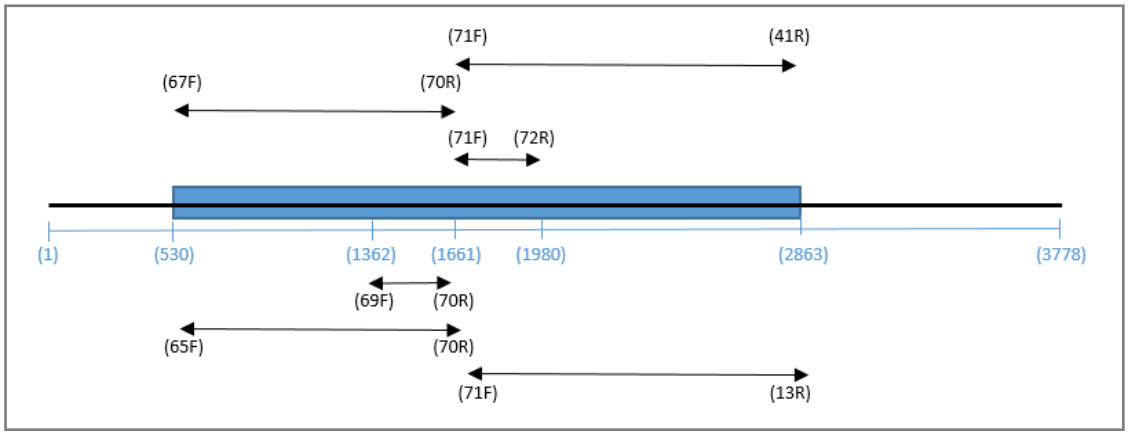


Figure 2. Representation of the ETR gene. Wide dark central line represents the complete sequence obtained from Spud DB database. Blue square indicates the ORF. Forward primer = F; reverse primer = R. Thin black lines between F and R primers represent the amplified gene fragment.

4.3 Ammonium transporter

Ammonium transporter gene according to transcript sequence from the Spud DB for potato consists of 2317 bp with an ORF of 1395 bp with a start codon on 21 bp and a stop codon 1415 bp. PCR amplification with primer combination 50F and 53R yielded after electrophoresis a weak band with the expected gene length (figure 3). PCR product was directly extracted from the gel and purified. Gene fragment was cloned into the pENTR™ TOPO® vector and selection after cloning was performed under LB solid medium plates containing Kanamycin. However, no colonies were observed suggesting a poor transformation efficiency. Modification of the manufacturer protocol on the concentration of PCR product in the TOPO Cloning reaction enzyme was attempted. Nevertheless, no colonies were observed on selection plates.

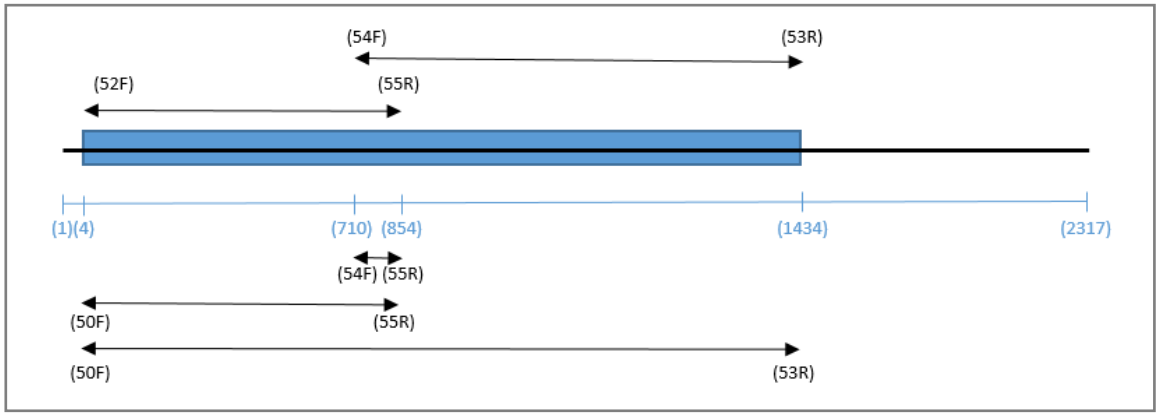


Figure 3. Representation of the AMT gene. The wide dark central line represents the complete sequence obtained from Spud DB database. Blue square indicates the ORF. Forward primer = F; reverse primer = R. Thin black lines between F and R primers represent the amplified gene fragment.

4.4 Transgenic plants overexpressing nitrate transporter 1.11

Potato plants transformed by Pablo Baldeon were used as starting material. Data analysis and phenotyping of different transformed lines were performed under greenhouse conditions Traits related to vegetative growth such as chlorophyll content in upper, and lower leaves, shoot height, tuber soluble protein content, tuber weight and *NRT1.11* gene expression in tubers are presented.

Two different time points were assessed for the phenotyping (half-way harvest and final harvest). Selected traits were analyzed separately for each time point, using Principal Component Analysis. With PCA assay, correlations between chlorophyll content in upper (UChlorophyll), and lower leaves (LChlorophyll), *NRT1.11* gene expression in tubers (NRT1.11), shoot height (HW.height) and soluble protein content in tuber (HW.SPC) were established at half-way harvest. On the other hand, at final harvest, correlations between tuber weight (F.Tub.Weight), soluble protein content in tubers (F.SPC) and shoot height (F.height) were established with the PCA assay. Results from both PCAs are presented separately.

Kaiser-Meyer-Olkin (KMO) test of the half-way recollected data shows a sample adequacy of 0.345 (Figure 4), KMO test values under 0.5 shows a deficiency in the number of samples of least one trait included in the PCA assay. Correlation matrix table (Figure 4) shows the correlations between traits during the half-way harvest. There is a significant correlation between UChlorophyll with the HW.SPC, and HW.height. In addition, the correlation matrix table shows that HW.SPC displays a stronger correlation with the LChlorophyll than with UChorophyll. Both chlorophyll contents (UChlorophyll and LChlorophyll) show no significant correlation between them. Meanwhile, *NRT1.11* gene expression in tubers shows no significant correlation with any other trait measured during the half-way harvest PCA.

KMO and Bartlett's Test

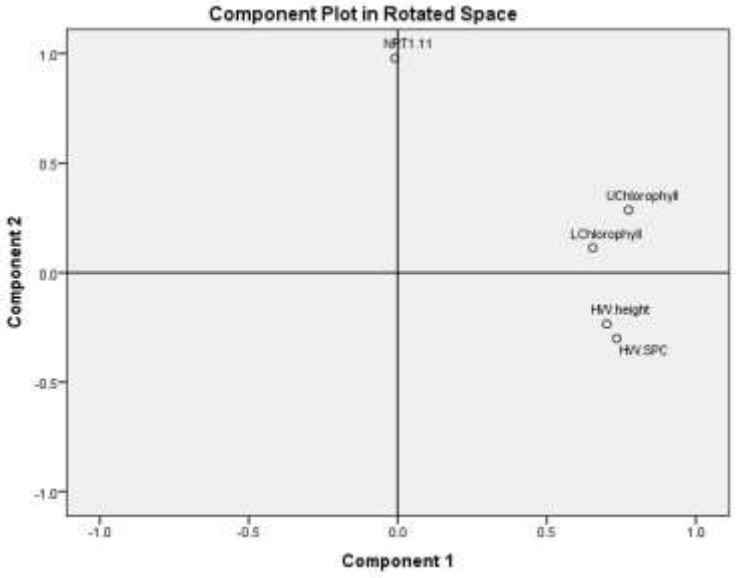
Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		.345
Bartlett's Test of Sphericity	Approx. Chi-Square	26.577
	df	10
	Sig.	.003

Correlation Matrix

		UChlorophyll	LChlorophyll	NRT1.11	HW.height	HW.SPC
Sig. (1-tailed)	UChlorophyll		.171	.171	.003	.032
	LChlorophyll	.171		.314	.136	.007
	NRT1.11	.171	.314		.148	.127
	HW.height	.003	.136	.148		.107
	HW.SPC	.032	.007	.127	.107	

Figure 4. KMO and Bartlett´s test, and correlation matrix of traits at half-way harvest.

The component plot in rotated space (figure 5) gives a visual representation of how physiology- and development-related traits are correlated to each other. This plot of components shows that UChlorophyll, LChlorophyll, HW.height and the HW.SPC load highly and positively on the first component (Component 1). However, NRT1.11 shows a loading close to zero on the first component, but it loads highly on the second component (Component 2). Component one explains 41% of the correlation among traits whereas, only 21% of the correlations are described by component two.



Rotated Component Matrix[a]

	Component	
	1	2
UChlorophyll	.774	.286
LChlorophyll	.654	.113
NRT1.11	-.010	.979
HW.height	.701	-.235
HW.SPC	.734	-.300

Figure 5. Component plot in rotated space and rotated component matrix of traits evaluated at half-way harvest.

A second PCA was performed to evaluate the correlation between traits during final harvest. KMO test of this time period shows a sampling adequacy of 0.411, which is higher when compared to the half-way harvest. Correlation matrix (Figure 6) shows a significant correlation between F.Tub.Weight and F.SPC in tubers during at final harvest. At this time point, there is a significant correlation between the F.SPC in tubers and F.height. A visual representation of these correlations on a component plot in rotated space is not possible since all traits are described and loaded in only one component.

KMO and Bartlett's Test

Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		.411
Bartlett's Test of Sphericity	Approx. Chi-Square	22.170
	df	3
	Sig.	.000

Correlation Matrix

		F.Tub.Weight	F.SPC	F.height
Sig. (1-tailed)	F.Tub.Weight		.004	.485
	F.SPC	.004		.000
	F.height	.485	.000	

Figure 6. KMO and Bartlett´s test, and correlation matrix of traits at final harvest.

