Cloning of genes *SULTR3;3*, *SULTR4;2* and *HEX* and studying the phenotype of overexpressed *NRT1.11* in potato

(PBR-80436)

MSc thesis

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Abbreviations

ABA	Abscisic acid
ANOVA	Analyses of variance
APR	APS reductase
ATPS	ATP sulphurylase
AUX	Auxin
CGS	Cystathionine γ-synthase
Cas9	CRISPR associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
CTAB	Cetyltrimethylammonium bromide
Cv	Cultivar
dCT	Delta Ct value
ddCT	Delta delta Ct value
FPKM	Fragments per kilobase million
GA	Gibberellin
GM	Genetically modified
GSH	Glutathione
GWAS	Genome wide association study
JA-ile	Jasmonoyl-isoleucine
Kardal WT	Wild-type Kardal potato cultivar
LB-agar	Lysogeny broth agar
LSD	Least significant difference
MS	Methionine synthase
OAS	O-acetylserine
PCA	Principle compenent analysis
PFJ	Potato fruit juice
PTR	Protein transport family
QTL	Quantive trait locus
SPAD	Soil-plant analysis development
SPAD-HL	SPAD measurement of higher leaves
SPAD-LL	SPAD measurement of lower leaves
SPC in PFJ	Soluble protein content in potato fruit juice
VIGS	Virus-induced Gene Silencing
ZCVK	Zeatin, cefotaxime, vancomycine and kanamycin

Abstract

Potato is a high nutritional value crop. It is mainly used for food consumption and for the starch industry. When potato is processed for the starch industry, two components are extracted starch and proteins. In the last decade, more research is done on extracting more high quality proteins. In this study, the focus is to increase the protein content in potato tuber with a forward genetic approach. From a GWAS of Michiel Klaassen four candidate genes were selected that could enhance the protein content in the tuber, these genes were *SULTR3;3, SULTR4;2, HEX* and *NRT1.11*. This thesis, focusses on the cloning and transformation of *SULTR3;3*, *SULTR4;2* and *HEX* to potato. Next to that, phenotypic differences were measured during plant development of thirty-two transgenic potato 35S:*NRT1.11* lines (GM Kardal NRT1.11). In this study, is shown that candidate gene *SULTR3;3* seems to be successfully cloned and transformed into Kardal. The sulphate transporter gene *SULTR4;2* appears to be cloned into a destination vector PK7GW2,0. However, still further verification of the presence of *SULTR4;2* into PK7GW2,0 should be done by sequencing of this vector. During this thesis, it was not possible to clone *HEX* into an entry vector. Furthermore, *HEX* seems to be either a pseudogene or a gene whereby the gene annotation is incorrect. In this study, a different functional hexose transporter *HEX6* was found in the same region of the annotated *HEX*. *HEX6* is therefore recommended as a candidate gene for cloning and transformation to potato in the future. In GM Kardal NRT1.11 lines was found that for eight of sixteen lines *NRT1.11* were higher expressed in the mature tuber than the Kardal wild-type. Furthermore, some phenotypic differences in GM Kardal NRT1.11 plants were seen. Chlorosis was seen on the younger leaves and transgenic plants showed possible anthocyanin accumulation in the stems. Thirteen of thirty-two transgenic lines showed significantly higher chlorophyll content in the sink leaves (SPAD-HL) than the wild-type. Next to that, thirteen of thirty-two GM Kardal NRT1.11 lines were found to be significantly higher in height. For the amount of proteins in the tuber, two transgenic lines, line 19 and 27 were found that had a significantly higher soluble protein content in the tuber. In the near future, with an improved experimental set-up, it can be seen if the protein content in potato can be enhanced by overexpression of *NRT1.11*, or with one of the other candidate genes *SULTR3;3*, *SULTR4;2* or *HEX6*. For more fundamental purposes, a reverse genetic approach can be done in the future. This to see how the mechanism of protein accumulation works in the tuber.

Keywords: *potato nitrate transport; amino-acids; soluble protein content; sulphate transport; hexose transport; agrobacterium-mediated transformation; chlorophyll production*

1. Introduction

1.1 Background

Potato (*Solanum tuberosum L.*) is an important food crop worldwide (Barrell, Meiyalaghan, Jacobs, & Conner, 2013). In 2013 the production of potatoes was around 376,5 million tonnes and they were cultivated on an area of 19,321,155 Ha worldwide (Faostat, 2013). Since 2013, the production of potatoes is still gradually increasing. Due to its increasing popularity, it is useful to invest more breeding efforts in the nutritional value of potato. A possible way to achieve this is by enhancing the protein content in the potato tuber. In this study, there will be a focus on cloning of candidate genes in potato, that possibly enhance the protein content in potato tubers.

Besides the importance of potatoes to the food industry, potatoes are mainly cultivated for extracting starch and proteins (Taskila, Ahokas, Jarvinen, Toivanen, & Tanskanen, 2017). A beneficial trait of potato proteins is that these proteins have a significantly lower allergenicity compared to other proteins such as egg, soy, nut, milk and gluten (Barsan, 2016; Amanda Waglay & Karboune, 2016). At this moment, proteins are mainly side-products obtained by processing potato tubers for starch. After starch extraction, a potato fruit juice (PFJ) residue is left over that is used for extracting proteins. On average, PFJ contains a small amount of 1 to 2,5% of industrial proteins (Lokra, 2008; Taskila et al., 2017). The most common techniques for separating proteins are precipitation techniques and adsorption chromatography. With these extraction methods, it is possible to recover high quality/pure potato proteins. The price for pure potato proteins for food consumption is around 6-8 Euro per kg, for feed consumption the price of proteins is around 0,40-0,80 Euro per kg (Lokra, 2008). It can therefore be economically profitable to increase protein content in potato tubers and focus on producing more pure protein from potatoes.

Potato proteins are a diverse group of proteins that are synthesized by different metabolic pathways. The majority of proteins are synthesized by nitrogen, carbon and sulphur pathways in plants (Shewry, 2003). These proteins support potatoes growth, survival under stress conditions and act as a storage sink for excess amounts of nitrogen and sulphur. Proteins in the potato tuber can be divided in three classes: patatins (35-40%,) protease inhibitors (25-50%) and higher molecular weight proteins (10-15%) (Lokra, 2008; Amanda Waglay & Karboune, 2016; A. Waglay, Karboune, & Alli, 2014). Patatins consist of 366 amino acids and are the main storage protein in the potato tuber. Next to that, patatins are functional in plant defences. The antioxidant activity of patatins are used to withhold pathogens for a certain amount of time (Barel & Ginzberg, 2008; Amanda Waglay & Karboune, 2016). Protease inhibitors are necessary to inhibit the degradation of proteins in the tuber, this stimulates the long term storage of proteins in the potato tuber (Amanda Waglay & Karboune, 2016; A. Waglay et al., 2014). The inhibition of degradation is against specific amino acids: aspartate, cysteine and serine. Higher molecular weight proteins can be protein kinases, enzymes for starch synthesis, lectins or phosphorylase isozymes.

In the last decade, more research has been done about genes that increase the production and storage of proteins in crops. Quantitative trait locus (QTL) mapping in pea and soybean showed that protein content in seed is a quantitate trait, this trait is associated with a various number of genes (Bandillo et

al., 2015; Barrell et al., 2013; Bourgeois et al., 2011; Qi et al., 2016; J. Wang et al., 2015). For potato, it can be useful to focus on a variety of QTL regions that have a positive effect on protein content. With potato it is even more difficult to find QTL regions than for other crops. Potatoes have high heterozygosity and are tetraploid (4n= 48) (Andersson et al., 2017; Bhaskar, Venkateshwaran, Wu, Ane, & Jiang, 2009). Fortunately, in 2010 the entire genome of potato was sequenced. The potato genome consist of 844 Mb with 39,031 protein-coding genes (Xu et al., 2011). With the availability of the potato genome, QTL regions for quantitative traits were more effortlessly found. In a genome wide association study (GWAS) by Klaassen, QTL regions for protein content in the potato tuber were found and candidate genes were selected (Klaassen, Unpublished work). In this study, the focus will be on cloning and transformation of three candidate genes in potato: *SULTR3;3* (sulphate transporter), *SULTR4;2* (sulphate transporter), *HEX* (hexose transporter) and assessing genetically modified (GM) potato cultivar Kardal lines with a overexpressed *NRT1.11* (nitrate transporter). Differences in the phenotype of GM Kardal NRT1.11 plants were compared to Kardal wild-type (WT).

1.2 Sulphate transporters

Sulphur is a macronutrient that is used for plant development and plants responses. Plant responses can relate to different kind of abiotic stresses and biotic stresses. As one of the macronutrients of the plant, sulphur is needed for biosynthesis of essential amino acids methionine and cysteine, different prosthetic groups, co-enzymes, glutathione (GSH), vitamins and biosynthesis of other proteins (Gigolashvili & Kopriva, 2014; Zuber, Davidian, Wirtz, et al., 2010). Sulphur has to be transported and relocated to create one of these products. Plants can retrieve sulphur by absorption of sulphate through the root systems. Reduction of retrieved sulphate molecules typically takes place in chloroplasts of the plant leaves, this reduction leads to biosynthesis of methionine and cysteine, these amino acids are needed for plant development (Fig. 1).

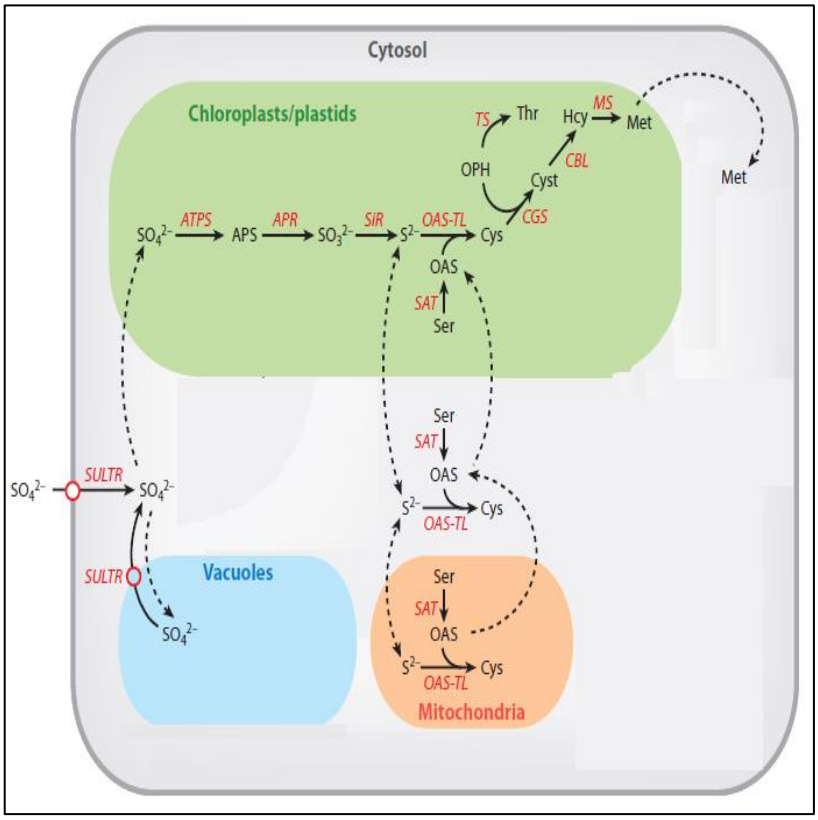

Figure 1 Sulphur metabolism of essential protein synthesis cysteine and methionine in plants cells. With dashed lines supposed transport of these metabolites is visualized. At the start sulphate is transported into the cytosol by SULTR and can be further transporter to the vacuole for storage or be transported to chloroplasts for amino acid synthesis. When sulphate is transported into the plastids, sulphate is adenylated by a ATP sulphurylase (ATPS) to get APS (adenosine 5'-phosphosulphate). APS is then reduced to sulphide by APS reductase (APR). Sulphide is further reduced to sulphur (S^{-2}), by combining an S^{-2} with a O-acetylserine (OAS) amino acid cysteine is formed. Cysteine biosynthesis can take place in the chloroplasts, cytosol and mitochondria of the plant cell. When methionine is needed in plant cells, methionine biosynthesis is possible by changing the amino acid cysteine. With cystathionine γ-synthase (CGS), cystathionine β-lyase (CBL) a homocysteine is synthesised from the cysteine. With a final step of methylation of this homocysteine by methionine synthase (MS) methionine is created in the plastids or cytosol of plant cells. Obtained amino acids cysteine and methionine are essential building blocks for plants development (Takahashi, Kopriva, Giordano, Saito, & Hell, 2011).

