Cloning and transformation of candidate genes to study the protein content in potato (*Solanum tuberosum* L.)

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Abbreviations

AAP	Amino acid permease gene family
BAP	6-Benzylaminopurine
bp	Base pair
cDNA	Complementary Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid
ETR	Ethylene receptor
GABA	Gamma amino butiric acid
HCP	Hexose carrier protein
LB	Lysogeny Broth medium
LHT	Lysine/histidine transporter
MS	Murashige & Skoog growing medium for vegetal material
NAA	1-Naphthaleneacetic acid
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
PINs	Protease inhibitors potato proteins
QTL	Quantitative trait locus
RNA	Ribonucleic acid
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
SOC	Super Optimal Broth with Catabolite repression medium
STR	Sulfate/bicarbonate/oxalate exchanger and transporter
TAE	Tris-acetate-EDTA buffer
TE	Tris-EDTA buffer
USDA	United States Department of Agriculture
ZCVK	Zeatin, Cefotaxime, Vancomycine and Kanamycin selection medium

Abstract

Potato proteins have become highly valuable for the Chemical and Food industries in The Netherlands. Protein content of potato (*Solanum tuberosum* L.) tubers is low (up to 3% in a fresh weight basis) and whether the protein content can be increased and its implications is a current research topic. Six candidate genes have been identified based on GWAS and homolog genes from other crops studies to investigate the protein composition and its function in potato. This work focused on the molecular cloning and transformation processes of the candidate genes aiming to produce transgenic potato plants. Experiments were performed to amplify and clone the candidate genes into the Gateway® cloning system which facilitates further transformation into *Agrobacterium tumefaciens*. The Gateway® cloning system consists of an entry clone (pENTR™ TOPO® vector) and a destination vector (PK7GW2,0 binary vector) which are recombined to produce an expression clone with the gene of interest. Kanamycin resistant and potential Nitrate transporter (NRT) transformed plants were generated through *Agrobacterium*-mediated transformation. However, verification for the presence of the NRT construct remains to be assessed. The new potential transgenic lines can be subjected to gene expression analysis to evaluate the effect of the NRT gene in these plants. Sulfate/bicarbonate/oxalate exchanger and transporter (STR) gene was partially confirmed in the pENTR™ TOPO® vector remaining for verification before performing further cloning steps. Ethylene receptor (ETR) and Lysine/histidine transporter (LHT) gene fragments were amplified from PCR. HCP and AAP genes were not amplified from cDNA. Cloning and transformation of the candidate genes towards the acquisition of valuable knowledge on the protein content in potato still remains to be completed.

Keywords: *Agrobacterium tumefaciens, Agrobacterium*-mediated transformation, Gateway, Molecular cloning, Potato proteins.

1 Introduction

1.1 Background

Potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world after rice, wheat and maize (Camire, 2016). Its nutritional value has been described extensively and variation can be found according the purpose of the cultivated varieties. Potatoes are mostly cultivated for different market segments such as processing, French fries, crisps, starch and fresh consumption (Singh *et al.,* 2016). Among the qualities of starch potatoes, starch content is the most known and studied property to date. Potatoes are known as a good source of energy as the predominant components are carbohydrates as well as proteins, minerals and vitamins (Navarre *et al.,* 2016). According to the USDA, the average nutritional content of potatoes show the following values per 100 grams of boiled potatoes with skin and peeled before consumption: water 77 grams; carbohydrates 20 grams; energy 87 kilocalories; protein 1.87 grams; fat 0.1 grams; calcium 5 milligrams; potassium 379 milligrams; phosphorus 44 milligrams; iron 0.31 milligrams; niacin 1.44 milligrams; thiamin 0.106 milligrams; riboflavin 0.02 milligrams. In The Netherlands, potato is one of the main starch producing crops for food processing with around 15% of the arable land covered with potatoes (Eurostat, 2016).