After the PCA, the above-mentioned traits were compared between WT potato plants and each individual transformed line. Based on significant differences between the WT and the transformed lines, correlations between traits can be established. Traits are considered separately by batch and harvesting time point. For half-way harvest, significant differences in total shoot height (Appendix 9.5.8 and 9.5.10), SPC in tubers (Appendix 9.5.2 and 9.5.4), SPAD chlorophyll content in upper leaves (Appendix 9.5.6 and 9.5.7), and *NRT1.11* gene expression in tubers (Appendix 9.5.12) were established.

Remarkably, values of the soluble protein content at half-way harvest come from a pooled value of 3-4 tubers (Appendix 9.5.2 and 9.5.4) with no standard deviation. Thus, correlations between traits and the SPC at half-way harvest can be only established in a tendency way.

For example, when looking at transformed lines 32, 47 and 52 from Batch 1 a negative tendency between *NRT1.11* gene expression in tuber and the SPC in tuber is observed (Figure 7). Moreover, we can observe that transforms 32 and 52 show a positive correlation between *NRT1.11* gene expressions in tubers and shoot height at half-way harvest (Figure 8).

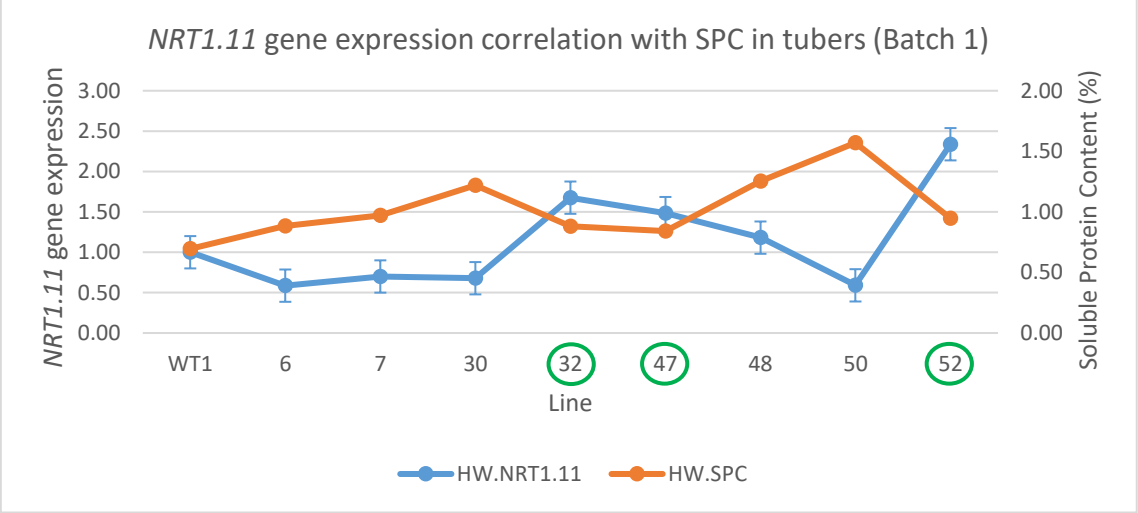


Figure 7. Batch 1 – *NRT1.11* gene expression in tubers comparison with SPC in tubers at half-way harvest. Gene expression values are displayed with a power of two. Green circle = *NRT1.11* gene expression significant different to WT1.

In Fig. 9, a comparison between chlorophyll content in upper leaves, shoot height and SPC in tubers at half-way harvest is illustrated. We observed a positive correlation between chlorophyll content and shoot height. Almost all lines showing incremented chlorophyll content in upper leaves shows higher shoot height. Furthermore, half-way SPC in tubers tends to follow the pattern of shoot height and chlorophyll content in upper leaves. At half-way harvest, transformed lines 48, 50 and 52 show higher SPC in tubers, higher shoot height and higher chlorophyll content in uppers leaves when compared to the WT1.

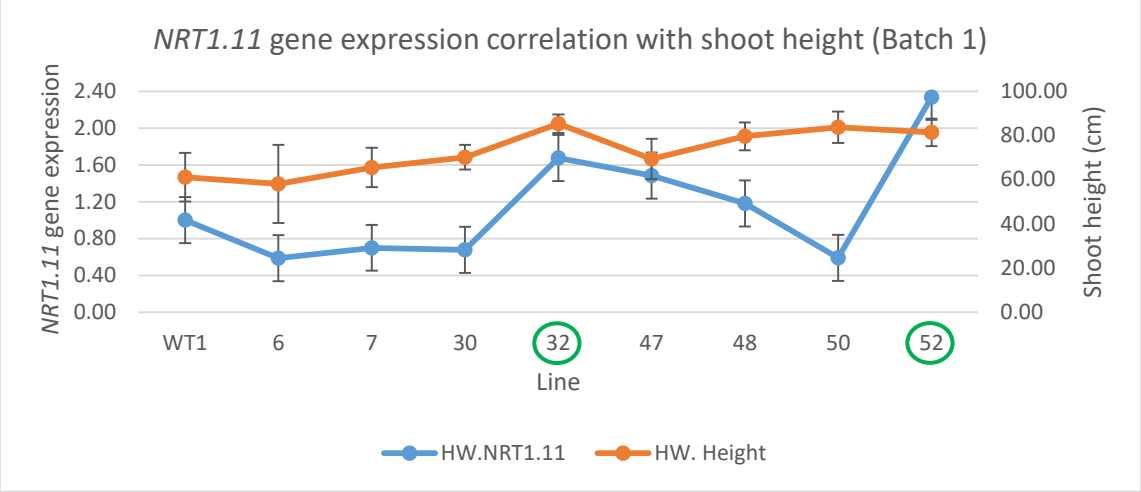


Figure 8. Batch 1 - *NRT1.11* gene expression in tubers compared to shoot height at half-way harvest. Gene expression values are displayed with a power of two. Green circle = both traits significant different to WT1.

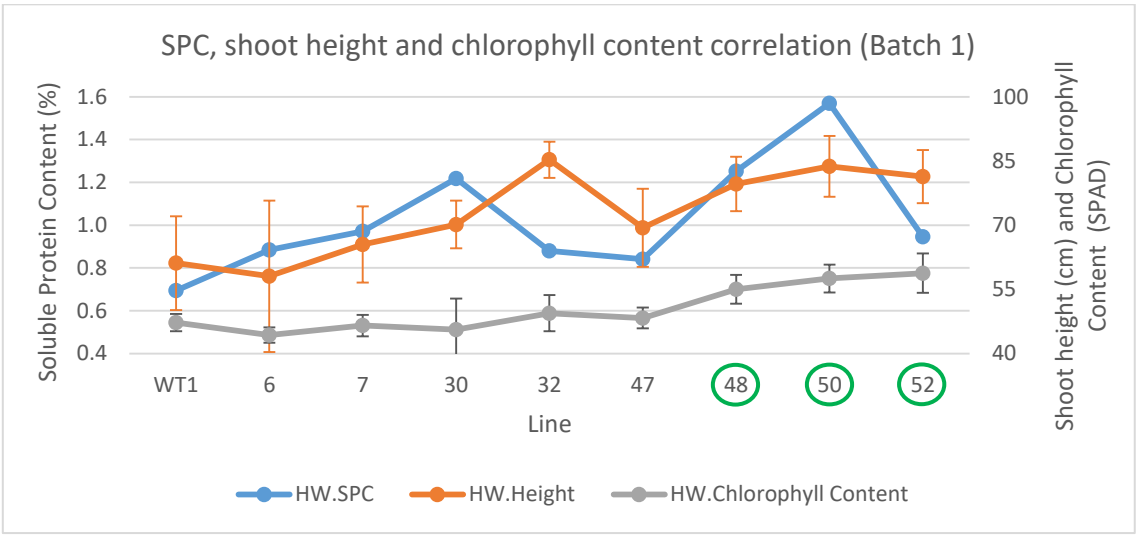


Figure 9. Batch 1 – Three-way comparison between SPAD chlorophyll content in upper leaves, shoot height and SPC in tubers at half-way harvest. Green circle = Shoot height and chlorophyll content significant different to WT1.

It is important to mention that transformed line 52 significantly differed from the WT1 in all traits investigated. Nevertheless, this line showed developmental phenotype with a curved, thin stem.

From Batch two, different results were obtained in comparison to Batch one. Only in Batch two transformed lines showed a significantly lower *NRT1.11* gene expression in tubers according to the RT-PCR analysis (Appendix 9.5.12). Transformed lines from Batch one with a significant *NRT1.11* overexpression in tubers correlate with an increased shoot height. Nevertheless, Batch two transformed lines 8 and 25 show the opposite pattern compared to Batch 1 (lower gene expression correlates with higher shoot height) (Figure 10). Transformant 49 showed a high expression value in the RT-PCR. However, no correlation can be inferred since the shoot height does not significantly differ from the WT (Figure 10). It should be mentioned, that the standard deviation in shoot height from transform 49 is almost three times bigger than the other transformants and WT2 (Appendix 9.5.10).

Batch two transformed lines 8, 25 and 49 show a significant higher shoot height and SPAD chlorophyll content in upper leaves at half-way harvest (Figure 11). Thus, a positive correlation between these two

traits can be inferred. This correlation between shoot height and SPAD chlorophyll content in upper leaves follows the same pattern as in Batch one (Figure 9). Also in transformants 8 and 49 plus transformant 52 a positive correlation between SPC in tubers and the chlorophyll content in upper leaves is observed (Figure 11). At half-way harvest, Batch one and two transformants tend to follow the same positive pattern in SPC in tubers, shoot height and SPAD chlorophyll content in upper leaves (Figures 9 and 11).