Sulphate molecules are transported from the root tissue to more aerial parts of the plant, where sulphate is altered to different kinds of amino acids, this transformation depends on which protein is needed. To transport sulphate to the right plant tissues plant, sulphate transporters (SULTR) are essential (Kataoka et al., 2004). SULTR's are present in higher plants as H^+ / sulphate transporters and react on plant cells with H^+/ sulphate symporters (Gigolashvili & Kopriva, 2014). SULTR groups consist of four groups: 1st group consist of high affinity SULTR, 2nd group are low affinity transporters, 3rd group are sulphate transporters of plastid membranes and the 4th group are considered to be vacuolar sulphate exporters and are found in the tonoplast of plant cells (Cao et al., 2013; Gigolashvili & Kopriva, 2014; Tombuloglu, Filiz, Aydin, & Koc, 2017).

1.3 Sulphate transporter SULTR 3:3

From the GWAS of Klaassen two specific QTL's were found for protein content (Klaassen, Unpublished work). From this region, two candidate genes of sulphate transporters were selected: *SULTR3;3* and *SULTR4;2*. *SULTR3;3* is particularly interesting, because the potato gene shares high similarity in sequence and physical position with the *SULTR3;3* of soybean (*Glycine max).* In soybean this *SULTR3;3*, has a positive impact with the accumulation of proteins in the seed (Bandillo et al., 2015). In potato, *SULTR3;3* is located on chromosome 5 position: 4,417,126 bp to 4,421,492 bp and corresponds with sequence identifier PGSC0003DMT400047403 (SPUDdatabase, 2018). RNA-seq data of doubled monoploid *S. tuberosum* (Group Phureja clone DM1-3) showed that the highest expression of *SULTR3;3* was found in the potato flower, fragments per kilobase million (FPKM) values were found from 40,4 to 47,0 (Hardigan et al., 2016). Other high expression of SULTR*3;3* was seen in the potato tuber, FPKM values vary from 18,9 and 19,2. Because of this high expression of *SULTR3;3* in potato tissue, it should be possible to clone *SULTR3;3* in potato.

1.4 Sulphate transporter SULTR 4:2

The second candidate gene, *SULTR4;2* is located in the vacuolar membrane of plant cells and is highly expressed in root cells of *Arabidopsis thaliana* (*A. thaliana*) (Fig. 2)(Zuber, Davidian, Wirtz, et al., 2010). In the vacuolar membrane, *SULTR4;2* is considered to have a function to maintain the gradient of sulphate efflux from the vacuole to the cytoplasm together with *SULTR4;1.*

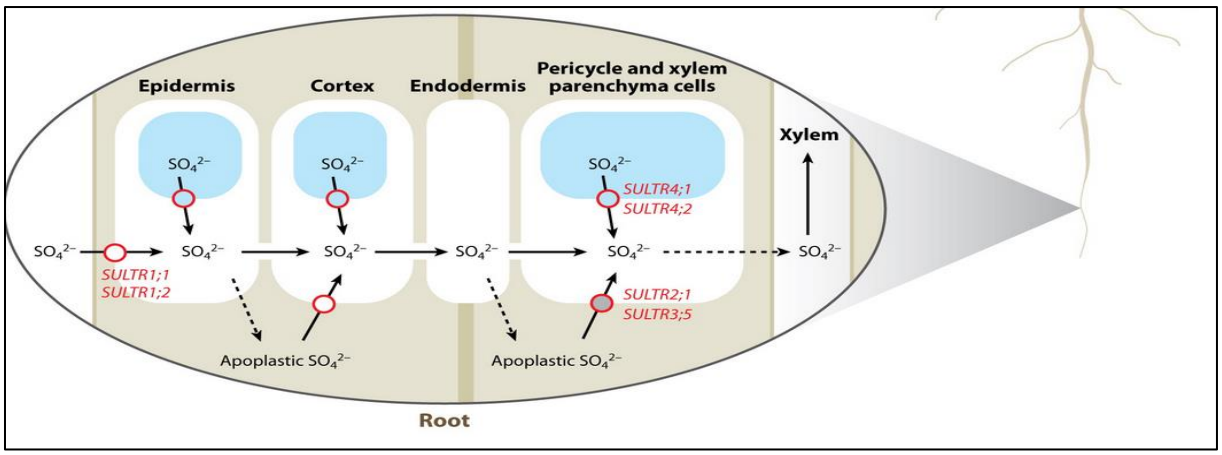

Figure 2 in A. thaliana SULTR4;1 *and* SULTR4;2 *function of this genes is to regulate transport of sulphate in plant cells. The release of sulphate from vacuole to cytoplasm is done by* SUTR4;1 *and* SULTR4;2. *Under sulphur limitation conditions accumulation of* SULTR4;1 *and* SULTR4;2 *enhances vacuoler sulphate release to the cytoplasm (Takahashi et al., 2011).*

In potato, *SULTR4;2* is located on chromosome 5 position: 50,492,402 bp to 50,591,214 bp and corresponds with PGSC0003DMT400060458 (SPUDdatabase, 2018). RNA-seq expression data of *SULTR4;2* in potato were especially high for potato flower and petiole, FPKM values vary from 17,8 to 34,4 (Hardigan et al., 2016). In the potato tuber, FPKM values of *SULTR4;2* were found varying from 1,1 to 2,3. The highest chance to find the *SULTR4;2* gene for cloning, seems to be with flower- or petiole tissue. When *SULTR4;2* is successfully cloned and overexpressed in potato, expectations are that changing the regulation of sulphate from the vacuole to the cytoplasm, can improve the efficiency of sulphate transport in potato.

1.5 Hexose transporter HEX

Hexose sugars are essential products that are retrieved after photosynthesis. Examples of hexose sugars are glucose, fructose and galactose, these sugars are monosaccharides containing a six carbon atoms (Engelking, 2015). Hexoses are essential for the carbohydrate metabolism and plant development (Granot, David-Schwartz, & Kelly, 2013). Hexoses transport goes from apoplast to symplast by hexose porters of the plant cell. Hexose porters are substrate specific, some transport glucose while other transport fructose or galactose (Granot et al., 2013; Reuscher et al., 2014). For what is known, the main function of hexose transport is considered to be distribution and maintaining of homeostasis of carbohydrates in plant cells (Granot et al., 2013). Ruan and colleagues found out that hexose transporters can increase the post-phloem transport of sugars to the outer pericarp of the tomato fruit (Ruan, Patrick, & Brady, 1997). If accumulating of sugars to the sink-tissue is highly conservative in plants, adjusting and overexpressing of the hexose transport mechanism can lead to an increase of accumulating sugars in the potato tuber, which can change the composition of metabolites in the tuber.

The third candidate gene that was selected for cloning to potato is the gene *HEX*. *HEX* is located on chromosome 3 position: 43,117,266 bp to 43,119,568 bp, this region corresponds to PGSC0003DMT400037213 (SPUDdatabase, 2018). RNA-seq expression data showed an overall low expression of *HEX* in all plants tissues. A FPKM value was found varying from 0,1 to 0,6 for leaf- and flower tissue (Hardigan et al., 2016). This suggest that *HEX* is not actively transcribed and translated under normal conditions. The low RNA expression of *HEX* can make it difficult to clone this gene in potato.

1.6 Nitrate transporter NRT1.11

Nitrate (NO_3^-) is an essential mineral for development of plants. In plant cells, NO_3^- is further reduced to ammonium (NH_4^+), which can be transformed in the GOGAT cycle to organic nutrient nitrogen (N) (Forde, 2000). For uptake and transport of NO_3^- in root cells, NO_3^- depends on nitrate transporter (*NRT*) gene family. In higher plant species, one major family for nitrate transport is the protein transport family (PTR), consisting of *NRT1* genes. The *NRT1* genes are essential for the nitrate transport in plant cells, nonetheless some of these nitrate transporter genes are also capable of transporting a variety of plant hormones, such as: jasmonoyl-isoleucine (JA-ile), auxin (AUX), gibberellin (GA) and abscisic acid (ABA) (Chiba et al., 2015; Forde, 2000).

The fourth candidate gene that is targeted for overexpression in potato is *NRT1.11*. From the GWAS study of Klaassen a high specific QTL peak region was found on chromosome 5, for this region the most likely gene was *NRT1.11 (Klaassen, Unpublished work)*. If the function of this gene is highly

conservative, it is expected that the general function of NRT1.11 in potato is similar to that of *A. thaliana*. In *A. thaliana* NRT1.11 and NRT1.12 have a positive influence on transferring nitrate from xylem to the phloem, this can have a positive effect on redistributing NO_3^- into developing leaves. The xylem to phloem system is essential for transporting amino acids obtained from the roots of the plant to the seed and/or plant leaves (Hsu & Tsay, 2013; Tegeder, 2014).

The nitrate transporter *NRT1.11* was previously cloned into potato cultivar Kardal driven by a constitutive CaMV 35S promoter (GM Kardal NRT1.11 lines) (Mendoza, 2017). *NRT1.11* is located on chromosome 5 position: 7,434,852 bp to 7,436,872 bp, this corresponds to PGSC0003DMT400040275 (SPUDdatabase, 2018). RNA-seq expression data of *NRT1.11* showed highest expression in leaf-, tuber- and root tissue, FPKM values varying from 4,9 to 14,0 (Hardigan et al., 2016). *NRT1.11* was lower expressed in flower tissue, FPKM values from 0,1 to 1,8. NO_3^- redistribution is necessary to increase the uptake of external nitrate, which can encourage faster plant development and storage of amino acids for later purposes.

2. Materials and methods

2.1 RNA extraction and cDNA synthesis of potato plant tissue

RNA was isolated from nine potato cultivars: Eigenheimer, Agria, Innovator, Desiree, Nicola, Bintje, Hansa, Posmo, and Kardal. This was done with two RNA isolation methods: cetyltrimethylammonium bromide (CTAB) RNA isolation method and the QIAGEN RNeasy Plant Mini Kit method. After RNA isolation, concentration of RNA was measured with a NanoDrop ND-1000 spectrophotometer. A DNase treatment was performed according to DNase I protocol of Thermo Fisher Scientific for removal of genomic DNA. cDNA was synthesised with either the support of iScript™ cDNA Synthesis Kit from Bio-rad or with TaqMan® Reverse Transcription Applied Biosystems kit. Synthesised cDNA was tested with a PCR with primer combination Pr76x77 on the presence of housekeeping gene *EF1A* (Appendix A Table. 1). The PCR conditions were as follows: 1 cycle of 94 °C for 5 min., 35 cycles of 94 °C 30 sec., 55°C 30 sec., 72°C 1.20 min and a final cycle of 72°C of 10 min.

2.2 Amplification of genes *SULTR3;3 , SULTR4;2 and HEX*

For *SULTR3;3*, *SULTR4;2* and *HEX* primers were designed with software of NCBI tool Primer BLAST and Primer3 software (version 0.4.0) (NCBI, 2018; Untergasser A, 2012). For amplification of a larger gene fragment >1.5 Kb, such as with genes *SULTR3;3* and *SULTR4;2,* the PCR was done with high specificity Phusion Taq polymerase. The PCR conditions were as follows: 1 cycle of 98 °C for 30 sec., 5 cycles of 98 °C 30 sec., 60 °C 30 sec., 72°C 3. min, followed by 30 cycles of 98 °C 30 sec., 62 °C 30 sec., 72°C 3. min and a final cycle of 72°C of 10 min. For the target genes a nested PCR or second PCR was done to obtain enough amplicon material for cloning to pENTR™ TOPO vector.

2.3 Cloning of genes to the entry- and destination vector

Genes that were successfully amplified, were purified with a Zymo Research DNA Clean & Concentrator™ kit. After purification, the protocol of pENTR™ Directional TOPO® Cloning Kits of Thermo Fisher Scientific was followed. Transformants cells grew on lysogeny broth (LB) agar-plates containing 50 µg/ml of kanamycin. Verification of target gene insert in pENTR™ TOPO® was done with a colony PCR. The colony PCR was performed with internal primers of target gene. After verification,

samples were purified with a QIAGEN QIAprep® Spin Miniprep Kit. Purified samples were send to GATC-Biotech® company for sequencing, this to confirm the gene insert.