Food and pharmaceutical industries use the starch as a basic component for the production of several items and food specialties for human consumption by different processes (Survase, *et al.,* 2016). The process of starch extraction involves different biochemical processes and the use of high amounts of water. In the past, after starch extraction, potato fruit juice (further referred as PFJ) remained as a waste of the process. Nowadays, it is mostly a low-value side-stream with an increasing value due to the protein content which can be extracted from the PFJ (Wageningen University, 2016; Michiel Klaassen, personal communication). Besides starch, potatoes also contain proteins which have been described to have interesting nutritional qualities when compared to proteins from egg and soy. One remarkable property of potato proteins (such as patatin – see paragraph below) is that it causes low allergic impact to humans in contrast to gluten or casein from wheat and milk respectively (Alting *et al.,* 2011).

The soluble protein content in potato is variable ranging from 1% up to 3% in some varieties on a fresh weight basis. The protein content in potato is contained in the PFJ which remains after starch extraction. Since approval of potato proteins as a source of food products, several extraction techniques and methodologies have been developed. Three major classes of proteins have been described for potato: Patatin, protease inhibitors (PINs) and other proteins (Waglay & Karboune, 2016). Patatin and PIN proteins are the most abundant accounting for around 40% for each of these classes of protein (Alting *et al.,* 2011). When compared to vegetal proteins obtained from crops such as wheat and soy, potato proteins have the highest quality and nutritional values. The nutritional value of potato proteins is comparable to animal originating proteins such as egg and milk proteins (Alting *et al.,* 2011). Therefore, an increasing interest of the pharmaceutical and food industries has arisen due to particular characteristic of potato proteins on their techno-functional properties (Creusot *et al.,* 2011), antioxidant behaviour (Kudo *et al.,* 2009) and high nutritional value (Bártová & Bárta, 2009).

For the food industry, protein from potatoes are becoming more and more important for many companies especially in the European Union. One clear example is the Dutch company AVEBE which mainly processes starch from potatoes. Their interest for the proteins contained in potato have promoted serious efforts in research aiming to improve the protein content in potatoes which have already started a couple of years ago (Rink Vegelin & Peter Bruinenberg, personal communication). The aim of the project is to study the protein composition of potato and its implications. To start investigating whether the protein content can be effected or not, cloning and transformation of the different candidate genes are the first steps to take. This thesis report focuses on aspects of molecular cloning and transformation of the candidate genes aiming to produce transgenic plants which allow us to elucidate the functioning of potato proteins in further studies.

1.2 Candidate genes

Genetic studies, functional genomics and proteomics analyses are available tools to study the factors involved in the protein content of potatoes (Barsan, 2016). These factors are genes present in the potato genome which have been identified within quantitative trait locus (QTLs) and a couple of candidate genes have been identified (preliminary results at the Bio-based Economy group). To date, two genetic studies have been reported on protein content in potato (studies from Acharjee, 2013 and Werij, 2011). The QTLs identified in these genetic approaches were shown to be present on chromosomes 1, 3 and 5. Based on GWAS studies in potato and other crops, Nitrate transporter (NRT), Sulfate/bicarbonate/oxalate exchanger and transporter (STR), Hexose carrier protein (HCP) and Ethylene receptor (ETR) genes were identified. Lysine/histidine transporter (LHT) and Amino acid permease (AAP) genes were found by searching for homologs in other crops (Wageningen University, 2016; Dianka Dees, personal communication). Moreover, the identified candidate genes are thought to influence the protein content in potato by different mechanisms. Research studies in the model plant *Arabidopsis thaliana*, rice (*Oriza sativa*) and wheat (*Triticum aestivum* L.) have provided insight in the potential roles of the candidate genes. Long transport of amino acids from source to sink organs or the accumulation of amino acids in leaves for protein formation are some examples of the roles identified for the different genes. In other crops, such as pea (*Pisum sativum* L.) and soy (*Glycine max* L. Merr.), the genes responsible for the protein content are known. Orthologous genes from pea and soy in potato have been selected to be studied on their role influencing the protein content in potato. Further studies with these candidate genes are necessary to elucidate whether the protein content can be effected. If protein content is increased in potato, one of the questions to address is: at what extent the protein content can be increased? The present work focuses on aspects of the molecular cloning of the six identified candidate genes.