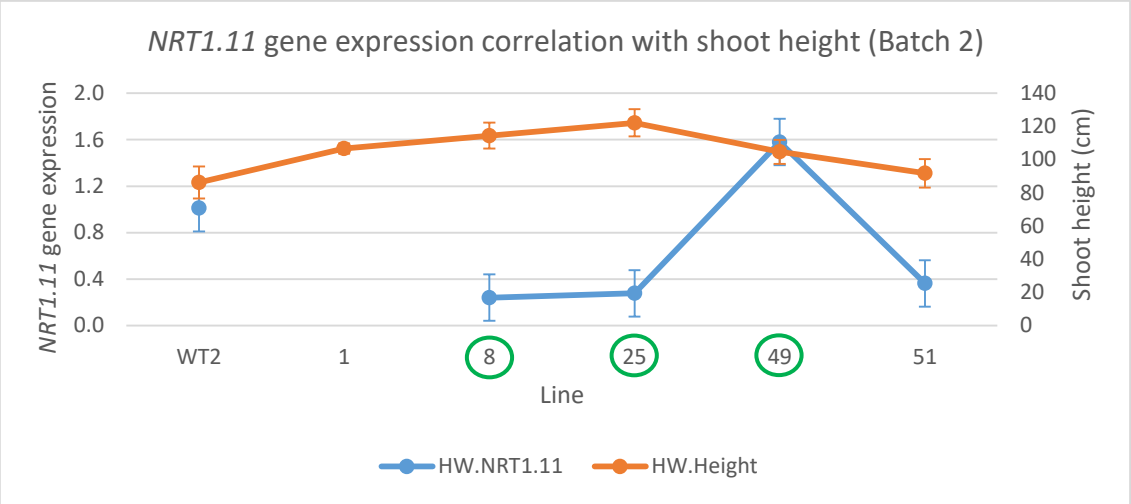


Figure 10. Batch 2 - *NRT1.11* gene expression in tubers comparison against shoot height at half-way harvest. Green circle = both traits are significantly different to WT2.

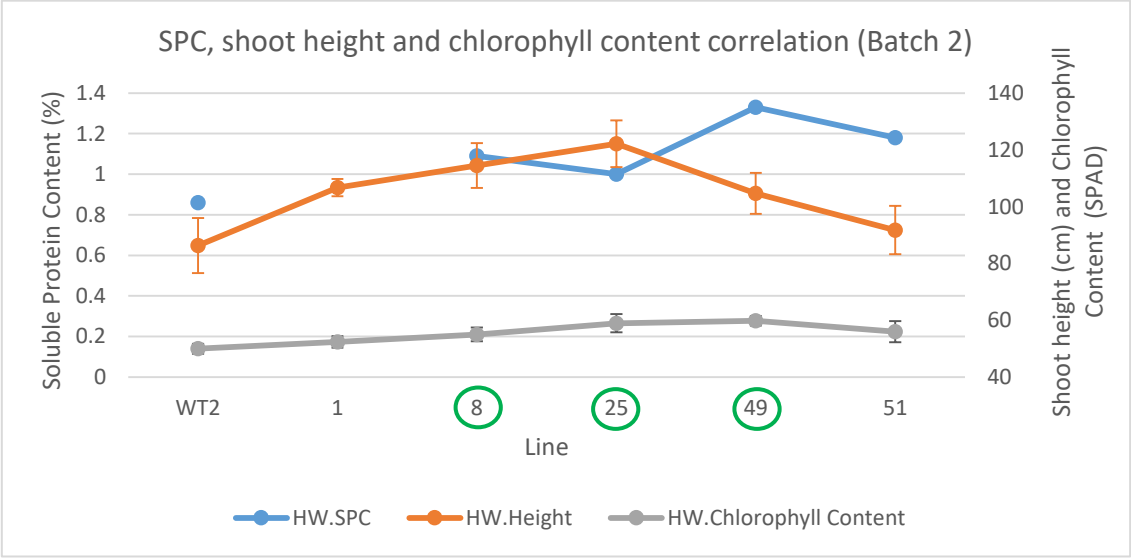


Figure 11. Batch 2 - Three-way comparison between SPAD chlorophyll content in upper leaves, shoot height and SPC in tubers at half-way harvest. Green circle = Shoot height and chlorophyll content significant different to WT1.

For the statistical analysis at final harvest only shoot height (Appendix 9.5.9 and 9.5.11), tuber weight (Appendix 9.5.13 and 9.5.14) and SPC in tubers (Appendix 9.5.3 and 9.5.5) were investigated. Tubers from the final harvest were sampled individually for the SPC measurement. Subsequently, statistical differences can be evaluated.

Interestingly, in Batch one, a negative trend between SPC in tubers and the final shoot height can be visualized (Figure 12). SPC correlation with shoot height differs between harvesting time points. At half-way harvest, transformed lines with higher shoot correlated with higher SPC in tubers, whereas at final harvest, shoot height correlate with a lower SPC in tubers. Statistical analysis in tuber weight shows

a lower significant difference of transformed lines 30 and 52 when compared to the WT2, whereas there was no transformed line showing a higher significant difference (Figure 13). Batch two transformed lines 1, 8 and 25 shows significant higher shoot height (Figure 14). Transformants from Batch 2 shows no significant difference in tuber weight compared to WT2 (Appendix 9.5.14).

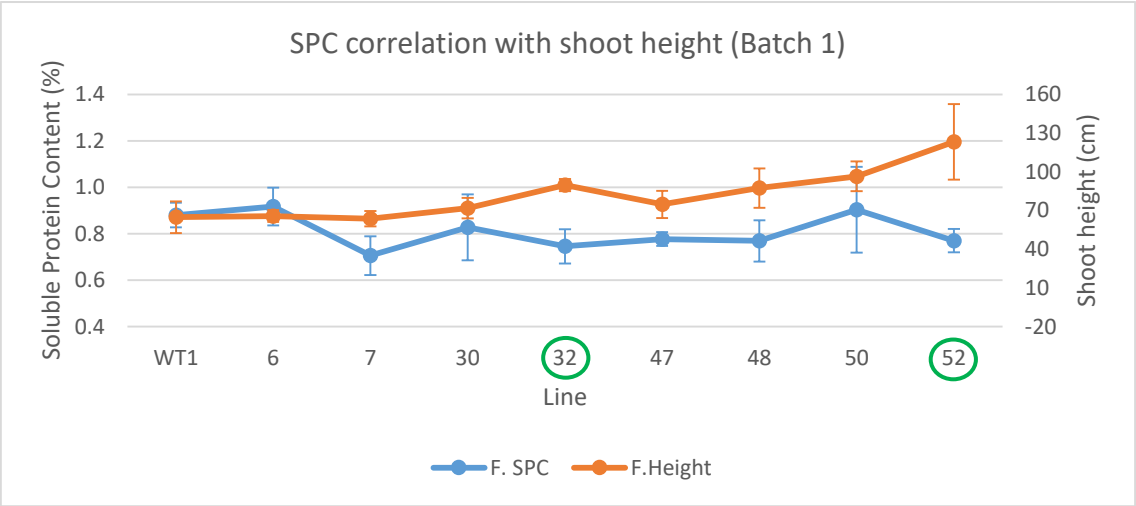


Figure 12. Batch 1 - SPC in tubers compared to shoot height at final harvest. Green circle = both traits are significantly different to WT2.

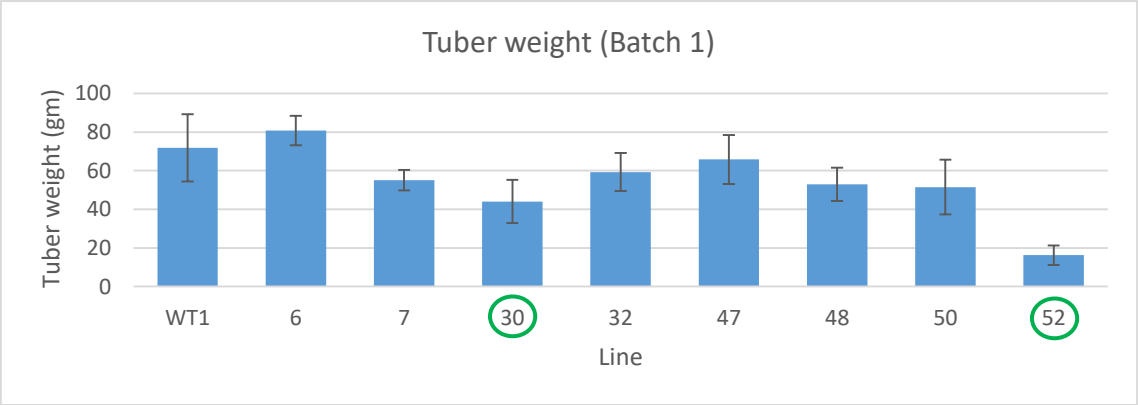


Figure 13. Batch 1 – Tuber weight at final harvest. Green circle = tuber weight is significantly different to WT2.

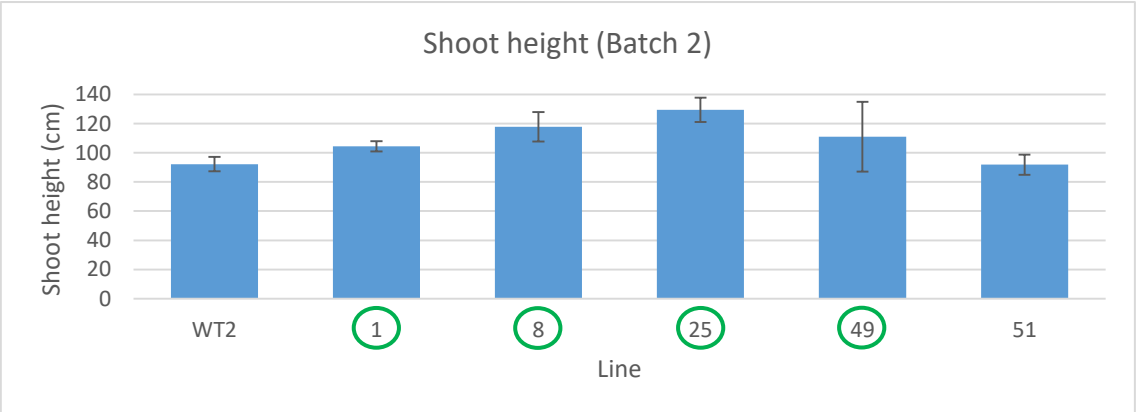


Figure 14. Batch 2 – Shoot height at final harvest. Green circle = tuber weight is significantly different to WT2.