A LR reaction was done with 50-150 ng of pENTR™ TOPO vector with target gene insert and with 150 ng of PK7WG2.0 destination vector. By recognition of flanking regions attR1 and attR2 ccdB gene was exchanged with the entry vectors gene insert. PK7WG2.0 with gene insert were put into competent TOP10 cells with a heat-shock. TOP10 cells that were transformed grew on LB agar-plates containing 200 µg/ml of spectinomycin. From these plates, colonies were selected for colony PCR. Colonies that showed positives results for the target gene, were verified by sequencing.

2.4 Cloning of *SULTR3;3* to the expression vector

PK7WG2.0 containing *SULTR3;3* was further put into *A. tumefaciens* AGL1 by electroporation. Electroporation was done with a Gene Pulser® II (Bio-Rad) at 1.4 kV, 200 Ohm resistance and 25 µF. After incubation of 2 days on 28 °C, AGL1 cells that were transformed grew on selective LB-agar plates containing chloramphenicol 50 µg/ml, carbenicillin 100 µg/ml and spectomycin 200 µg/ml. Colonies of these LB- agar plates were verified on the presence of *SULTR3;3* with a colony PCR.

2.5 Agrobacterium-mediated transformation to Kardal

One day before agrobacterium-mediated transformation, small stems of Kardal (2-5 mm) were placed on regeneration media of R3B agar (MS-30 medium, BAP 1 mg/L, NAA 2mg/L + PACM). Kardal stems were exposed to AGL1 with PK7WG2.0 and *SULTR3;*3 ($O.D._{600}$) for ten minutes. After exposure to the AGL1 suspension, Kardal stems were transferred back on R3B agar + PACM and were incubated at 24 ^{o}C for two days. After two days, Kardal stems were transferred to selection medium ZCVK (MS-20 medium, zeatin 1 mg/L, cefotaxime 200 mg/L, vancomycin 200 mg/L and kanamycin 100 mg/L). Every Kardal explant is a possible transgenic line with overexpressed *SULTR3;3*. Kardal explants were transferred to fresh ZCVK media after fourteen days. To monitor the agrobacterium-mediated transformation controls were made. Three controls were used: MS-20 agar plates without AGL1, MS-20 agar plates with AGL1 and ZCVK agar plates without AGL1.

2.6 Gene expression of GM Kardal NRT1.11

Gene expression of GM Kardal NRT1.11 lines were measured using a CFX96 Touch™ Real-Time PCR Detection System Bio-Rad®. SYBR Green of Bio-Rad was used as a fluorescence dye to track the amplification the gene in the samples. Different potato tissues were tested: mature tuber, young leaf and stem. *NRT1.11* gene expression was measured with either Pr23x24 or Pr119x120. The following qPCR conditions were used for amplification: 95 °C for 3 min., 40 cycles of 95 °C 30 sec., 55 °C (for Pr119 x 120) 30 sec. or 60 °C (for Pr23xPr24) 30 sec., 72 °C 1.20 min and a final step, 72°C of 10 min. Melt curves of samples were measured from 65 °C to 95 °C with a increment of 0,5 °C for 0,05 sec. For every qPCR run a reference gene *EF1A* was used (Pr76xPr77).
Calculations of *NRT1.11* gene expression were done as followed:
$\Delta Ct\ (gene\ expression\ NRT1.11) = Ct\ \overline{x}_{(\mathrm{NRT1.11})}$- $Ct\ \overline{x}_{(\mathrm{EF1A})}$
After correcting for the reference gene, the data was transformed to see differences in expression.
$dCt = 2^{-\Delta Cq}$
A relative gene expression ($\mathrm{dd}Ct$) difference was measured by subtracting the *NRT1.11* expression of a GM Kardal NRT1.11 line to the wild-type (Kardal WT).
$\mathrm{dd}Ct\ = dCt(GM\ Kardal\ \mathrm{NRT1.11}) -\ dCt(Kardal\ WT)$

1.1 Greenhouse experiments of GM Kardal NRT1.11

Greenhouse experiments were conducted at Wageningen University Unifarm with GM Kardal NRT1.11 lines (Appendix A Table 2) in pots. These plants were planted in three series. 1st series was transferred to pots in week 36, 2nd series in week 37 and 3rd series in week 40. After nine weeks of growing GM Kardal NRT1.11 lines relative chlorophyll content was measured with a SPAD 502 plus chlorophyll meter. SPAD-higher leaf (SPAD-HL) was measured of every fourth fully expanded leaf from the top of the plant (Zheng, Liu, Qin, Chen, & Fan, 2015), the leaf was measured three times to get an average SPAD value. SPAD-lower leaf (SPAD-LL) was measured from the fifth leaf of the bottom. Both SPAD measurements were done on 2/3 of the leaf next to the major nerf, as seen in research of (Li, Qin, Liu, Hu, & Fan, 2012; Yuan et al., 2016; Zheng et al., 2015). After nineteen weeks of growing of the plants, five additional measurements were done: plant height (cm), soluble protein content (SPC) in PFJ (%), soluble protein content (%) and total weight of the tubers (g).

1.2 Statistical analysis on GM Kardal NRT1.11

Statistical analyses were performed using software IBM SPSS Statistics Data Editor version 25 ®. For GM Kardal NRT1.11 lines outliers in normal distribution histograms, QQ-plots and box-plots of different traits were removed from the data-set (data not shown). This was from GM Kardal NRT1.11 line 13 plant no. 6 and 7, from line 19 plant no. 8, from line 22 plant no. 5, from line 38 plant no. 8, from line 6 plant no. 8 and from line 30 plant no. 7. These outliers showed data points for a trait either 1,5 interquartile ranges below the first quartile or above the third quartile.

The SPAD-LL, SPAD-HL, height, SPC in PFJ, soluble protein content and total weight of the tubers were checked for normal distribution with a histogram (Appendix B Fig. 1). A Levene's test was done for normality, results showed that this was not significant (p-value >0,05), this result showed that the normality could be assumed for every measurement. For further statistical analyses weight of the tubers was removed, because this trait doesn't have a direct relation to the tuber that is analysed for the soluble protein content.

Analyses of variance (ANOVA) with post-hoc Fisher's least significant difference (LSD) tests were done, to test if there were differences between the Kardal WT of the three different series. Kardal WT were significant different from each other for height, SPAD-HL, SPC in PFJ and soluble protein content (ANOVA, p-value <0,05) (Appendix B Table 1-2). For SPAD–LL was found that the Kardal WT were not different from each other between the three series (ANOVA, p-value >0,05). In the report, the three series with GM Kardal NRT1.11 lines were analysed separately or it will be mentioned otherwise. Correlation between the different traits was verified with a Pearson correlation test or with a principle component analysis (PCA).

1.3 Soluble protein content measurement

After harvesting GM Kardal NRT1.11 tubers for soluble protein content, fresh weight and dry weight of these tubers was determined. Tubers were dried at 70 °C for 2-3 days and then weight to obtain the dry weight. A part of fresh tubers were grinded to obtain PFJ. For measuring SPC in PFJ a SPRINT™ Rapid Protein Analyser (CEM Corporation, NC, U.S.A.) assay was performed. Soluble protein content was calculated as follows: $\text{Soluble protein content } (\%) = \text{SPC in PFJ } (\%) * \text{tuber fresh weight } (\%)$

2. Results

2.1 *SULTR3;3*

SULTR3;3 was previously amplified and cloned in an entry vector (pENTR™ TOPO®) (Mendoza, 2017). Amplification of *SULTR3;3* was done with primer combination Pr18x20 (1990 bp). In this research, further verification of the presence of *SULTR3;3* was done by sequencing of three pENTR™ TOPO® colonies (Mendoza, 2017). Aligning of the Sanger sequencing results showed that approximately 640 bp of *SULTR3;3* was built into the entry vector, instead of the whole *SULTR3;3* (Fig. 3).

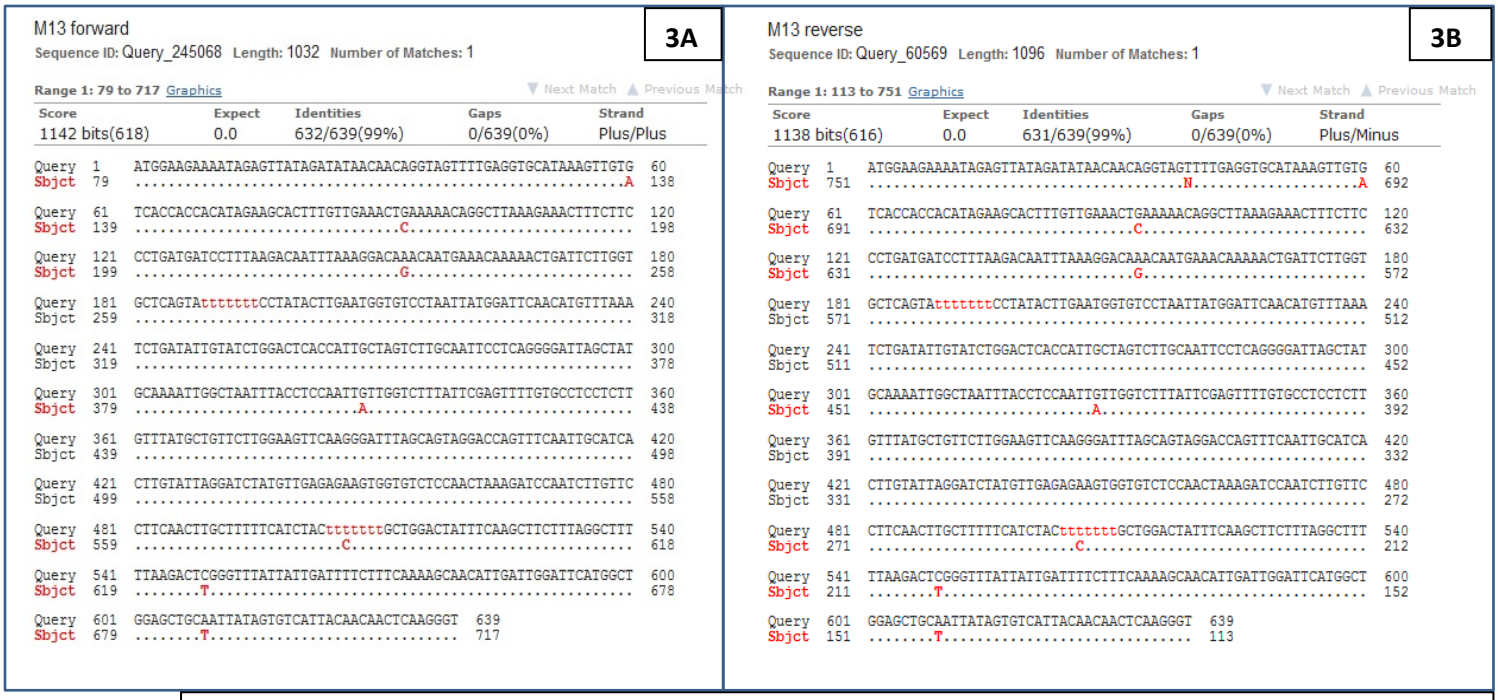

Figure 3 Highlighted in red are nucleotide differences of the sequence of SULTR3;3 PGSC0003DMT400047403 (query) aligned to the sequence of the colonies SULTR3;3 in the entry vector (subject). Dots mean that the nucleotides are the same as the query. 3A= sequence of subject with M13 forward primer and 3B= sequence of subject with M13 reverse primer

As the results showed that *SULTR3;3* wasn't successfully built into the entry vector, amplification and cloning of *SULTR3;3* had to be redone in this study. First, cDNA of different potato tissues was checked on the presence of *SULTR3;3* with Pr21x22 (310 bp). After amplification of the cDNA, amplicons ±310 bp were seen on the agarose gel for the different samples (Appendix A Fig.1). The brightest amplicons were seen with cDNA of Kardal stolon tissue and with cDNA of Bintje mature tuber tissue. The cDNA of these samples was further used for a nested PCR of the whole *SULTR3;3*. First a PCR was done with primers Pr113x114 (2219 bp), this resulted in amplicons ±2200-2400 bp (Appendix A Fig. 2). Then a second PCR was done on these samples with Pr18x20. The result of the second PCR showed a bright amplicon ±2000 bp for Bintje and for Kardal (Fig. 4.). It was almost certain that the bright amplicons were the target *SULTR3;3*. These amplicons were purified and used for cloning to the entry vector.