1.2.1 Nitrate transporter (NRT).

Nitrate transporter genes belong to a big family composed by several genes with diversity of functions. A profile study on the expression patterns in roots and shoot of three weeks old wheat plants was performed by Buchner & Hawksford (2014). Transcript analysis showed differences between the NRT gene family, for example, TaNPF6.1 and TaNPF6.2 genes were highly expressed in the roots but in low amounts in the shoots whilst other genes showed similar transcript quantities in roots and shoots (Buchner & Hawksford, 2014). Gene expression studies in potato

were performed by M'hamdi *et al.,* (2016) using tubers for its assays. StNRT1.2, StNRT1.5 and StNRT2.1 genes were tested in different cultivars and assessed for levels of nitrate accumulation. The analysed genes were identified based on similarities with the *Arabidopsis* NRT homologs. At harvesting time the expression of the genes mentioned above were also expressed in leaves and tubers suggesting a role on mobilization of nitrate from source to sinks in potato (M'hamdi *et al.,* 2016). Starvation treatments to wheat seedlings revealed a reduction of nitrate content by 10% in roots and less than 1% in shoots compared to the controls. Intriguingly, the expression of NRT1 in roots was not affected by nitrate starvation nor induction. Moreover, the expression levels of NPF (Nitrate Transporter 1/Peptide Transporter family) genes such as TaNPF6.3 was found to be decreased in shoots treated with nitrate starvation. Conversely, the expression of TaNPF4.1 was up-regulated by nitrate starvation as well as nitrate induction treatments (Buchner & Hawksford, 2014). Furthermore, genes AtNRT2.1, AtNRT2.4, AtNRT2.5 and AtNRT2.6 (NTR2 gene family) from *Arabidopsis* were generally expressed in the roots. However, AtNRT2.7 was found to be expressed in shoots and aerial plant parts (Orsel *et al.,* 2002). These studies are examples of the importance of NRT genes and the mobilization and allocation of nitrate through the whole plant system.

Wang *et al.,* (2009) studied the NRT1.1 gene function in *Arabidopsis* mutants and concluded from that NRT1.1 gene not only plays a role in transport of nitrate but also may function as a nitrate sensor. These findings were supported by a reduction of nitrate uptake observed in the cells where the nitrate sensors are located. Since NRT1.1 is able to transport and sense nitrate, a proposed function as a "transceptor" was addressed. However, when nitrate-restricted experiments were performed, the NRT1.1-dependent regulation was lost while expression of endogenous genes was observed. The latter suggests the presence of other nitrate sensing systems and nitrate sensing function of NRT1.1 could not be attributed only to this gene (Wang *et al.,* 2009). Accumulation of nitrate was credited to the expression levels of NRT genes, showing differences between cultivars suggesting a potential function on nitrate accumulation in tubers (M'hamdi *et al.,* 2016). In potato, enhanced transport of nitrate likely influence the biomass of the plants. Whether the protein content in tubers can be effected still needs to be evaluated. Nevertheless, the findings from the study of M'hamdi *et al.,* (2016) suggests that an increased accumulation of nitrate in tubers possibly effects the protein content. Transgenic pea (*P. sativum*) plants increased the biomass suggesting a modified metabolism in the Nitrogen uptake and transport from roots to shoots. The NRT pea homolog of the NRT1 from *Arabidopsis* was upregulated in transgenic plants by up to 14-fold. The total Nitrogen content in roots was increased by 19% and the free amino acids content was enhanced to 22% and 56% higher than the wild type in transgenic pea lines (Zhang *et al.,* 2015). In case that enhanced accumulation of nitrate in tubers or higher biomass production by higher uptake of nitrate were possible and the protein content indeed were effected, the immediate question to address is what would be the impact and counter effects of NRT genes in potato. Several NRT genes have been found to be active within the whole plant, consequently, experiments with transgenic lines expressing one single NRT gene from potato may not be sufficient to conclude a definitive role of NRT genes.