SPC at half-way and final harvest

In Batch one, half-way harvest SPC analysis shows that transformants tend to possess a higher protein content than WT1 (Figure 15). However, over time SPC in the WT1 tubers continues to increase, whereas transformed tubers exhibit a decrease in SPC between half-way and final harvest. Finally, at final harvest, the majority of the transformed lines (7, 30, 48, 50, and 52) show a lower tuber SPC compared to the wild-type tubers (Figure 15).

Batch two transformants and WT2 were also analyzed at two different harvest times. Potato tuber SPC results from Batch 2 are shown in Figure 16. Transformed line 1 contained only 4 developed clones, all clones were only used for the final harvest SPC assessment. Transformed lines in Batch 2, display a more stable SPC between half-way and final harvests, no significant difference in SPC in tubers between harvest times was found. On the other hand, only in WT2, a significant increase in SPC was observed. WT2 increasing behavior, in terms of SPC, correlates with WT1. In the end, during the final harvest, there was no significant difference in SPC between transformed lines and the WT2 (Figure 16).

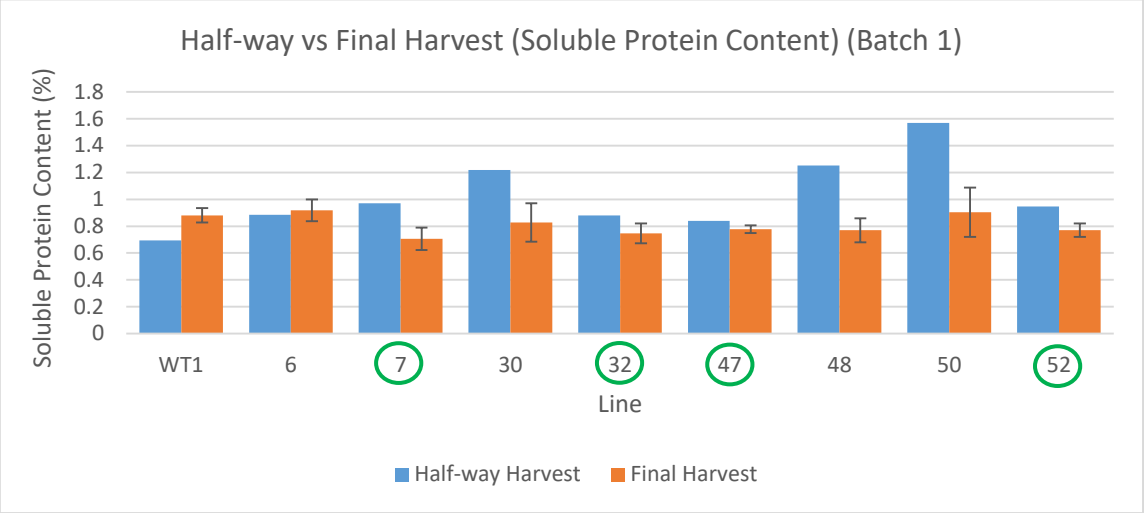


Figure 15. Batch 1 SPC results from WT1 and transformed lines in half-way (blue) and final (orange) harvest. Transformed lines circled in green present a significant different SPC in the final harvest when compared to the WT1.

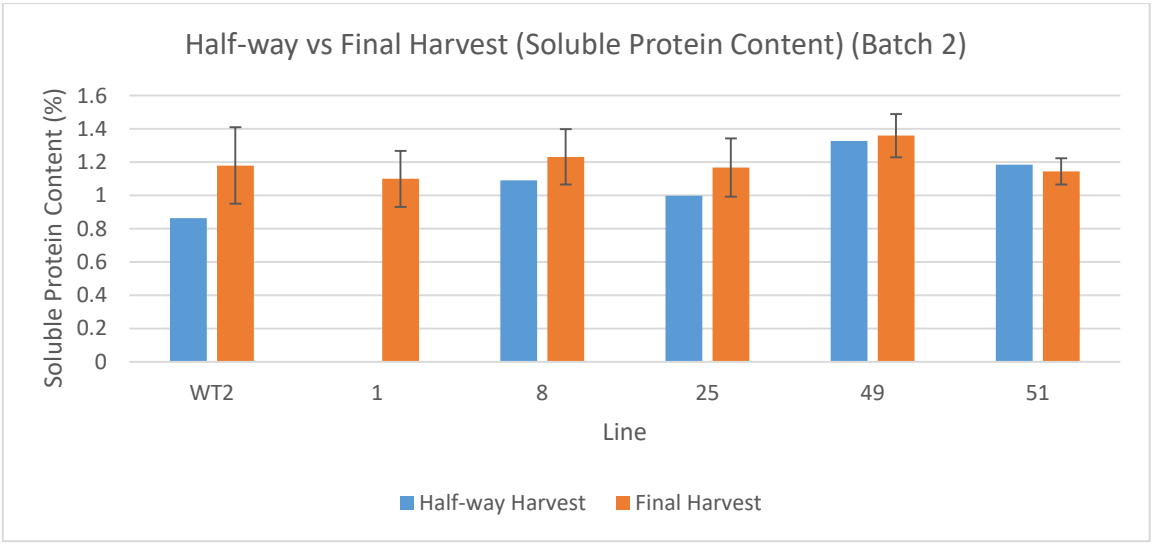


Figure 16. Batch 2 SPC results from WT2 and transformed lines in half-way (blue) and final (orange) harvest.

Finally, it is important to mention that WT2 at final harvest shows almost 33% higher SPC in tubers (Figures 15 and 16) and nearly 40% higher shoots (Figures 12 and 14) than WT1.

5 Discussion

SPC in transgenic tubers decreases during harvest periods meanwhile, in WT increases.

Nitrogen forms are transported and redistributed by vascular tissues to developing organs depending on the growth stage. During vegetative growth organic and inorganic N is transported from the root tissue or older leaves to younger upper leaves and to reproductive organs during flowering (Diaz *et al*., 2008 and Fan *et al*., 2009)). According to a study of Masclaux-Daubresse´s research group (2010), depending on plant species, roots-mediated N uptake may be partially or completely compromised during the reproductive stage. N redistribution from source to sink is essential to overcome the high N demand of reproductive organs. N uptake inhibition causes N deficiency and the senescence of older leaves. Leaf protein proteolysis releases amino acids that can be reallocated to sink tissues via phloem vessels (Fan *et al*., 2009). Keeping this knowledge in mind and considering that NRT 1.11 facilitates the reallocation of inorganic and organic N from older leaves (source) to the youngest tissue (sink) via xylem-phloem, we can raise the following hypothesis. Transformed plants overexpressing the *NRT1.11* present enhanced N transport in form of amino acids to tubers at the initial stage of tuberization. However, since the N uptake is at least partially inhibited (Masclaux-Daubresse *et al*., 2010), available N in the plant becomes limited. Transformants will reallocate this limited N from the leaves to the tubers faster and at halfway harvest a higher protein content will be observed in the tubers, in comparison to the WT. In this situation, without arresting growth and development of the tubers, transformed plants will exhaust the available N and they will show a decrease in the percentage of the SPC in the tubers at final harvest. In the WT, N in form of amino acids will be translocated to the tubers in similar amounts but at a lower flow rate when compared to the overexpressing lines, until the N available from mature leaves is completely exhausted. This assumption might explain why at half-way harvest the obtained transformants show a higher SPC (%) against the WT. However, at final harvest, transformants will show a decreased SPC (%) in comparison to WT, because tuber growth will continue in terms of sugars and water accumulation but not in protein content.

A positive correlation between *NRT1.11* expression, SPC, shoot height and chlorophyll content at reproductive stage.

Nitrogen amounts have a direct significant effect on the growth and photosynthesis of plants (Zhou *et al*., 2011). N content induces leave and shoots growth by increasing the leaf area influencing directly the photosynthesis (Pate, 1973). Evans, 1983 concludes that the chlorophyll content is approximately proportional to leaf N content. Thus, higher shoots will present higher chlorophyll content. Obtained results are in line with our expectations. Transformant lines presenting an increased shoot growth exhibit also a higher chlorophyll content in the upper leaves. Later on, *NRT1.11* gene expression in transformants is expected to be constant over all the different plant tissues since a 35S promoter (Seternes *et al*., 2016) was utilized in the construct for *Agrobacterium* transformation. Therefore, we could expect that RT-PCR measurements in different tissues show a similar level of expression in each plant, for example, leaves. At halfway harvest (reproductive stage), RT-PCR analysis assay, shoot height measurements, SPC in tubers and chlorophyll content showed that a significant increase and decrease in *NRT1.11* expression in tubers correlates with plants having a higher shoot height, higher SPC and higher chlorophyll content in upper leaves. We were expecting that high gene expression in tubers will correlate with higher shoots, SPC in tubers and chlorophyll content in upper leaves. However, results show that either a higher or lower expression of the *NRT1.11* in tubers produce transgenic plants with higher shoots, SPC in tubers and chlorophyll content in upper leaves. We believe that alteration in *NRT1.11* expression somehow enhances the rate flow of translocation of nitrate from the source into sink tissues. Since very limited information about the *NRT1.11* is available, it is difficult to generate a viable hypothesis. These results do not exclude the possibility that additional NRTs or NRT coupled regulatory elements may be involved in N utilization. At the same time, in some transformant lines and in the WT used as a control, averages from samples present a high standard deviation between clones.

This is not sufficient to confirm that some lines are statistically different to the WT in some traits. Evaluation of *NRT1.11* gene expression in other plant tissues is needed to confirm that the *NRT1.11* gene expression values in tubers are comparable to those in other plant tissues.

SPC and shoot height on maturation stage.