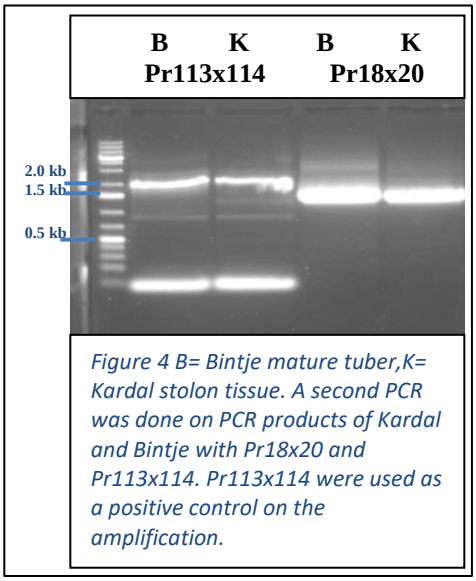

Figure 4 B= Bintje mature tuber,K= Kardal stolon tissue. A second PCR was done on PCR products of Kardal and Bintje with Pr18x20 and Pr113x114. Pr113x114 were used as a positive control on the amplification.

Cloning of *SULTR3;3* to the pENTR TOPO vector was only successful with the purified amplicon of Bintje. pENTR TOPO vector containing a gene insert grew on LB-agar plates with kanamycin. From these LB-agar plates, a number of colonies were tested for the presence of *SULTR3;3* with a colony PCR with Pr21x22. All tested colonies gave a desired amplicon ±310 bp (data not show). Ten colonies were further sequenced for verification of the *SULTR3;3* insert. Sequence results showed a query coverage of 88% with an E-value of 0 to the *SULTR3;3* of potato, this re-counts for 1920 bp of the 1990 bp of *SULTR3;3* (Appendix A Fig. 3).

One of the ten colonies was selected for the LR reaction of *SULTR3;3* to the destination vector PK7WG2.0. PK7GW2.0 were put into TOP10 cells with a heat-shock. TOP10 cells with PK7WG2.0 and gene insert grew on LB-agar plates with spectomycin. These colonies were verified for the presence of *SULTR3;3* with a colony PCR (data not shown). Four colonies that showed positive results from colony PCR, were further selected for verification with sequencing with Pr21F and Pr22R. Sequence results showed a high query coverage of 86% with an E-value of 0 to *SULTR3;3*, this corresponds with 1859 bp of *SULTR3;3* (Fig. 5). Therefore, it was verified that *SULTR3;3* was inserted in PK7WG2.0 for these four colonies.

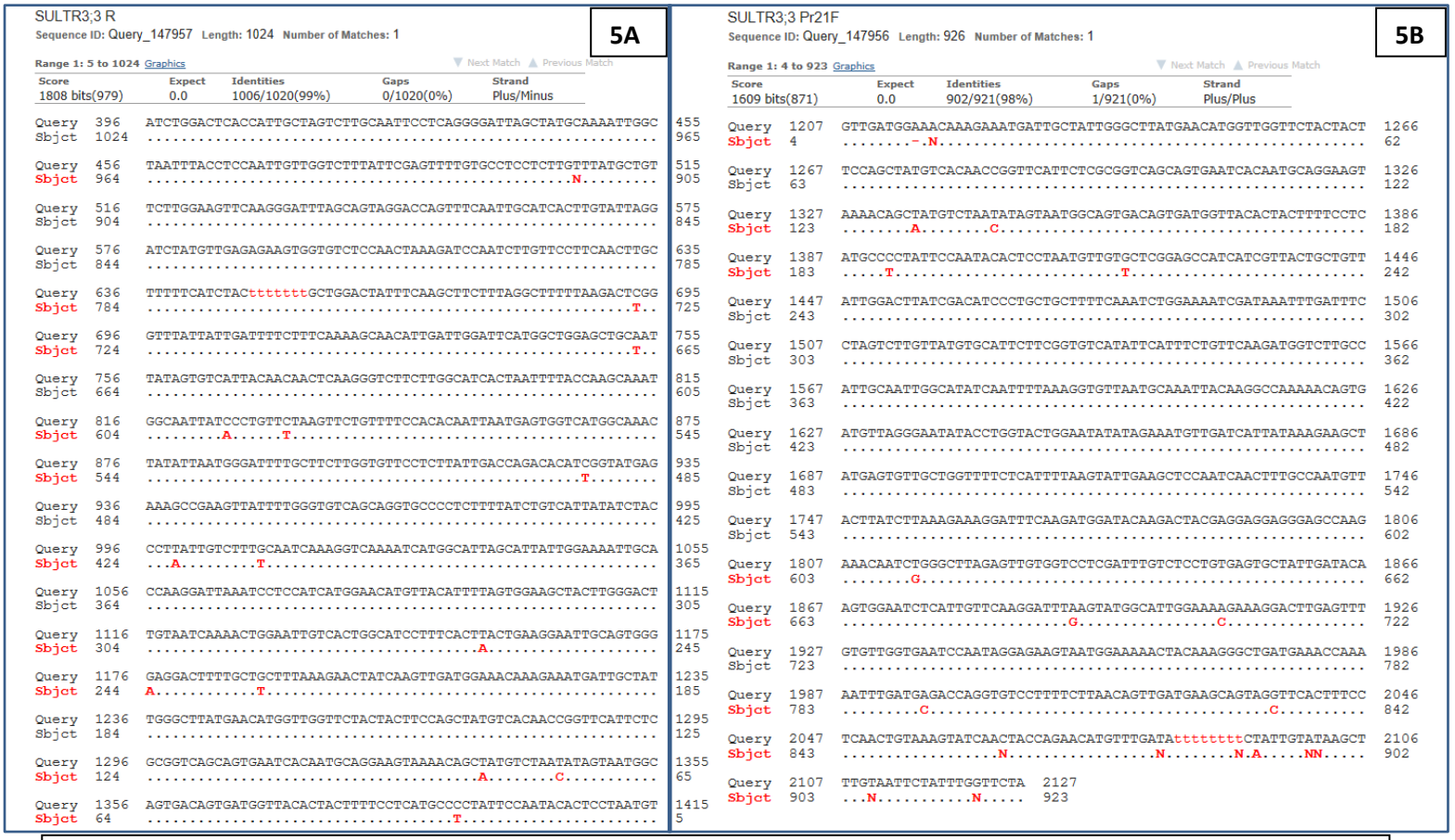

Figure 5: sequence SULTR3;3 PGSC0003DMT400047403 (query) aligned to sequence data of colony PK7WG2.0 with SULTR3;3 insert of Kardal (subject). 5A= sequence of subject with Pr22R reverse primer and 5B= sequence of subject with Pr21F forward primer.

The plasmid of the destination vector should be introduced to the agrobacterium expression vector. By electroporation, PK7WG2.0 with *SULTR3;3* was cloned into the expression vector AGL1. Transformed AGL1 cells grew on LB-agar plates with chloramphenicol, carbenicillin and spectomycin. With a colony PCR, colonies of AGL1 were confirmed for the presence of *SULTR3;3* (data not shown). AGL1 containing PK7WG2.0 with *SULTR3;3* were further cloned into small stems of Kardal. At this

moment, GM Kardal SULTR3;3 explants develop shoots and callus on ZCVK media, which was expected. In a few months transformed Kardal plants can be analysed on expression levels of *SULTR3;3*. For the agrobacterium-mediated transformation the control plates showed good results. Whereas explants on M20-plates grew fastest, these explants developed roots and shoots. M20-plates with AGL1, but without antibiotics showed a fast development of AGL1. ZCVK-plates without AGL1 and with antibiotics showed the most disruption of plant development. In the future, with GM Kardal SULTR3;3 phenotypic traits related to the protein content of the plants can be measured.

2.2 *SULTR4;2*

The second candidate gene for cloning to Kardal was *SULTR4;2*. Specific internal primers were designed: Pr105x106 (210 bp) and for the whole *SULTR4;2*: Pr115x116 (2460 bp). cDNA of different potato tissues were checked on the presence of *SULTR4;2* by amplification with Pr105x106. After amplification, for potato cDNA of the tuber, stolon, leaf and flower amplicons ±210 bp were visible on the agarose gel (Appendix A Fig.1 and Fig. 4).

Amplification of the whole *SULTR4;2* was only possible with cDNA of the flower. For flower sample Fl 2 a weak amplicon ±2400-2600 bp was seen on the agarose gel (Appendix A Fig. 5). A second amplification on this product showed a brighter amplicon ±2400-2600 bp (Fig. 6). The size of this amplicon was comparable to the size of the amplicon of previous amplification and was considered to be *SULTR4;2*. The amplicon of sample Fl 2 was further cloned into pENTR™ TOPO® vector.

After transformation of *SULTR4;2* to the entry vector, six colonies grew on LB-agar plates with kanamycin. These colonies A to F were verified for *SULTR4;2* insert with a colony PCR with Pr105x106 (Fig.7). Verification of these colonies was done by sequencing with primers M13F, M13R, Pr105F or Pr106R. The sequencing results were negative for the six colonies A to F.

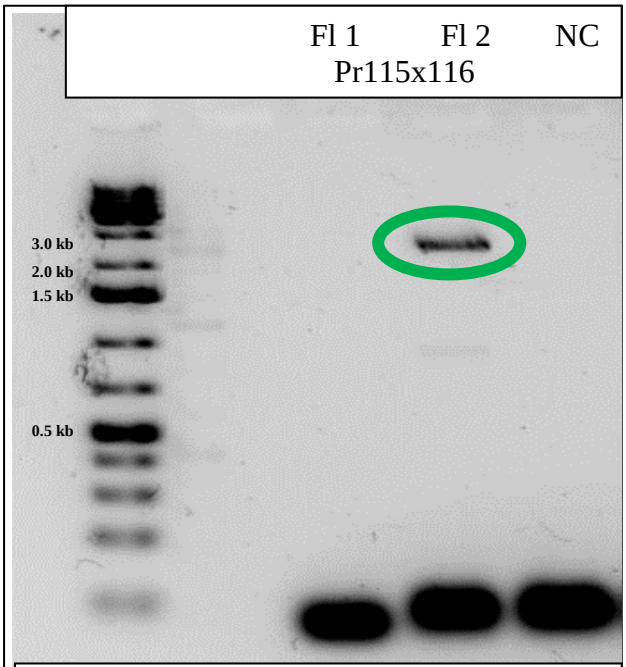

Figure 6: amplification of SULTR4;2 was done with a PCR with Pr115x116. Fl 1= cDNA Kardal flower, Fl 2= 2nd amplification on PCR products of Kardal flower. NC= negative control. Amplicon is highlighted with a green circle for Fl 2. Amplicon size was around 2400-2600 bp.

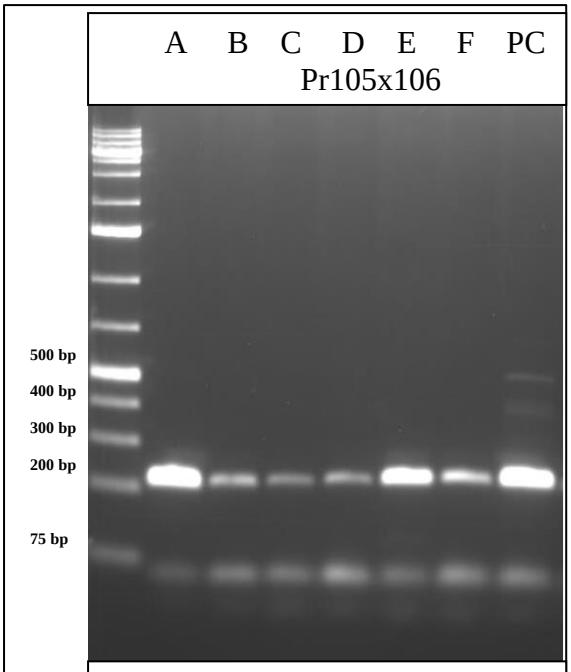

Figure 7 colonies A to F were selected from LB- plates with kanamycin. Positive control (PC) was cDNA of Kardal flower. For samples A-F one amplicon ±210 bp was visible on the agarose gel.