1.2.2 Sulfate/bicarbonate/oxalate exchanger and transporter (STR).

Sulfate/bicarbonate/oxalate exchanger and transporter STR genes are thought to be related to transport activity of sulfate, to activate sulfate transmembrane transport and have been found in plasmodesma and membrane (Spud DB, 2016). Genes related to sulfate/bicarbonate/oxalate exchanger and transporters have been described in chickpea (*Cicer arietinum*), wheat (*T.*

aestivum), Indian mustard (*Brassica juncea* L.), tobacco (*Nicotiana tabacum*) and soybean (*G. max*). The genes Ca_02835 and Ca_13487 were found to be expressed in roots, flower buds, mature flowers, pods and stems (Li *et al.,* 2012). In soybean, Glyma07g00840.1, Glyma08g22120.1, Glyma13g43670.1 and Glyma15g.01710.1 were found to be expressed in roots, hypocotyls, seeds, leaflets, seedlings, root hair cells, seed coats and few on mature leafs (Li *et al.,* 2012). Sulfate accumulation was assessed on 2 and 3 weeks old hydroponic wheat plants under sulfate-sufficient and sulfate-restricted conditions. Generally, under sulfate-sufficient conditions the biomass was increased 2 and 3-fold in shoots and roots respectively. Under sulfate-restricted conditions, only the root biomass was found to be increased. The plants were grown until anthesis under sulfate-sufficient conditions during four weeks to evaluate the gene expression. The four-week period correspond to grain filling stage. After the first week from anthesis, a reduction of the sulfate content in glume/lemma tissues was detected, however, the reduction of sulfate was not spotted in leaf and sheath tissues until the second week after anthesis. When compared to the sulfate-sufficient plants, sulfate-restricted conditions treated plants did not decrease the sulfate content in grains, suggesting that accumulation of sulfate needed for grain filling occurred before anthesis (Buchner *et al.,* 2010).

Abdin *et al.,* (2010) transferred the *S. lycopersycum* STR gene (LeST1.1) to indian mustard (*Brassica juncea* L.) through *Agrobacterium*-mediated transformation. Almost all transformed plants were found to be higher in sulfate uptake capacity compared to untransformed plants within three sulfate concentrations (25, 50 and 1000 uM SO_4^{2-}). One of the generated transgenic lines presented a 2-fold increase in the sulfate uptake capacity which was suggested to be a consequence of high expression levels of the LeST1.1 gene. The chlorophyll content was measured to detect any possible symptom of sulfur deficiency. Transgenic plants showed no visible symptoms whereas the untransformed plants presented severe deficiency symptoms (Abdin *et al.,* 2010). Similarly, Ding *et al.,* (2016) studied the GmSULTR1;2b gene to elucidate the effect of sulfate accumulation in soybean and tobacco. The expression of GmSULTR1;2b was upregulated 7-fold under sulfate-restricted conditions compared to the controls. Quantitative real time PCR revealed upregulation of the GmSULTR1;2b only in the roots. Transformed tobacco plants showed an increase of 11% in seed yield from overexpressing GmSULTR1;2b plants. Moreover, the biomass of two-month-old tobacco plants was 18% higher under sulfate-sufficient conditions compared to control plants. Interestingly, the overexpressing GmSULTR1;2b plants showed less severe chlorosis under sulfate-restricted conditions suggesting an enhanced sulfate uptake capacity of the plants (Ding *et al.,* 2016). Sulfate as an important nutrient in potato can be determinant under low supply conditions causing yield losses. Sulfate deficiency in potato might affect negatively the synthesis of sulfur-based amino acids altering the production of proteins. In contrast, enhanced uptake and assimilation of sulfate might promote a better performance in potato. Besides playing roles in the synthesis of amino acids, sulfate have also been related to tolerance under nutrient limited conditions. Overexpressing STR genes in potato can be expected to enhance the biomass production of the plants. Whether an increment in biomass might lead to higher protein accumulation remains to be addressed.