At maturation stage (final harvest), transformants showing a low SPC in tubers present a higher shoot height. Based on obtained results, we can assume that N is principally translocated to upper leaves enhancing the chlorophyll content in leaves and increasing the leaf and shoot development meanwhile, the translocation of N to tubers decrease producing a lower SPC in tubers. The reason why these transformants prefer to translocate N forms to shoots over tubers as mentioned above could be addressed to the favorable conditions provided by the greenhouse which enhances the development of shoots over tubers. This is consistent with the findings of Tekalign and Hammes (2005) who showed that a favorable temperature promoted shoot growth and plant development in potato by decreasing the translocation of assimilated to the tubers. A comparison between the SPC in tubers and leaves in transformants would be of interest in order to comprehend better the N translocation process.

WT1 and WT2

In *S. tuberosum* L. traits as, dry weight, the number of tubers per plant, tuber yield and N accumulation in potato tubers are significantly affected by the growing season (M'hamdi *et al*., 2016). Environmental factors, mainly light intensity, significantly influence the nitrate content in plants resulting in a higher N accumulation in the plant with light superior quality (Anjana & Iqbal 2007). Transformed lines from Batch one and two present several differences. However, a comparison between transformants, from different batches, is not consistent since variations in phenotype can be related to the transformed genotype. Interestingly, WT1 and WT2 present a significant difference in the SPC in tubers and shoot height. WT2 present almost a 33% higher SPC in tubers and a 40% higher shoot height than WT1. These results contradict the expected since Batch two was placed in the greenhouse 3 weeks after Batch one. Therefore, Batch one benefited of a higher natural light quality was supposed to exhibit a higher SPC in tubers and shoot height. Two possible hypothesis might explain these differences between WT1 and WT2. The first one relay on the reasoning that artificial light used in the greenhouse possess a better quality than the natural light provided during autumn and winter seasons. Since Batch one was placed 3 weeks before, plants form this batch were irradiated with shorter periods of artificial light and longer periods of natural light. Meanwhile, Batch 2 was irradiated with shorter periods of natural light and longer periods of artificial light since natural daylight was decreasing as time pass. Thus, as second hypothesis, it might be also reasonable to assume that WT2 plants clones were in a further development stage were available N within the plant has been totally translocated to sink tissues promoting a higher SPC in tubers and shoot height. Meanwhile, in Batch one N translocation was still in process. The difference in development stages between batches might also explain why in Batch two transformed lines shows no significant difference at the final harvest on SPC in tubers. This raises the question if transformed lines, from Batch one, will still present a higher SPC than WT1 if having left longer time. Finally, we were expecting to find a significant difference in WT1 and WT2 tuber yield. Results show no significant differences between wild-types. However, wild-type yield from both batches shows a high standard deviation between samples. An increase in the number of samples will provide us more reliable results.

***NRT1.11* gene expression and final SPC in tubers**

At mature stage, transformant lines under-/ overexpressing the *NRT1.11* gene in tubers shows an equal/lower SPC in tubers and tuber yield. Consequently, obtained results show that an overexpression of the *NRT1.11* gene will cause no increment in the final SPC in tubers.

6 Conclusion

My thesis aimed at studying the involvement of candidate genes in N utilization both in form of inorganic and organic N in potato tubers and to establish a possible correlation between the expression patterns of the candidate genes and the SPC of tubers. Two main tasks were intended: 1) clone *AMT*, *LHT* and *ETR* genes and introduce them, into potato plants via *Agrobacterium tumefaciens* transient assays (ATTA); 2) phenotype and analyze transformed potato plants overexpressing the *NTR1.11* gene in comparison to WT potatoes. AMT gene was fully amplified, however insertion of the AMT gene sequence into the pENTR™ TOPO® vector was unsuccessful. LHT and ETR were not amplified from cDNA. Transformed potato plants overexpressing *NRT1.11* gene were phenotyped and analyzed. PCA results showed correlations between chlorophyll content in upper leaves, shoot height, SPC. *NTR1.11* expression levels in tubers show no direct correlation with other traits. Obtained results showed that *NRT1.11* expression levels accelerate the N translocation and accumulation in potato tubers at reproductive stage. At the maturation stage, transformed lines show lower or equal amounts of SPC in tubers. However, more information is needed to fully understand the influence of *NRT1.11* gene on N utilization in potato plants. Preparing a cDNA library and cloning genes form these cDNA can be laborious and time-consuming, therefore appropriate planning and good practices in the laboratory are needed.

7 Recommendations

After six months of work in this thesis project, some recommendations can be suggested for improving the results quality. During the development of transgenic lines *AMT*, *LHT* and *ETR* potato genes presented difficulties in the amplification and cloning process. An alternative feasible way to study the effect of gene overexpression might be the use of *Arabidopsis* homologs genes. This alternative approach might speed the cloning task. For future transgenic lines is suggested to place the plant material in the greenhouse following a random block design to reduce the environmental interactions among phenotyping results. An increase in the number of clones per transformed line will enhance the statistical power of the analysis. Moreover, is suggested to weight and measure the diameter and sugar content of the tubers before the SPC analysis. Obtained measurements might provide valuable information about the interaction between these traits. It is highly recommended to evaluate the SPC in tubers individually per clone plant for obtaining a standard deviation per transformed line and consequently, use this data in a statistical analysis. Furthermore, evaluation of the *NRT1.11* gene expression in leaves and roots at half-way and final harvest might provide information to understand and elucidate the role of this gene in potato plants. Finally, through the project, it is recommended the use of the same set of pipettes, PCR and RT-PCR machines for decreasing the error in laboratory practices.

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9 Appendix

9.1 DNA extraction and cDNA synthesis

9.1.1 Plant DNA extraction

1. Place the sample on ice immediately after detached. A sample of 0.5 cm2 of young leaf is usually enough.
2. Place the sample in plastic tubes and add 20 to 60 µl 0.5 M NaOH depending on the leaf area and add one bullet per tube.
3. Place the samples for 1 min in tissue shaker.
4. If needed centrifuge the samples at 3000 rpm for a 1- 2 min.
5. Place the samples on ice and add 20 µl 100nM Tris pH 7.4
6. After a couple of seconds carefully take 5 µl of the supernatant and collocate it in 100 µl 100mM Tris pH 7.4
7. Extraction samples can be stored in freezer at -20°C.

9.1.2 RNA isolation protocol and Cetyltrimethyl Ammonium Bromide (CTAB) buffer preparation

1. Place plant material stored at -70 °C into a mortar with liquid nitrogen, start grinding the tissue until the nitrogen has evaporated and then continue grinding until a fine powder remains.
2. With a cooled spatula transfer carefully the powder into a 2 mL pre-cooled Eppendorf tube and then add 800 µl of pre-warmed 2% CTAB buffer, vortex for 30 sec and incubate for 15-30 min at 60°C and shake every 5 min.
3. Add 800 µl of chloroform and vortex for 30 sec.
4. Spin in the centrifugation at 12000 rpm and 4°C for 5min.
5. Extract 700 µl of supernatant with an equal volume if chloroform (700 µl) in a new 2.0 mL tube and vortex for 30 seconds.
6. Spin in the centrifugation at 12000 rpm and 4°C for 5min.
7. Carefully transfer the top phase (600 µl) to a new 1.5 mL tube and add 0.8 of the total volume of isopropanol and vortex for 30 seconds.
8. Spin in the centrifugation at 12000 rpm and 4°C for 5min.
9. With extremely carefully pour of supernatant since the pellet will be a little loose and add 500 µl of 80% EtOH.
10. Spin in the centrifugation at 12000 rpm and 4°C for 5min. Dry pellet using a pipette and resuspend in 100 µl of Milli-Q.
11. Add 1/3 volume (33.3 µl) of 8 M LiCl and incubate overnight at 4°C.
12. Spin for 30 min at 14000 rpm at 4°C.
13. Using 80% EtOH carefully wash pellet and spin for 5 min under the same conditions.
14. Dry the pellet briefly, in case of needed spin for another 5 min.
15. Dissolve pellet in 25 µl Milli-Q

Notes: Always use RNA free pipettes and Eppendorf tubes. For steps 3-9 is recommend to do it in a cabinet flow.

9.1.3 Thermo Scientific DNase I, RNase-free

1. 1 µl 10x DNase I reaction buffer
2. 1 µl DNase I
3. 1 µg of RNA (adapt the volume to fulfill 1 µg of RNA)

4. Add Milli-Q water for a 10 µl final volume
5. Mix the reaction gently and incubate for 15 min at room temperature (20 -22 °C)
6. Add 1 µl of EDTA (25 Mm)
7. Mix the reaction gently and incubate for 10 min at 65 °C

9.1.4 BIO-RAD iScript™ cDNA Synthesis Kit

1. 11 µl RNA (DNase treated)
2. 4 µl 5x iScript reaction buffer
3. 1 µl iScript reverse transcriptase
4. 4 µl RNase free water
5. Mix the reaction gently and run a PCR (5 min at 25 °C; 30 min at 42 °C, 5 min at 85 °C, 5 min at 4 °C, 5 min at 85 °C; hold at 10 °C)

9.2 Plasmid DNA isolation and DNA purification

9.2.1 Zymoclean ™ Gel DNA Recovery Kit Protocol

All centrifugation steps should be performed between 10,000 - 16,000 x g.

1. Excise the DNA fragment from the agarose gel using a razor blade, scalpel or other device and transfer into 1.5 mL microcentrifuge tube.
2. Add 3 volumes of **ADB** to each volume of agarose excised from the gel.
3. Incubate at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved.
4. Transfer the melted agarose solution to a **Zymo-Spin ™ Column** in a **Collection Tube**.
5. Centrifuge for 30-60 seconds. Discard the flow-through.
6. Add 200 µl of **DNA Wash Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.
7. Add 6 µl **DNA Elution Buffer** or water directly to the column matrix. Place column into 1.5 mL tube and centrifuge for 30-60 seconds to elute DNA.