Because the colony PCR confirmed a *SULTR4;2* insert, the possible *SULTR4;2* insert of samples C and F was further placed into PK7GW2.0 and in TOP10 cells. Colonies that grew on LB-agar plates with spectomycine were verified with a colony PCR on the presence of *SULTR4;2* (data not shown). Only sequence results couldn't confirm a possible *SULTR4;2* insert in PK7WG2.0. Since sequence results couldn't confirm the presence of *SULTR4;2,* single colonies were checked for the whole *SULTR4;2* with Pr115x116 (Fig 8.). Results of sample S showed an desired single amplicon ±2400-2600 bp, this amplicon could be the whole *SULTR4;2* insert in PK7GW2.0. Next to that, colonies P and R showed an amplicon ±2000-2500 bp and one amplicon ±1000-1500 bp. The higher amplicon could be *SULTR4;2* insert in PK7GW2.0. The lower amplicon was probably caused by non-specific amplification during PCR. In the future, the presence of *SULTR4;2* in PK7GW2.0 should be confirmed by sequencing, before continuing the cloning experiments.

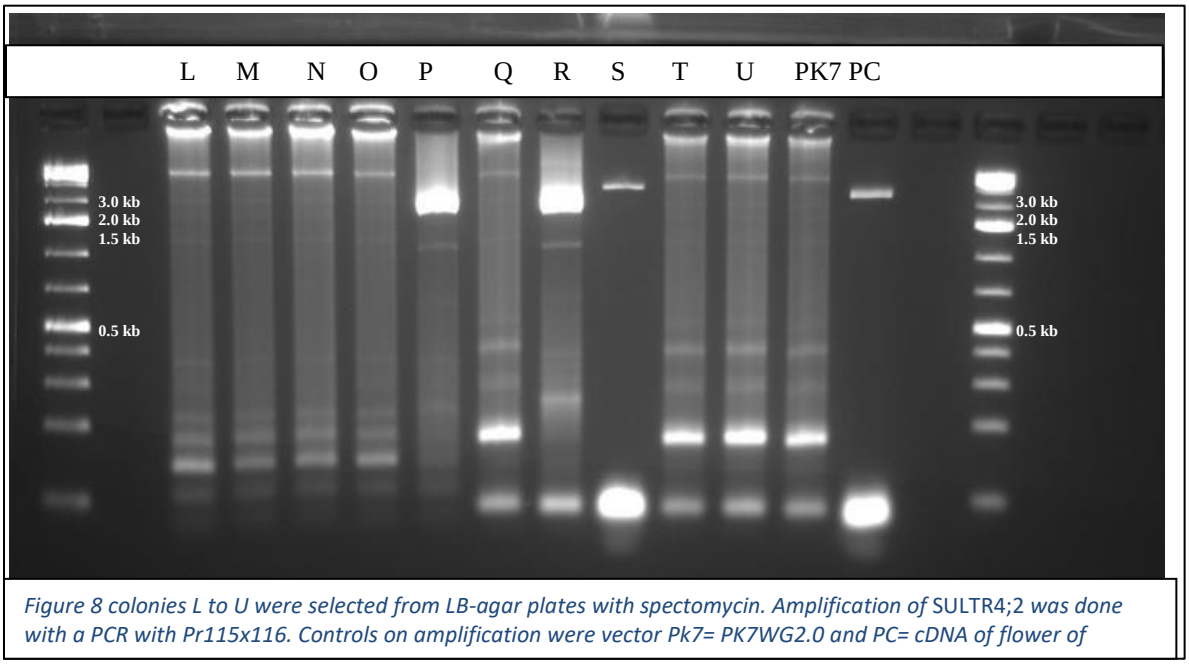

Figure 8 colonies L to U were selected from LB-agar plates with spectomycin. Amplification of SULTR4;2 *was done with a PCR with Pr115x116. Controls on amplification were vector Pk7= PK7WG2.0 and PC= cDNA of flower of*

2.3 *HEX* and *HEX6*

The third candidate gene for cloning to Kardal was *HEX*. For amplification of the whole *HEX,* one set of primers were designed, Pr101xPr102 (237 bp). After amplification of potato cDNA, several amplicons were visible on the agarose gel (Fig. 9). The size of these amplicons vary between 75 bp to 700 bp. One possible amplicon was seen for every cultivar ±200-250 bp that could be the gene *HEX*. Because of non-specific amplification during PCR, further cloning of *HEX* to the entry vector wasn't possible.

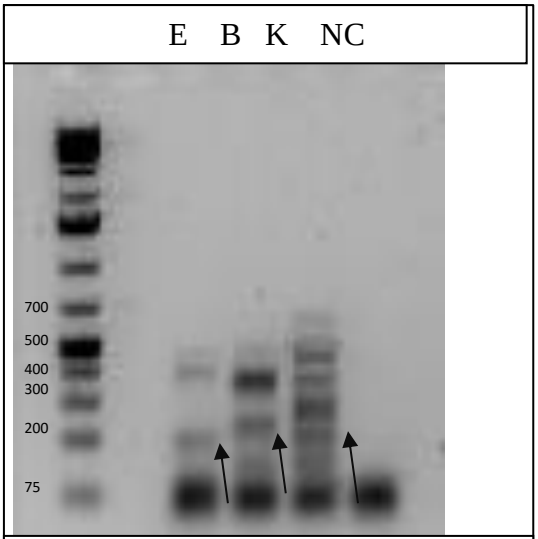

Figure 9 PCR was done on cDNA of three potato cultivars. E=Eigenheimer MT, B=Bintje MT and K= Kardal MT with Pr101x102. NC showed no amplification on the agarose gel. Highlighted with black-arrows are amplicons ±200-250 bp.

With HEX one other problem was seen, the protein sequence *of HEX* seems to be too short to create a functional hexose transporter. Luckily, another gene *HEX6* was found that is located on the same chromosome and same region as *HEX* and could therefore be the target for enhancing protein content in the tuber. For *HEX6* primers were designed that could amplify the whole gene, Pr117x118 (936 bp). For amplification of *HEX6* cDNA of flower, leaf ,stolon, immature tuber

and mature tuber was used. Only, amplification of *HEX6* wasn't possible with the used cDNA (Appendix A Fig. 4 and 6).

2.4 *NRT1.11* presence in GM Kardal NRT1.11 lines

In total there were three series of GM Kardal NRT1.11 lines, these lines presumably contain a *NRT1.11* insert driven by a CaMV 35S promoter. In this study, series 2 of GM Kardal NRT1.11 lines were checked on the presence of *NRT1.11*, the other series 1 and 3 were checked by Jorge Aleman Baez (data not shown). This was done with a PCR with Pr23xPr24 (165 bp/246 bp) on the DNA of GM Kardal NRT1.11 lines. Amplicons ±165 bp on agarose gel showed the cloned insert of *NRT1.11* to Kardal and amplicons ±246 bp showed *NRT1.11* that was present in the genomic DNA of potato.

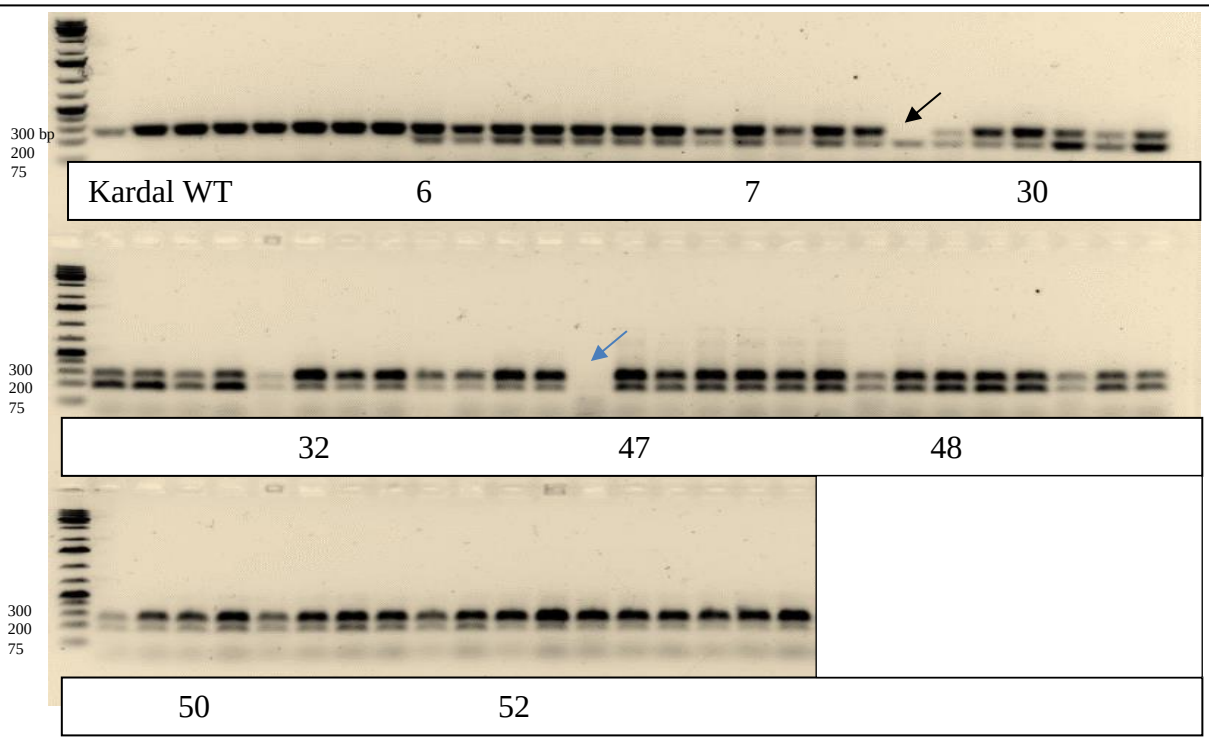

Figure 10 PCR was done on DNA of GM Kardal NRT1.11 lines (series 2) with Pr23x24. The first eight plants were Kardal WT (negative control). GM Kardal NRT1.11 lines 6, 7, 30, 32, 47, 48, 50 and 52 were tested. Every line consist of eight unique plant samples.

Every GM Kardal NRT1.11 line 6, 7, 30, 32, 47, 48, 50 and 52 of series 2 showed at least for one plant an amplicon ±165 bp and an amplicon ±246 bp on agarose gel (Fig.10). The amplification of GM Kardal NRT1.11 line 32 plant 8 didn't succeeded (blue arrow) and for one other GM Kardal NRT1.11 line 7 plant 5 no visible amplicon ±246 bp was seen (black arrow). Probably, amplification failed because of an unsuccessful DNA isolation.

2.5 Gene expression of *NRT1.11* in GM Kardal lines

In previous chapter the insert of *NRT1.11* was proven in GM Kardal lines of series 2 (series 1 and 3 were verified by Jorge Aleman Baez, data not shown). In this chapter, sixteen GM Kardal NRT1.11 lines from series 1 were measured for dCt differences in *NRT1.11* expression compared to Kardal WT. Eight lines: 3, 5, 17-19, 24, 27 and 38 showed a higher dCt expression of *NRT1.11* (Appendix B Table 3). For these eight lines a ddCt difference was found varying between 1,3-9,3 ddCt (Fig. 11).

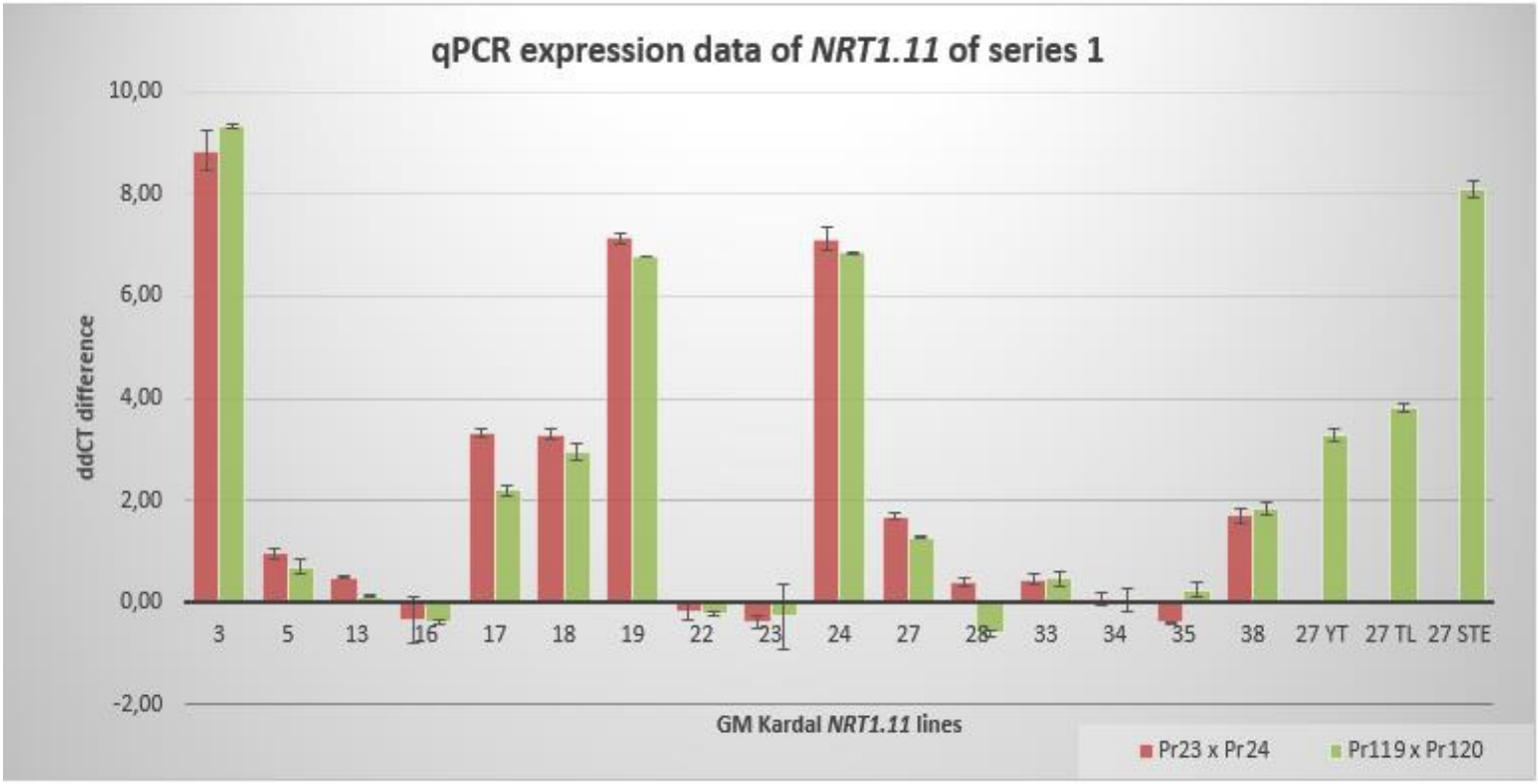

Figure 11 shows ddCt difference of GM Kardal NRT1.11 lines 3-38 mature tuber expressions level of NRT1.11. In red color qPCR data with Pr23x24 and in green color qPCR data with Pr119x120 is seen. Standard deviation is shown for every line (n=2). For line 27 expression of NRT1.11 immature tuber (YT), young leaf (TL) and stem (stem) were also measured.

To asses expression of *NRT1.11* throughout the plant, different tissues of line 27 were analysed. For line 27 was found out that immature tuber had a 3,3 ddCt, young leaf had a 3,8 ddCt and stem had a 8,1 ddCt difference with the *NRT1.11* expression of Kardal WT (Fig. 11). For line 27, an overall higher ddCt was found for these tissues , than for the mature tuber that was only 1,3 ddCt.

The melt-curve data of qPCR run with Pr23x24 and with Pr119x120 were compared to each other, to see if the specificity of qPCR data was correct. For a number of samples two peaks were seen by the qPCR run with Pr23x24. For these samples one melt curve peak was seen at 76 ^{o}C and the other melt curve peak at 81 ^{o}C. The additional lower peak at 76 ^{o}C can affect the expression data of this qPCR run. The expression values of this qPCR run can be slightly overestimated for these samples. The melt-curve data of Pr119x120 showed one singular melt curve peak at 76,5 ^{o}C. The specificity of the *NRT1.11* expression data seems to be better for Pr119x120.

To estimate if there was any relationship between gene expression and the different phenotypic traits , a Pearson correlation was done for the relative gene expression of *NRT1.11* and six traits of the potato: height, SPAD-LL, SPAD-HL, SPC in PFJ, soluble protein content and weight of tubers per plant. No significant correlation was found for these six traits compared to the gene expression data of GM Kardal NRT1.11 lines of series one (Pearson, p-value >0,01)(Appendix B Table 4).

2.6 Phenotype differences in GM Kardal NRT1.11

Phenotypic changes were visible after five to eight weeks of growing of GM Kardal NRT1.11 plants. The main visible changes of GM Kardal NRT1.11 were chlorosis of parts of younger leaves (Fig. 12. A-D) and gradually change of color of stems to a green stem with purple pigmentation (Fig.12 E-F).

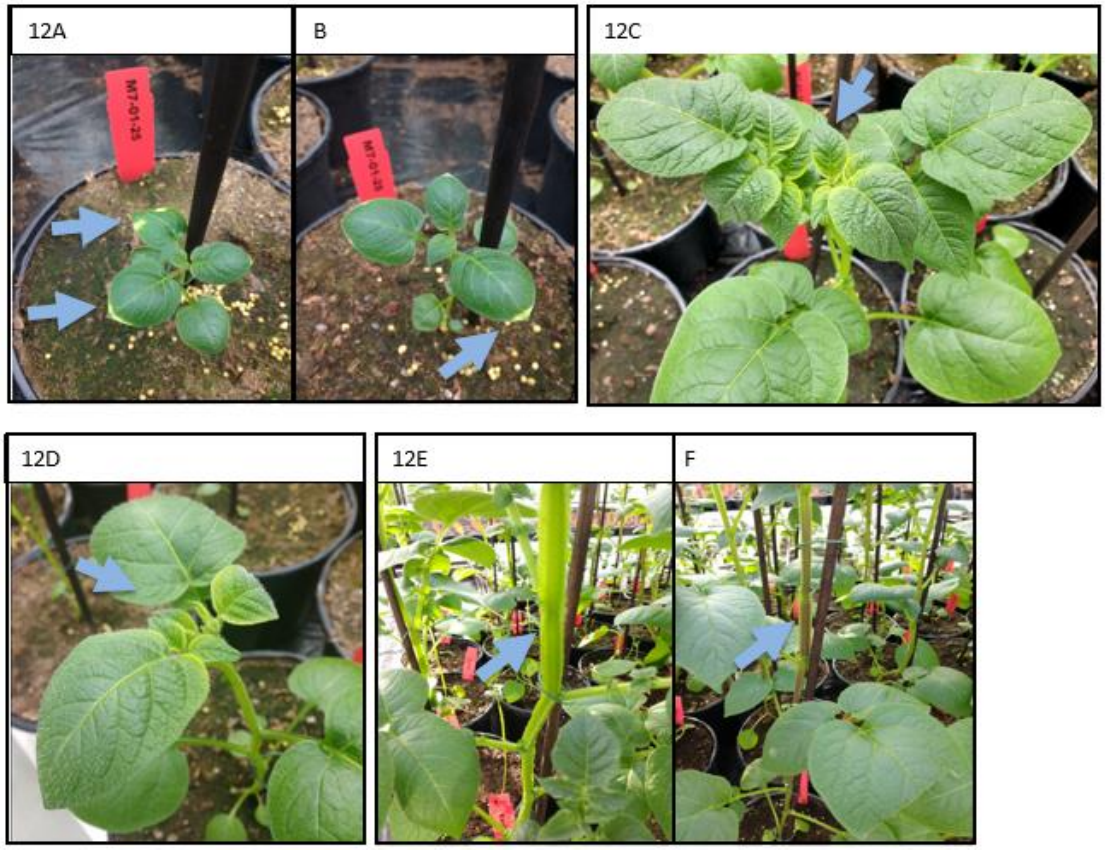

Figure 12 pictures 12A-D show a variety of GM Kardal NRT1.11 lines with places of chlorosis on the young leaves. 12E-F stem color of Kardal WT (12E) and GM Kardal NRT1.11 (12F). Stem color of most GM Kardal NRT1.11 lines showed purple pigmentation after eight weeks of growing

Relative chlorophyll content (SPAD) measurements of GM Kardal NRT1.11 lines were done because of the relation of nitrate metabolism and a possible enhancement of amount of chlorophyll in the leaves (Cardoso, Alvarenga, Melo, Viana, & Matsumoto, 2011; Silva, Fontes, & Miranda, 2009; Zheng et al., 2015). When senescence occur of the source leaves, proteins are transported to these sink tissues (Hsu & Tsay, 2013; Masclaux-Daubresse et al., 2010). Therefore, I expect that developing sink leaves will have a higher chlorophyll content than the source leaves. Thirty-two lines of GM Kardal NRT1.11 were divided in three series. Statistical tests were performed for every series independently. Two out of thirty-two lines: 3 and 30 had a lower SPAD-LL (ANOVA, p-value <0,05) (Fig.13, Appendix B Table 4-6). On the other hand , six lines were found, lines 6, 35 and 48-50, that were higher for SPAD-LL (ANOVA, p-value <0,05). Expectations were that more negative lines would be found for SPAD-LL. Due a possible increase of nitrate transport, foliar senescence in lower leaves can occur sooner, when this happens it can result in lower SPAD-LL values (Have, Marmagne, Chardon, & Masclaux-Daubresse, 2017; Hsu & Tsay, 2013; O'Brien et al., 2016).

In this study, thirteen of thirty-two GM Kardal NRT1.11 lines had higher SPAD-HL than Kardal WT (Fig. 13) (ANOVA, p-value <0,05). No lines were found that had a lower SPAD-HL. There was considered that there would be a negative correlation between SPAD-LL and SPAD-HL.

Instead, when SPAD-LL was compared to the SPAD-HL this gave a weak positive correlation r=0,254 (Pearson correlation, p-value <0,001).

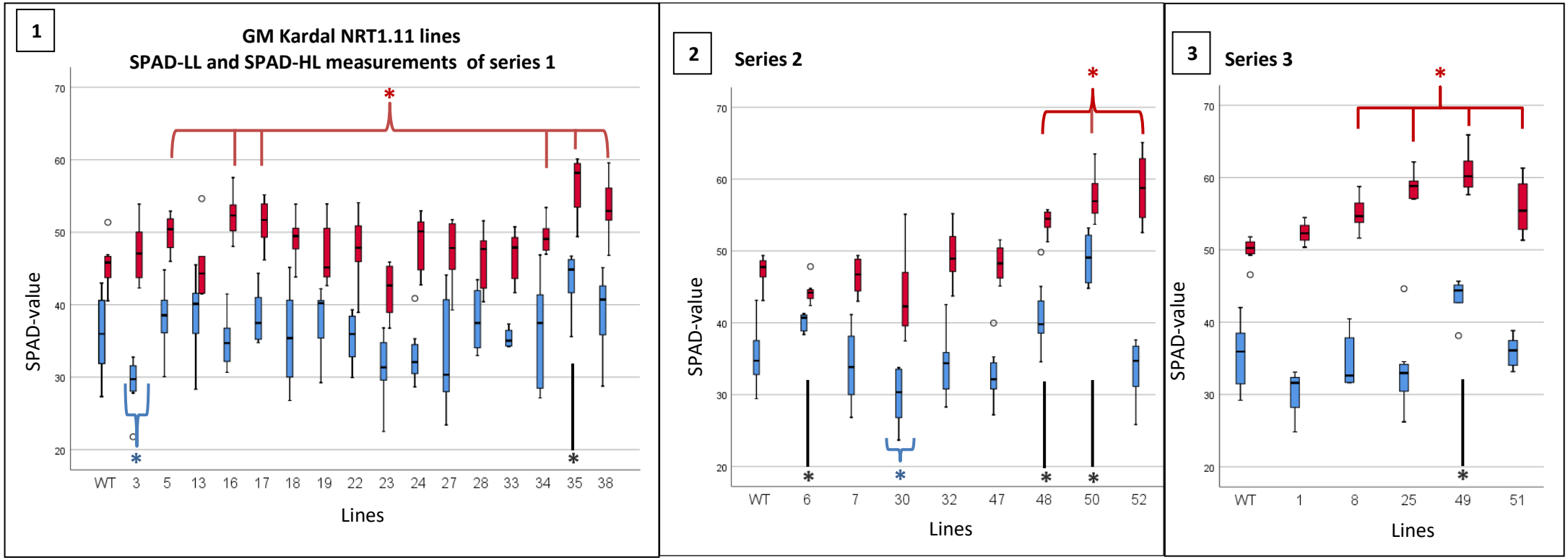

Figure 13 Box-plot representation of the data of SPAD-LL (in red) and SPAD-HL (in blue) of GM Kardal NRT1.11 lines of different series 1-3 (13-1 to 13-3). Error bars cover 95% confidence interval (CI). *Significant differences were marked with an asterisk p-value <0,05. Error bars cover 95% CI.*

Nitrogen and carbon balance is essential for plants growth. Increased nitrogen supply in the plant can positively affect the photosynthesizes, when photosynthesis is more efficient, the transport of carbohydrates to the sink tissue is increased (Tegeder, 2014). One important factor to monitor plant growth is the height of the plant. In this study, three GM Kardal NRT1.11 series were evaluated on the trait height, compared to the Kardal WT. Thirteen lines of thirty-two lines were found to be higher for height (ANOVA, p-value <0,05) (Fig. 14, Appendix B Table 8-10). One line, 22, was found to be lower for height (ANOVA, p-value <0,05).