9.2.2 QIAGEN QIAprep® Spin Miniprep Kit

1. Pellet 1–5 mL bacterial overnight culture by centrifugation at >8000 rpm (6800 x g) for 3 min at room temperature (15–25°C).
2. Resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube.
3. Add 250 µl Buffer P2 and mix thoroughly by inverting the tube 4–6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times. If using LyseBlue reagent, the solution will turn colorless.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
6. Apply the supernatant from step 5 to the QIAprep spin column by decanting or pipetting. Centrifuge for 30–60 s and discard the flow-through, or z apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum source.
7. Recommended: Wash the QIAprep spin column by adding 0.5 mL Buffer PB. S Centrifuge for 30–60 s and discard the flow-through.
8. Wash the QIAprep spin column by adding 0.75 mL Buffer PE. Centrifuge for 30–60 s and discard the flow-through. Transfer the QIAprep spin column to the collection tube.
9. Centrifuge for 1 min to remove residual wash buffer.

10. Place the QIAprep column in a clean 1.5 mL microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the QIAprep spin column, let stand for 1 min, and centrifuge for 1 min.

9.3 pENTR™ TOPO® cloning protocol

Pablo Baldeon cloning protocol modification.

Use the following procedure to perform the TOPO® Cloning reaction. Reminder: For optimal results, be sure to use a 0.5:1–2:1 molar ratio of PCR product: TOPO® vector in your TOPO® Cloning reaction.

Note: The blue colour of the TOPO® vector solution is normal and is used to visualize the solution.

Reagents*	**Chemically Competent *E. coli***		
Fresh PCR product	0.5 µl	0.5 µl	1.5 µl
Salt Solution	1.0 µl	0.5 µl	0.5 µl
Dilute Salt Solution (1:4)	-	-	-
Sterile Water	3.5 µl	1.5 µl	0.5 µl
TOPO® vector	1.0 µl	0.5 µl	0.5 µl
Final volume	6.0 µl	3.0 µl	3.0 µl

*Store all reagents at –20°C when finished. Salt solution and water can be stored at room temperature or 4°C.

1. Mix the reaction gently and incubate for 5 minutes at room temperature (22–23°C).

Note: For most applications, 5 minutes will yield a sufficient number of colonies for analysis. Depending on your needs, the length of the TOPO®. Cloning reaction can be varied from 30 seconds to 30 minutes. For routine subcloning of PCR products, 30 seconds may be sufficient. For large PCR products (> 1 kb) or if you are TOPO® Cloning a pool of PCR products, increasing the reaction time may yield more colonies.

2. Place the reaction on ice and proceed to Transforming One Shot® Competent *E. coli*.

Note: You may store the TOPO® Cloning reaction at –20°C overnight.

Transform One Shot® Competent *E. coli*

After performing the TOPO® Cloning reaction, you will transform your pENTR™ TOPO® construct into competent *E. coli*. One Shot® TOP10 or Mach1™-T1R. Chemically Competent E. coli are included with the kit to facilitate transformation,

Required materials

Components required but not supplied:

- TOPO® Cloning reaction
- LB plates containing 50 µg/mL kanamycin (2 for each transformation)
- 37°C shaking and non-shaking incubator
- general microbiological supplies (i.e. plates, spreaders)
- 42°C water bath (or electroporator with cuvettes, optional)

Components supplied with the kit:

- One Shot® TOP10 or Mach1™-T1R chemically competent *E. coli*

- S. O.C. Medium

There is no blue-white screening for the presence of inserts. Most transformants will contain recombinant plasmids with the PCR product of interest cloned in the correct orientation. Sequencing primers are included in the kit to sequence across an insert in the multiple cloning site to confirm orientation and reading frame.

Prepare for transformation

For each transformation, you will need 1 vial of One Shot® competent cells and 2 selective plates.

- Equilibrate a water bath to 42°C or set up your
- Warm the vial of S.O.C. Medium to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® cells for each transformation.

One Shot® chemical transformation protocol

Use the following protocol to transform One Shot® TOP10 or Mach1™-T1R chemically competent *E. coli.*

1. Add 2 µl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, into a vial of One Shot® Chemically Competent *E. coli* and mix gently. Do not mix by pipetting up and down.
2. Incubate on ice for 15–30 minutes.

Note: Longer incubations on ice seem to have a minimal effect on transformation efficiency. The length of the incubation is at the user's discretion.

3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250 µl of room temperature S.O.C. Medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 50–200 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. We recommend that you plate 2 different volumes to ensure that at least 1 plate will have well-spaced colonies.
8. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5–10 colonies for analysis.

9.4 RT–PCR protocol

Reagents*	Volume
cDNA	2.0 µl
Buffer	5.0 µl
Forward Primer	0.3 µl
Reverse Primer	0.3 µl
Sterile Water	2.4 µl
Total	10.0 µl

Cycling Conditions

1. 94°C for 15 min
2. 94°C for 20 seconds
3. 61°C for 60 seconds
4. (Repeat steps 2 and 3 for a total of 10 cycles. Decrement -0.6°C / per cycle)
5. 94°C for 20 seconds
6. 55°C for 60 seconds
7. (Repeat steps 5-6 for a total of 26 cycles)
8. Add read step at 37°C for 1 min.

9.5 Phenotyping of traits and statistical analysis of data

9.5.1 Batch 1 and 2 clones per line

Batch 1	Line	WT1	6	7	30	32	47	48	50	52
	Clones	8	7	8	7	7	8	8	8	8
Batch 2	Line	Wt2	1	8	25	49	51			
	Clones	7	3	6	7	6	8			

9.5.2 Soluble Protein Content (SPC) in tubers at half-way harvest (Batch 1)

	Half-way Harvest	
Line	No. pooled tubers used (> 2cm)	*Soluble protein content (%)*
WT1	4	0.69
6	3	0.88
7	3	0.97
30	3	1.22
32	3	0.88
47	3	0.84
48	4	1.25
50	2	1.57
52	1	0.95

9.5.3 Soluble Protein Content (SPC) in tubers at final harvest (Batch 1)

		End Harvest				
Line	*Replicate*	*Soluble protein content*				
		SPC (%)	*St. Dev*	*Average*	*p.value*	*Sig. Diff*
WT1	1	0.92	0.05	0.88	1.00	No
	2	0.81				
	3	0.93				
	4	0.86				
6	1	0.91	0.08	0.92	0.48	No
	2	0.87				
	3	1.03				
	4	0.86				
7	1	0.71	0.08	0.71	0.02	Yes
	2	0.63				
	3	0.82				
	4	0.66				

30	1	0.62	0.14	0.83	0.52	No
	2	0.90				
	3	0.94				
	4	0.85				
32	1	0.75	0.07	0.75	0.03	Yes
	2	0.73				
	3	0.84				
	4	0.66				
47	1	0.79	0.03	0.78	0.02	Yes
	2	0.74				
	3	0.78				
	4	0.81				
48	1	0.68	0.09	0.77	0.09	No
	2	0.71				
	3	0.87				
	4	0.81				
50	1	0.66	0.18	0.90	0.82	No
	2	0.95				
	3	0.89				
	4	1.11				
52	1	0.76	0.05	0.77	0.02	Yes
	2	0.76				
	3	0.72				
	4	0.84				

9.5.4 Soluble Protein Content (SPC) (%) in tubers at half-way harvest (Batch 2)

	Half-way Harvest	
Line	*No. pooled tubers used (> 2cm)*	*SPC (%)*
WT2	3.00	0.86
1	-	-
8	2.00	1.09
25	3.00	1.00
49	2.00	1.33
51	3.00	1.18

9.5.5 Soluble Protein Content (SPC) in tubers at final harvest (Batch 2)

		Final Harvest				
Line	*Replicate*	*Soluble protein content*				
		SPC (%)	*St. Dev.*	*Average*	*p.value*	*Sig. Diff.*
WT2	1	1.05	0.23	1.18	1.00	No
	2	0.94				
	3	1.27				
	4	1.46				
1	1	1.27	0.17	1.10	0.62	No
	2	0.93				
	3	1.10				
	4	-				
8	1	1.25	0.17	1.23	0.73	No
	2	1.32				
	3	0.99				
	4	1.36				
25	1	1.43	0.18	1.17	0.94	No
	2	1.05				
	3	1.07				
	4	1.13				
49	1	1.49	0.13	1.36	0.24	No
	2	1.34				
	3	1.42				
	4	1.19				
51	1	1.20	0.08	1.14	0.79	No
	2	1.07				
	3	1.22				
	4	1.08				

9.5.6 Soil Plant Analysis Development (SPAD) Chlorophyll content in upper leaves at halfway harvest (Batch 1)

	Chlorophyll measurements with SPAD meter						Average		Pooled Average		Standard Deviation		p. value	
	Low Leave			High Leave			Low Leave	High Leave	Low Leave	High Leave	Low Leave	High Leave	Low Leave	High Leave
Line	1	2	3	1	2	3								
WT1	36.5	33.3	30.4	42.9	42.7	43.7	33.40	43.10	35.33	47.25	4.21	2.03	1.00	1.00
	39	38.7	38	48.3	46.5	47	38.57	47.27						
	32.4	34.2	34.3	44.3	48.5	44.4	33.63	45.73						
	36.3	35.5	35.6	49.4	47.5	49.1	35.80	48.67						
	43.2	43.5	42.7	50.7	48.1	49.3	43.13	49.37						
	30	28.5	29.8	46.5	46.9	47.7	29.43	47.03						
	38.3	34.7	36.4	47.7	49.3	48.8	36.47	48.60						
	32.1	32.1	32.3	48	48.3	48.4	32.17	48.23						