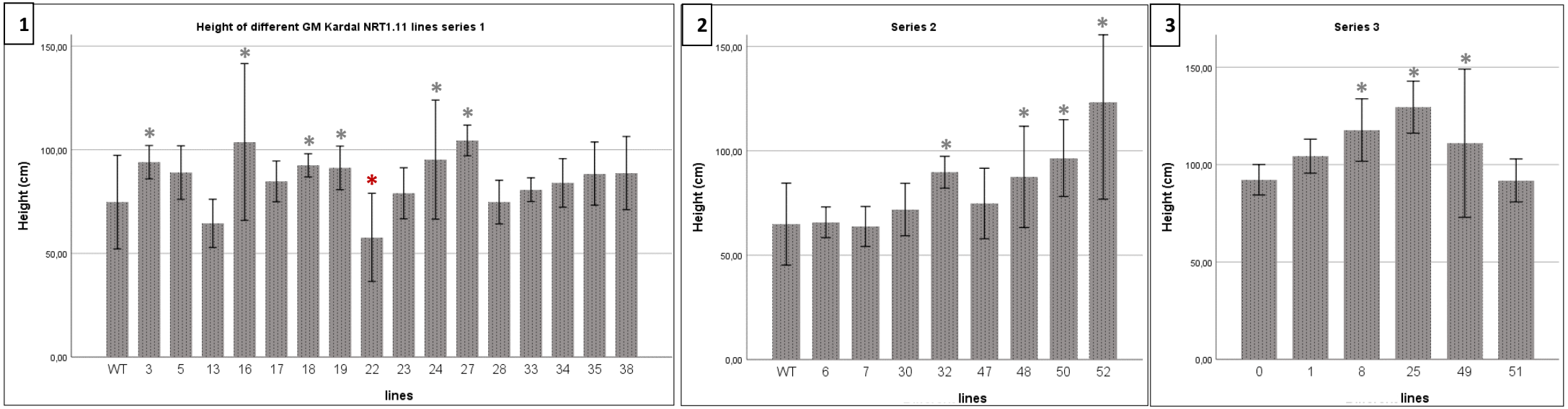

Figure 14 distribution of the mean height data of GM Kardal NRT1.11 lines of different series 1-3 (14-1 to 14-3). Error bars cover 95% CI. Significant differences were marked with an asterisk p-value <0,05.

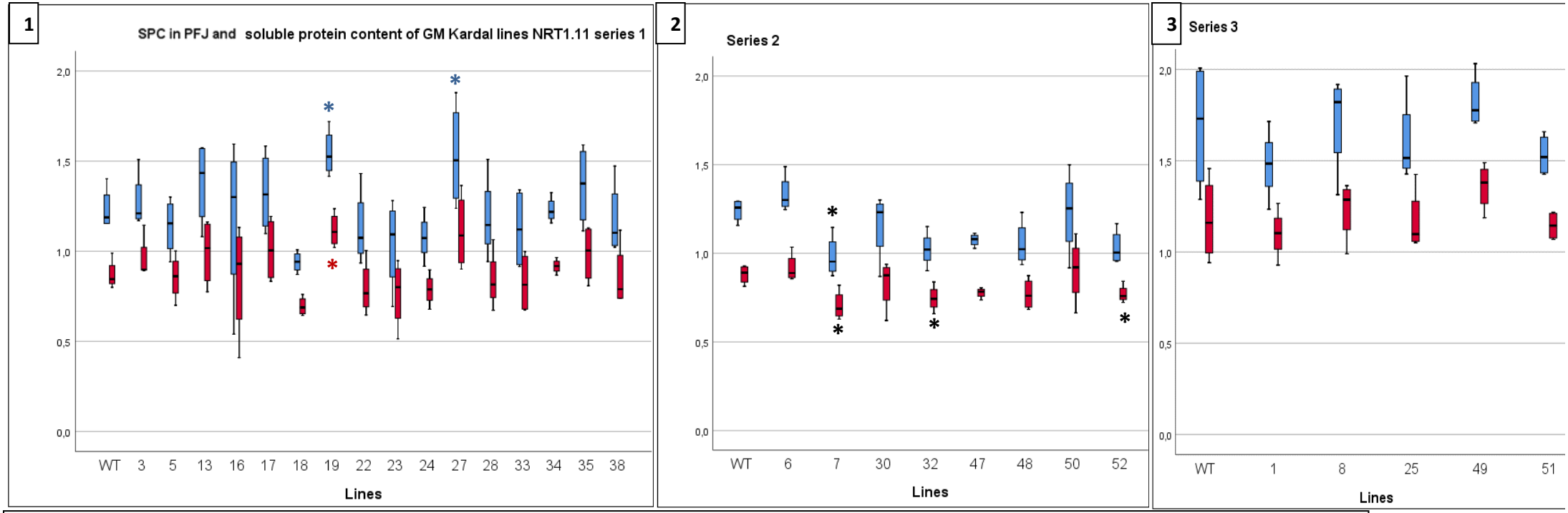

Figure 15 Box-plot representation of the data of soluble protein content (in red) and the SPC in PFJ (in blue) of GM Kardal NRT1.11 lines of different series 1-3 (15-1 to 15-3). Significant differences were marked with an asterisk p-value <0,05. Error bars cover 95% CI.

Overexpressed *NRT1. 11* can be important for enhancing remobilization of nitrogen from source- to sink tissues, nitrogen deficiency in source leaves will lead to senescence, the leaf proteins will undergo proteolysis (Hsu & Tsay, 2013; Masclaux-Daubresse et al., 2010). Available amino acids from this proteolysis are possibly transported and accumulated inside the tuber. In this study, differences between the soluble protein content of GM Kardal NRT1.11 lines and Kardal WT were found. Higher levels of soluble protein content were found for line 19 and 27 (ANOVA, p-value <0,05) (Fig. 15, Appendix B Table 8-10). For only one line, line 7, a lower amount of soluble protein content was found (ANOVA, p-value <0,05). For a higher concentration of SPC in PFJ line 19 was found (ANOVA, p-value <0,05). Lower levels of SPC in PFJ were found for lines 7, 32 and 52 (ANOVA, p-value <0,05).

With a PCA on GM Kardal NRT1.11 lines, possible correlations between the following five traits were evaluated: SPAD-LL, SPAD-HL, height, soluble protein content and the weight of the tubers. With a KMO of 0,541 and Bartlett's test (p-value <0,001), relationships between these five components could be found. For the PCA model, three of the five components could explain 82,3% of the variation (Appendix B Table 11). The 1st principal component showed that the soluble protein content, SPAD-HL and height were correlated with each other (Fig. 16). This correlation of these three traits can occur because overexpression of *NRT1.11* possibly increased the nitrate transport to the sink tissues in the potato. The 2nd component showed only a weak correlation between SPAD-LL and SPAD-HL. The second component showed a possible relationship with the amount of nitrogen that was present in the leaves. For the 3rd principal component a weak correlation was found for the weight of the tubers and the soluble protein content, this is possible because for measuring of the soluble protein content only one potato tuber was used. A correlation between these two traits is therefore possible and can relate to the tuber biomass that is produced belowground.

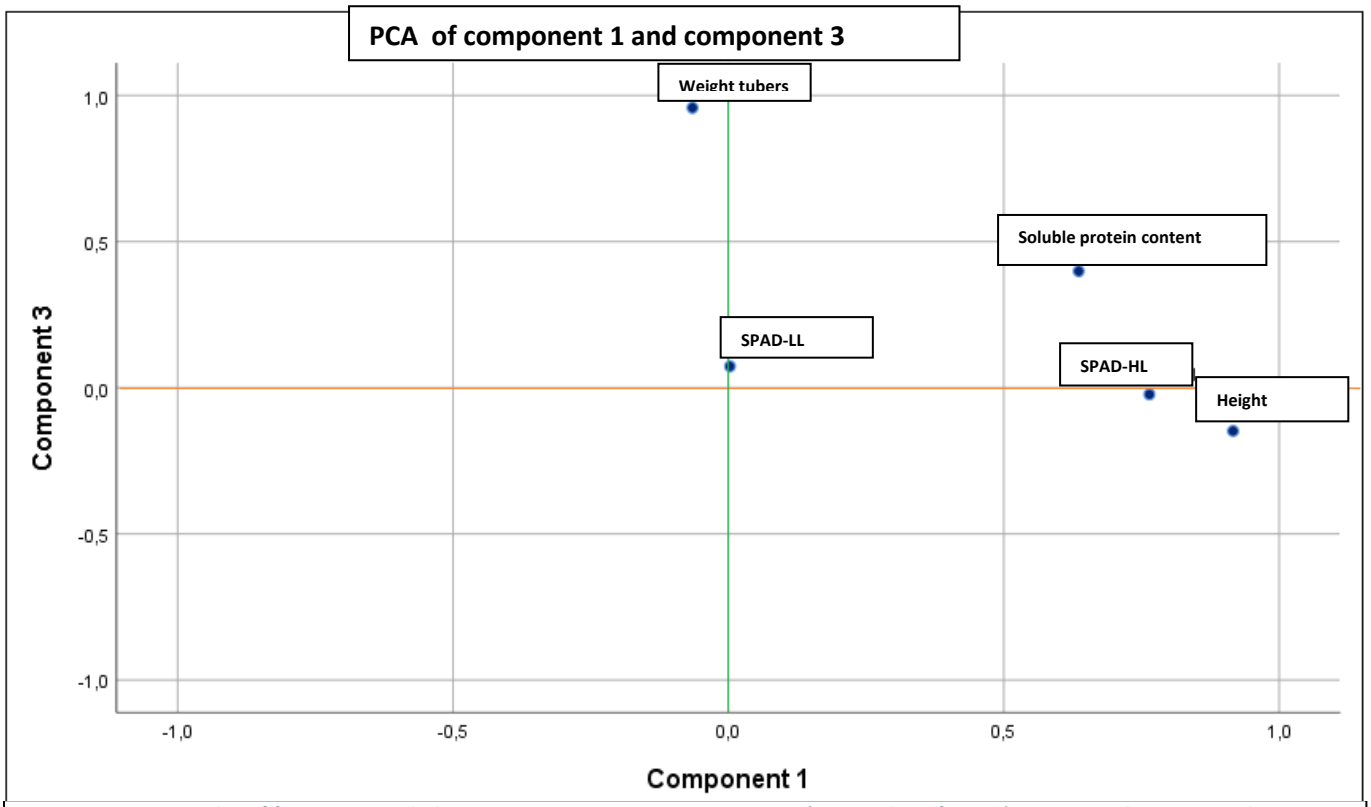

Figure 16 PCA score plot of five traits, each dot represents one trait. Component 1 (eigenvalue of 2,037) is seen on the x-axis and component 3 (eigenvalue of 1,014) is placed on the y-axis.

3. Discussion and conclusions

3.1 *SULTR3;3*

Cloning of *SULTR3;3* in Kardal seems to be successful. At this moment, GM stem explants with a possible *SULTR3;3* insert are growing on selection media. For every GM explant one to four copies of *SULTR3;3* can be present in the genome. These inserts *SULTR3;3* are driven by a CaMV 35S promoter to maintain high expression of *SULTR3;3* during plant development. In a few weeks, gene expression of *SULTR3;3* in different tissues can be measured in these lines. The overexpression of *SULTR3;3* could lead to an increase of transportation of sulphate from source-to-sink compartments. At this moment, there is little known about the exact functioning of *SULTR3;3* in potato.

For what is known in other plants, Zuber and colleagues have found out that *AtSultr3;3* mutants had a decrease of cysteine synthesis in the seeds, because of this finding they suggested that overall sulphur assimilation was reduced in these plants (Zuber, Davidian, Aubert, et al., 2010). In another study of Liao and colleagues was found out that the function of *SULTR3;3* was related to storing of proteins in developmental stadia IV of *P. vulgaris* (Liao et al., 2012). With this knowledge, I expect that overexpression of *SULTR3;3* can have an positive effect on the storage of proteins in the potato tuber. Future experiments, should focus on measurements of cysteine and methionine levels in GM Kardal SULTR3;3 lines. Next to that, *in vitro* experiments should be done with GM Kardal SULTR3;3 lines that grow on MS20-media with different sulphate concentrations, this to see if any phenotype differences are seen.

3.2 *SULTR4;2*

Amplification of *SULTR4;2* was more challenging than of *SULTR3;3*. Only when a different cDNA reverse transcriptase kit was used of Applied Biosystems, an amplicon of *SULTR4;2* was found with flower cDNA of Kardal. The results showed that cloning of *SULTR4;2* to the entry vector and to destination vector seemed to be successful. Only sequence results couldn't confirm an *SULTR4;2* insert into the vectors. The most likely cause of this is that a part of the *SULTR4;2* internal primers have a high similarity to the sequence of the vectors, which gave overlapping peaks in the sequence data. Another factor could be that insufficient amount of purified vector with insert was available for sequencing. Further experiments should be done to see what fragment of *SULTR4;2* is inserted in the destination vector. Primers should be designed on the outer edges of *SULTR4;2* with a low similarity to the vector sequence. In the near future, it should be possible to create GM Kardal SULTR4;*2* lines.

In research of Gallardo and colleagues was seen that *SULTR4;2* accumulation allowed transport of stored sulphate in the vacuole to the cytoplasm, this to manage the plant stress when the uptake of sulphate is limited (Gallardo, Courty, Le Signor, Wipf, & Vernoud, 2014). When *SULTR4;2* is overexpressed into Kardal, a possible function can be that additional sulphate is delivered to chloroplasts and mitochondria, which could increase the protein synthesis in these plant cells. Therefore, for GM Kardal SULTR4;2 lines it can be useful to analyse these lines on MS-20 media with different sulphate concentrations. After *in vitro* experiments of these lines experiments should be done in the greenhouse, this to see if any phenotypic changes are stable in a more natural environment.

3.3 *HEX*

Last target for cloning to potato was the *HEX* gene. The amplification of *HEX* gave some difficulties. Different PCR conditions, potato cultivars, reverse transcriptase kits and different tissues were used to overcome this problem. In the end, presumably *HEX* and non-specific products were amplified during PCR. *HEX* is currently annotated as PGSC0003DMT400037213 and consist of only 79 amino acids, this suggest that *HEX* is a pseudogene in potato. This explains why *HEX* is low expressed in potato tissue (Appendix A Table 3) (SPUDdatabase, 2018). Another option is that the annotation of *HEX* is not correct. The RNA-seq expression patterns of *HEX* support this option. The RNA-seq expression of *HEX* doesn't correspond to the exon region of the gene (Fig. 17).

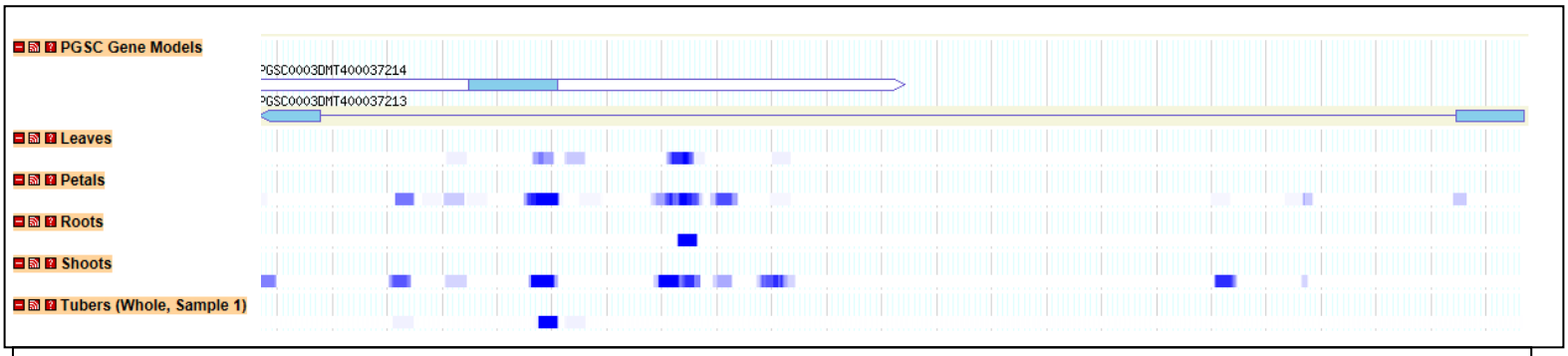

Figure 17 HEX *of PGSC0003DMT400037213 RNA-seq expression data of potato leaves, petals, roots, shoots and tubers. Higher expression is shown in a brighter color blue, white means no expression was seen in that region of the genome (SPUDdatabase, 2018).*

In the case that the annotation of *HEX* is incorrect, another gene should be present in the same region that can have a positive effect on the protein content in the potato tuber. After comparing sequence data of potato, the most likely other candidate gene in this region is *HEX6*. *HEX6* corresponds to sequence identifier Sotub03g014170.1.1 and is located on chromosome 3 position 43,117,444 bp to 43,119,568 bp. When *HEX6* (311 amino acids) of potato is alignment with crops such as: crops castor bean (*Ricinus communis)*, grapevine (*Vitis vinifera)* and tomato (*S. lycopersicum)*, a high similarity in protein sequence is found for 245-308 amino acids. In these crops, *HEX6* is translated and seems to be functional as hexose transporter (Hayes, Davies, & Dry, 2007; Reuscher et al., 2014; Weig, Franz, Sauer, & Komor, 1994). A phylogenetic tree was made to see the relationship of the *HEX6* of potato compared to other crops (Appendix A Fig. 7) (NCBI, 2018). *HEX6* of potato seems to be most related to *S. lycopersicum STP15 (HEX6)*. It seems that *HEX6* is quiet conservative between species. In this study, primers were designed to amplify the predicted *HEX6*. Only, the amplification of *HEX6* with cDNA of the potato tissue wasn't successful during this study. With future experiments amplification of *HEX6* should be done on genomic DNA of potato, this to be certain that *HEX6* is present in potato.

Currently little is known about the function of *HEX6*. To study the function of *HEX6*, it should be beneficial to create GM Kardal HEX6 lines. For what is known of hexose transporters is that in *V. vinifera*, hexose transporters correlate positively with an increase accumulation of proteins during fruit development (Lecourieux, Lecourieux, Vignault, & Delrot, 2010). In another crop, cucumber (*Cucumis sativus*), was seen that hexose transporters contribute to phloem unloading of hexoses to the fruit *(Cheng et al., 2015).* If the function of hexose transporters is conservative between plant species, then it could be that by overexpressing of a hexose transporter *HEX6* in potato, more proteins will accumulate in the tuber.

3.4 *NRT1.11*

From every GM Kardal line it was possible to confirm the *NRT1.11* insert. On the other hand, it was not possible to see how many copies of *NRT1.11* were present in the GM Kardal genome. The amount of copies of *NRT1.11* are related to the gene expression of *NRT1.11*, more copies of the gene means a higher gene expression level (Bubner & Baldwin, 2004; Pappas, 2008). For series 1 of GM Kardal NRT1.11 was found that eight of sixteen lines have higher expression of *NRT1.11* in the mature tuber. For line 27 was found that in young leaf, immature tuber and stems higher expression levels of *NRT1.11* were present than in the mature tuber of this line. In the near future, it is important to see if this characteristic is seen in all other GM Kardal NRT1.11 lines. High expression of *NRT1.11* in the potato, presumably explain phenotypic differences, such as green stems with a purple pigmentation and chlorosis of younger leaves (Fig. 12). In potato the accumulation of anthocyanins in the stems can be the cause of the change in stem color (Payyavula, Navarre, Kuhl, & Pantoja, 2013). The chlorosis of younger leaves could occur by a disruption of the nitrate transport in apical meristems during plant development (Ojala, Stark, & Kleinkopf, 1990).

In this study, it seems that with GM Kardal NRT1.11 lines two plant traits were positively affected SPAD-HL and height. For SPAD-HL and height thirteen of thirty-two transgenic lines gave significant higher values than the Kardal WT. These significant lines were not the same for the traits. It could be that overexpressed *NRT1.11* improves the overall nitrogen transport in potato. SPAD-HL and height are partially depended on the efficiency of nitrogen transport and distribution of nitrogen in plants. A higher accumulation of nitrogen in the sink tissues could increase the synthesis of chlorophyll in the plant cell and increase the plant development (Tegeder & Masclaux-Daubresse, 2018).

It was expected that overexpression of *NRT1.11* in potato increased senescence of source leaves. With SPAD-LL measurements was found that only two GM Kardal NRT1.11 lines were significant lower than the Kardal WT. For this moment, this number of lines is too low to estimate if there was an negative effect on the chlorophyll levels in the leaves. Measuring of senescence of the leaves was difficult, because of finding the correct time point for foliar senescence in all source leaves simultaneously. These difficulties reduces the specificity for SPAD-LL measurements in this study.

On the other hand, two GM Kardal NRT1.11 lines, lines 19 and 27 were found that had a higher soluble protein content in the potato tuber. Because of the low number of lines that enhance the protein content, it is uncertain if this is caused by overexpression of *NRT1.11* in the plants or by environmental conditions. The performed PCA showed a correlation between traits height, SPAD-HL and soluble protein content. Increased nitrogen uptake and transport to the potato sink tissue, could have an positive affect on the protein content in the tuber (Bartova, Barta, Svajner, & Divis, 2012; Tegeder, 2014). For this study, the experimental set-up of GM Kardal NRT1.11 lines in the greenhouse made it more difficult to correct for any environmental factors (circadian differences, gradient of fertilizer differences, pathogens in the greenhouse etc.) or to correct for any errors (errors concerning measuring and analysing the traits). To overcome some limitations of the experimental set-up, statistical analyses were done for three separate series, but the cost for this was that the statistical tests had lower statistical power to find any significant differences for the traits. In the near future, with an improved experimental set-up, there is a chance that more GM Kardal NRT1.11 lines will be found that have a higher protein content in the tuber than the wild type.

NRT1.11 is important for further assimilation of amino acids and transport to sink leaves through the stems (Hsu & Tsay, 2013). Higher expression of *NRT1.11* in potato can therefor positively affect traits such as height, chlorophyll content and protein content in the potato. Because of the complexity of the nitrogen transport, other nitrate transporters and regulators can be the limiting factor for increasing protein content in the tuber. In *A. thaliana* is known that genes *NRT1.1*, *NRT1.2* are involved for retrieval of NO_3^- from soil to roots (O'Brien et al., 2016). These genes could perhaps be bottlenecks for the nitrate transport GM Kardal NRT1.11 lines. Further understanding of interaction of *NRT1.11* to other NRT genes can gave more insight of possible bottlenecks of the nitrate transport in potato.

4. Recommendations experimental set-up

For fundamental purposes, it can be useful to monitor the protein accumulation in the tuber by developing a reverse genetic approach in potato. A possible way to do this is by silencing target genes *SULTR3;3*, *SULTR4;2*, *HEX6* and *NRT1.11* with virus induced silencing (VIGS) in potato leaves or tubers (Faivre-Rampant et al., 2004; Senthil-Kumar & Mysore, 2011). Another option for silencing of these genes is with an Agrobacterium-delivered CRISPR/Cas9 system, this can be an excellent tool for targeted mutagenesis of genes in potato (Andersson et al., 2017; Butler, Atkins, Voytas, & Douches, 2015; S. H. Wang et al., 2015).

Furthermore, greenhouse experiments with GM Kardal NRT1.11 lines should be done with a complete randomized block design (Jensen, Schaarschmidt, Onofri, & Ritz, 2018). For these greenhouse experiments, the number of plants per GM Kardal NRT1.11 line should increase and these lines should be planted on the same date. This will increase the power of statistical tests that can be done afterwards. At this moment, the most promising lines for a higher soluble protein content in the potato tuber, seems to be GM Kardal NRT1.11 line 19 and 27.

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