6	40	40.5	42.4	43	41.5	42.7	40.97	42.40	39.72	44.34	1.48	1.78	0.02	0.01
	39.3	40.4	42.4	45.6	44.3	44.4	40.70	44.77						
	41	40.4	42.2	41.8	42.4	43.9	41.20	42.70						
	37.7	40.1	39.9	48.7	48.1	46.7	39.23	47.83						
	41.2	42.2	40.4	44.7	45.4	42.5	41.27	44.20						
	38	39.5	37.9	44.4	44.5	43.3	38.47	44.07						
	37.8	39.2	38.1	44.1	45.1	44.1	38.37	44.43						
	37.9	38	36.8	-	-	-	37.57	-						
7	26.8	27.4	26.3	49.4	48.6	50	26.83	49.33	33.99	46.55	5.00	2.50	0.57	0.55
	33.6	34.2	33	48.3	50	49.8	33.60	49.37						
	38.4	38.7	38.3	44.4	46.7	46.3	38.47	45.80						
	35.1	34.7	32.4	49.6	47.4	48.1	34.07	48.37						
	34.1	39.7	39.6	43.1	43.2	42.7	37.80	43.00						
	29.1	28.8	26.7	43.1	43	44.8	28.20	43.63						
	31.2	31.5	32.7	47.8	48.2	47	31.80	47.67						
	40.4	42.6	40.4	44.3	46.3	45	41.13	45.20						
30	33.9	33	33.7	40	39.4	39.3	33.53	39.57	28.10	45.58	5.85	7.25	0.02	0.57
	24	23.4	23.6	54.1	57.2	54	23.67	55.10						
	33.1	33.6	32.8	37.4	36.6	38.4	33.17	37.47						
	27.2	27.2	28.1	41.8	40.8	40.7	27.50	41.10						
	27	26.5	26.9	47	47	47	26.80	47.00						
	34.5	32.9	33.9	44.8	42.8	42.8	33.77	43.47						
	16	19.2	19.7	57.3	53.5	55.3	18.30	55.37						
	-	-	-	-	-	-	-	-						
32	36.8	36.3	37.2	54.6	56.3	54.7	36.77	55.20	34.07	49.45	4.76	4.23	0.60	0.24
	34.7	35.2	35	43.4	42.6	45.2	34.97	43.73						
	41.8	41.4	44.4	46.3	45.4	48	42.53	46.57						
	31.8	31.8	30.8	47.9	46.7	48.5	31.47	47.70						
	26.6	29.1	29.1	50.4	47.7	48.7	28.27	48.93						
	33.8	34.5	34.8	54.2	56.7	53.9	34.37	54.93						
	31.4	29.6	29.3	49.3	46.8	51.1	30.10	49.07						
	-	-	-	-	-	-	-	-						
47	31.4	31.9	30.2	44.8	46.3	45.3	31.17	45.47	32.73	48.31	3.76	2.45	0.22	0.36
	34.2	36.5	35.1	48.2	46.7	47.6	35.27	47.50						
	33.9	34.3	32.6	50.5	51.7	52.4	33.60	51.53						
	31.1	30	30.1	48.2	49.8	50.8	30.40	49.60						
	40	40.8	39.1	48.3	51	47.8	39.97	49.03						
	31.8	32.4	33.3	45.8	46.3	43.2	32.50	45.10						
	31.5	31.4	32.4	44.5	46	50.5	31.77	47.00						
	29.7	24.3	27.6	51.8	49.8	52.1	27.20	51.23						
48	34.4	35.8	33.5	50.3	52.1	51.4	34.57	51.27	40.90	55.04	4.65	3.39	0.03	0.00
	44.1	46.4	44.7	53.4	55.9	55.9	45.07	55.07						
	39	42.1	42	54.1	54.6	54.2	41.03	54.30						
	39.7	39	38.1	52.7	53.7	53	38.93	53.13						
	41.1	39.1	40.6	55.2	57.2	54.8	40.27	55.73						
	37.2	38.6	38.7	55.2	55.7	53.1	38.17	54.67						
	48.6	50.1	50.8	64.3	61.3	62.5	49.83	62.70						
	39.1	39.4	39.5	52.6	55.3	52.4	39.33	53.43						
50	44.7	45.1	46.1	51.1	57.9	58.2	45.30	55.73	48.96	57.54	3.58	3.28	0.00	0.00
	47.1	44.8	48.6	58.7	56.9	57.6	46.83	57.73						
	44.1	45.3	45	54.8	58.2	55.6	44.80	56.20						
	50.1	54.4	50.5	62.5	62.8	65.2	51.67	63.50						
	51.1	51.7	51.2	57.3	58.5	57.1	51.33	57.63						
	53.4	55.6	50.6	55.3	51	54.8	53.20	53.70						
	49.5	50.4	58.2	54.7	56.6	53	52.70	54.77						
	47.1	45.1	45.3	61.6	59.4	62.1	45.83	61.03						

52	35.2	36.5	38.4	65.5	64.3	65.5	36.70	65.10	33.57	58.77	4.05	4.61	0.41	0.00
	33.9	34.4	32.2	61	60.6	63.6	33.50	61.73						
	29.4	33	33.3	53.7	53.8	55.9	31.90	54.47						
	25.1	25.6	26.8	58.6	59.5	59	25.83	59.03						
	36.5	37.4	33.8	66.2	63.4	62.3	35.90	63.97						
	37.5	36.6	38.7	57.6	59.3	58.5	37.60	58.47						
	30.1	29.9	31.1	52.6	52.5	52.6	30.37	52.57						
	35.7	37.4	37.2	54.2	54.4	55.9	36.77	54.83						

9.5.7 Soil Plant Analysis Development (SPAD) Chlorophyll content in upper leaves at half way harvest (Batch 2)

	Chlorophyll measurements with SPAD meter						Average		Pooled Average		Standard Deviation		p. value	
	Low Leave			High Leave			Low Leave	High Leave	Low Leave	High Leave	Low Leave	High Leave	Low Leave	High Leave
Line	1	2	3	1	2	3								
WT2	31	31	29.6	47.2	46.2	46.3	30.53	46.57	35.30	49.97	4.73	1.74	1.00	1.00
	38.8	38.4	38.1	49.1	49.5	49.2	38.43	49.27						
	42.4	40.3	43.4	49.8	48.5	50.7	42.03	49.67						
	39.1	38	38.5	51.3	50.2	51.7	38.53	51.07						
	29.3	28	30.3	51.9	52	51.5	29.20	51.80						
	36.6	36.5	34.7	51.6	49.3	49.9	35.93	50.27						
	32.4	32.8	32	51.7	51.7	50	32.40	51.13						
	-	-	-	-	-	-	-	-						
1	33.6	32.1	33.6	52.3	53	51.6	33.10	52.30	29.84	52.38	4.40	2.05	0.15	0.16
	24.8	25.1	24.6	49.9	50.7	50.5	24.83	50.37						
	30.7	32	32.1	55.7	55.4	52.3	31.60	54.47						
	-	-	-	-	-	-	-	-						
	-	-	-	-	-	-	-	-						
	-	-	-	-	-	-	-	-						
	-	-	-	-	-	-	-	-						
	-	-	-	-	-	-	-	-						
8	40.6	41.3	39.5	56.9	54.2	54	40.47	55.03	34.47	55.00	3.78	2.43	0.73	0.00
	37.8	38.5	37.2	55.6	56.7	57.1	37.83	56.47						
	31.2	31.4	32.4	53.4	55.4	54.1	31.67	54.30						
	31.1	32.2	32.3	59.3	59	58	31.87	58.77						
	33.4	32.7	34	51.8	51.6	51.5	33.37	51.63						
	31.5	31.7	31.6	52.8	54.8	53.8	31.60	53.80						
	-	-	-	-	-	-	-	-						
	-	-	-	-	-	-	-	-						
25	26.8	25.4	26.4	57.4	57	57.1	26.20	57.17	33.29	58.95	5.84	3.16	0.49	0.00
	32.1	32.8	32.4	56.2	57.4	57.6	32.43	57.07						
	34.1	32.8	34.4	57.7	57.3	56	33.77	57.00						
	28.5	28.8	28.1	61.1	58.2	58.6	28.47	59.30						
	32.4	33.4	33.1	59.1	59.1	58	32.97	58.73						
	35.1	35.5	63.3	66.2	65.3	66	44.63	65.83						
	35	34	34.6	57	58.4	57.3	34.53	57.57						
	-	-	-	-	-	-	-	-						
49	45.1	45.2	45.1	62.1	59.4	61.7	45.13	61.07	43.41	59.80	2.78	1.65	0.00	0.00
	44.2	47.2	45.6	62.2	61.8	62.8	45.67	62.27						
	38.7	37.3	38.4	57.7	56.7	58.5	38.13	57.63						
	44.3	43.7	46.1	59.6	58.7	59.6	44.70	59.30						
	43.6	41.7	42.8	58.4	58.2	59.9	42.70	58.83						
	44.1	44.1	44.1	59.1	60	60	44.10	59.70						
	-	-	-	-	-	-	-	-						
	-	-	-	-	-	-	-	-						

51	36.7	35.7	35.7	60.2	60.2	61.1	36.03	60.50	35.90	55.92	2.06	3.72	0.76	0.00
	34.1	32.3	33.4	56	56.7	55.2	33.27	55.97						
	32.8	33.2	33.5	56.9	59.6	56.7	33.17	57.73						
	35.7	36.1	36.7	55.2	55	54.4	36.17	54.87						
	35.2	34.2	35	54	53.9	53.8	34.80	53.90						
	39.4	39.4	37.7	52.6	53	49.7	38.83	51.77						
	37.5	36.5	37.1	61.7	62.9	59.3	37.03	61.30						
	38.6	37.1	38	52.4	51.3	50.3	37.90	51.33						

9.5.8 Plant shoot height (cm) at half-way harvest (Batch 1)

	Half-way Harvest					
Line	Plant	Height	St. Dev	Average	p. value	Sig. Dif.
WT1	1	46	10.99	61.13	1.00	No
	2	58				
	3	57				
	4	70				
	5	73				
	6	46				
	7	69				
	8	70				
6	1	68	17.70	58.06	0.69	No
	2	72				
	3	44.5				
	4	62				
	5	61				
	6	72				
	7	65				
	8	20				
7	1	65	8.93	65.50	0.40	No
	2	71				
	3	76				
	4	77				
	5	50				
	6	62				
	7	61				
	8	62				
30	1	72	5.58	70.14	0.07	No
	2	63				
	3	72				
	4	68				
	5	79				
	6	73				
	7	64				
	8	-				
32	1	87	4.23	85.29	0.00	Yes
	2	87				
	3	80				
	4	80				
	5	86				
	6	92				
	7	85				
	8	-				

47	1	55	9.10	69.38	0.12	No
	2	64				
	3	80				
	4	76				
	5	79				
	6	62				
	7	74				
	8	65				
48	1	88	6.32	79.63	0.00	Yes
	2	83				
	3	75				
	4	79				
	5	83				
	6	80				
	7	82				
	8	67				
50	1	89	7.09	83.75	0.00	Yes
	2	81				
	3	92				
	4	73				
	5	80				
	6	78				
	7	84				
	8	93				
52	1	90	6.21	81.38	0.00	Yes
	2	73				
	3	80				
	4	87				
	5	77				
	6	82				
	7	75				
	8	87				

9.5.9 Plant shoot height (cm) at final harvest (Batch 1)

	Final Harvest					
Line	Plant	Height	St. Dev.	Average	p. value	
WT1	5	73	12.33	64.88	1.00	No
	6	46.5				
	7	70				
	8	70				
6	4	64	4.63	65.75	0.90	No
	5	62				
	6	72.5				
	7	64.5				
7	5	56.5	6.01	63.75	0.88	No
	6	65				
	7	71				
	8	62.5				

30	4	66.5	7.89	71.88	0.38	No
	5	80.5				
	6	76.5				
	7	64				
32	4	83	4.79	89.75	0.02	Yes
	5	90				
	6	94				
	7	92				
47	5	83	10.63	74.75	0.27	No
	6	62				
	7	84				
	8	70				
48	5	106	15.24	87.50	0.06	No
	6	85				
	7	90				
	8	69				
50	5	107	11.56	96.50	0.01	Yes
	6	86				
	7	87				
	8	106				
52	5	120	29.18	123.25	0.02	Yes
	6	164				
	7	95				
	8	114				

9.5.10 Plant shoot height (cm) at half-way harvest (Batch 2)

	Half-way Harvest					
Line	Plant	Height	St. Dev	Average	p. value	Sig. Dif.
WT2	1	73	9.69	86.29	1.00	No
	2	90				
	3	75				
	4	88				
	5	84				
	6	99				
	7	95				
	8	-				
1	1	104	3.06	106.67	0.00	Yes
	2	110				
	3	106				
	4	-				
	5	-				
	6	-				
	7	-				
	8	-				
8	1	115	7.87	114.50	0.00	Yes
	2	110				
	3	120				
	4	122				
	5	119				
	6	101				
	7	-				
	8	-				

25	1	123	8.21	122.14	0.00	Yes
	2	111				
	3	129				
	4	122				
	5	130				
	6	129				
	7	111				
	8	-				
49	1	107	7.26	104.67	0.00	Yes
	2	108				
	3	93				
	4	114				
	5	100				
	6	106				
	7	-				
	8	-				
51	1	80	8.53	91.75	0.27	No
	2	96				
	3	108				
	4	94				
	5	92				
	6	92				
	7	89				
	8	83				

9.5.11 Plant shoot height (cm) at final harvest (Batch 2)

	Final Harvest					
Line	Plant	Height	St. Dev.	Average	p. value	Sig. Dif.
WT2	4	93	4.92	92.25	1.00	No
	5	86				
	6	98				
	7	92				
1	1	108	3.51	104.33	0.01	Yes
	2	101				
	3	104				
		-				
8	3	118	10.08	117.75	0.01	Yes
	4	121				
	5	128				
	6	104				
25	4	138	8.39	129.50	0.00	Yes
	5	132				
	6	130				
	7	118				
49	3	84	23.92	111.00	0.22	No
	4	142				
	5	112				
	6	106				
51	5	95	6.93	91.88	0.93	No
	6	95				
	7	96				
	8	81.5				

9.5.12 RT-PCR Data and Analysis of *NRT1.11* at half-way harvest (Batch 1 and 2)

RT-PCR Data							
NRT gene primers (23-24)	Line	#1	#2	Housekeeping gene primers (76-77)	Line	#1	#2
	6	26.3	26.3		6	19.3	19.3
	7	25	24.9		7	17.8	17.5
	30	25.1	25.1		30	17.9	17.9
	32	26.4	26.3		32	17.8	17.8
	47	26.9	26.7		47	18.4	18.4
	48	25.2	25		48	17.1	17.1
	50	24	23.8		50	16.9	16.8
	52	27.5	27.6		52	18.5	18.6
	K	26.2	26.3		K	18.5	18.4
	8-1	25.1	25.4		8-1	18.7	18.9
	25-1	26.7	25.8		25-1	19.4	19.5
	25-2	25.3	25.1		25-2	18.8	18.8
	25-3	25.3	25.3		25-3	18.4	18.5
	48-2	24.3	24.3		48-2	17.7	17.7
	49-1	26.6	26.9		49-1	17.4	17.5
	49-2	27.3	27.2		49-2	17.1	17.2
	51-2	24.1	23.9		51-2	16.9	16.9
	51-3	24.1	24		51-3	17.1	17.1
	51-4	24.7	24.7		51-4	17.3	17.5
	K-1	25.6	25.6		K-1	17.3	17.3
	K-2	25.7	25.8		K-2	17	16.9
	K-3	26.6	26.5		K-3	18	17.9

	Line	Elongation Factor (76-77)	NTR (23-24)	dCT	ddCT	2^{-ddCT}
Batch 1	6	19.29	26.325	-7.035	-0.77	0.59
	7	17.66	24.95	-7.29	-0.515	0.70
	30	17.86	25.105	-7.245	-0.56	0.68
	32	17.79	26.34	-8.55	0.745	1.68
	47	18.395	26.77	-8.375	0.57	1.48
	48	17.075	25.12	-8.045	0.24	1.18
	50	16.835	23.88	-7.045	-0.76	0.59
	52	18.53	27.56	-9.03	1.225	2.34
	WT1	18.445	26.25	-7.805	0	1.00

	Line	Line	Elongation Factor (76-77)	NTR (23-24)	dCT	ddCT	2^{-ddCT}	St. Dev (2^{-ddCT})	2^{-ddCT}
Batch 2	8 (pl.1)	8	18.79	25.27	-6.48	-2.09	0.24	0	0.24
	25 (pl.1)	25	19.44	26.23	-6.79	-1.78	0.29	0.52	0.28
	25 (pl.2)		18.79	25.21	-6.425	-2.14	0.23		
	25 (pl.3)		18.42	25.31	-6.895	-1.67	0.31		
	49 (pl.1)	49	17.72	24.32	-6.6	-1.97	0.26	1.27	1.58
	49 (pl.2)		17.42	26.75	-9.335	0.77	1.70		
	49 (pl.3)		17.18	27.22	-10.045	1.48	2.79		
	01-51 (pl.2)	51	16.88	24.00	-7.12	-1.45	0.37	0.04	0.36
	01-51 (pl.3)		17.12	24.05	-6.93	-1.64	0.32		
	01-51 (pl.4)		17.42	24.67	-7.25	-1.32	0.40		
	WT 3 (pl.1)	WT2	17.32	25.61	-8.29	-0.28	0.83	0.18	1.01
	WT 3 (pl.2)		16.95	25.77	-8.82	0.25	1.19		
	WT 3 (pl.3)		17.96	26.55	-8.59	0.02	1.02		

9.5.13 Tuber weight (gm) at final harvest (Batch 1)

	Final Harvest Harvest					
Line	Clone	Weight Tubers	St. Deviation	Pooled Weight	T. Test	Sig. Dif.
WT1	5	96.6	17.43796146	71.825	1	No
	6	56.7				
	7	63.6				
	8	70.4				
6	4	89.5	7.577323626	80.775	0.398635579	No
	5	71				
	6	81.4				
	7	81.2				
7	5	59	5.325723738	55.05	0.148495176	No
	6	47.3				
	7	58				
	8	55.9				
30	4	42.6	11.12426028	44.075	0.042824725	Yes
	5	52.1				
	6	52.7				
	7	28.9				
32	4	72.9	9.858118482	59.275	0.268458131	No
	5	49.6				
	6	58.8				
	7	55.8				
47	5	53.3	12.71377206	65.8	0.598614926	No
	6	73.3				
	7	79.7				
	8	56.9				
48	5	62.1	8.597286394	52.9	0.117275594	No
	6	41.4				
	7	55.1				
	8	53				
50	5	60.2	14.21111654	51.525	0.123145612	No
	6	65.2				
	7	47.2				
	8	33.5				
52	5	12.3	5.098038839	16.25	0.005483678	Yes
	6	12.9				
	7	16.4				
	8	23.4				

9.5.14 Tuber weight (gm) at final harvest (Batch 2)

	Final Harvest					
Line	Clone	Weight Tuber	St. Deviation	Pooled Weight	T. Test	Sig. Dif.
WT2	4	71.8	27.32451585	97.025	1	No
	5	76.1				
	6	127.2				
	7	113				
1	1	98.6	10.30404451	88.13333333	0.582623103	No
	2	78				
	3	87.8				
8	3	92.3	11.97507829	79.325	0.299397342	No
	4	81.3				
	5	63.3				
	6	80.4				
25	4	67.8	18.33564379	94.625	0.889465277	No
	5	97.9				
	6	107.1				
	7	105.7				
49	3	66.4	18.33255301	67.175	0.126606299	No
	4	47.8				
	5	62.6				
	6	91.9				
51	5	112.9	11.55173147	102.125	0.748129505	No
	6	110.3				
	7	88.3				
	8	97				