1.2.3 Ethylene receptor (ETR).

Ethylene receptor genes (ETR) are known to be involved in several processes and different life stages of plants from seed germination, root development to senescence of plants. In potato, ETR genes have been reported to be expressed in tubers (young and mature), stolons, stems,

leaves and flower parts such as petals, sepals, petioles and stamen. Furthermore, ethylene receptor genes might be also functioning in callus formation of young plantlets. ETR genes also respond under abiotic stress for example to drought conditions (Spud DB, 2016). The hormone ethylene has been described to function as a signal molecule under biotic stress caused by wounding by insects or mechanical wounding and Ethylene receptors are classified in two subfamilies (Spud DB, 2016). ETR1 genes have been described to be involved in signalling roles related to defense responses of *Arabidopsis* (Pajerowska *et al.,* 2005) and a wide range of studied plants. Ethylene genes have also been studied for its influence and interaction with other genes. For example, ethylene-mediated signalling pathways in *Arabidopsis* showed a similar phenotype in wild type plants treated with ethylene and mutants lacking glucose sensitivity. Faster germination, smaller plants and darker coloured rosettes were similar characteristics observed in *Arabidopsis*. Contrasting to the insensitive glucose plants, mutants were found to be insensitive to ethylene which were also demonstrated to be hypersensitive to glucose. The early study of Zhou *et al.,* (1998) already suggested a close interaction between the sugar and ethylene signalling pathways, both playing roles in overall and initial stage performance of *Arabidopsis* mutants.

Phenotypic differences were found in transgenic rice overexpressing ETR2 genes, RNAi lines and wild type plants. Overexpressing ETR2 plants were found to have shorter coleoptiles than the wild type plants suggesting an important role of ethylene in coleoptile growth. Additionally, heading time was evaluated resulting in a delay of 1 week on average for overexpressing ETR2 plants while earlier heading time was observed for RNAi lines (Wuriyanghan *et al.,* 2009). Furthermore, overexpressing ETR2 showed a larger amount of starch nodules in analysed internodes compared to the controls. In contrast, RNAi plants showed occasionally less or no difference in the starch granules number compared to the control plants. However, the density of starch granules measured by Iodine staining were found to be lower in the controls and RNAi plants (Wuriyanghan *et al.,* 2009). Gene expression studies of the ETR2 genes reported downregulation of overexpressing lines, contrasting with upregulation of genes by RNAi lines. It was suggested that ETR2 genes may play a role in gene expression by inhibition of a number of genes related to Ethylene (Wuriyanghan *et al.,* 2009). Since ETR genes have been shown to effect other genes, it may be difficult to determine the influence of ETR genes in potato. Ethylene is known to play diversity of functions in several stages of plant development and ETR genes might be important in potato influencing the vegetative period as reported by Wuriyanghan *et al.,* (2009) in rice plants. The vegetative period is meant for the synthesis of substances required for development including amino acids. Differences on the effects of ETR genes and the relation in expression of other genes may serve as guide to elucidate the effects of ethylene in potato.

1.2.4 Lysine/histidine transporter (LHT).

Lysine/histidine transporter (LHT) genes have its place into a group of seven ancestry genes closely related to AAP genes known to transport neutral and acid amino acids. Molecular studies in *Arabidopsis* reported that a mutation of the LHT1 was strong enough to inhibit plant growth. Mutant plants presented variation in phenotype and further studies demonstrated a ~20% reduction of the amino acids uptake capacity compared to wild type plants. Consequently, it was suggested that LHT1 genes may play a role in the uptake of amino acids from soil to the leaf mesophyll (Hirner *et al.,* 2006). LHT4 and LHT7 *Arabidopsis* genes were suggested to be involved in reproduction, particularly in pollen and anthers development. Nevertheless, LHT4 gene was observed to be expressed in roots and stems (Winter *et al.,* 2007). LHT genes expression was also

reported in reproductive and floral tissue such as anthers, pollen tubes and pistils (Tegeder & Ward, 2012). LHT1 genes were found mostly expressed in non-vascular tissue including leaf mesophyll and root surfaces. Overexpressing *Arabidopsis* LHT1 mutants were observed with an increased concentration of amino acids in the apoplasm (Hirner *et al.,* 2006). Gene expression in roots at early stages such as emerging roots and lateral roots excluding the principal root were described for LHT1 by histochemical GUS studies. Other analysis showed stronger expression of LHT1 in the mesophyll cells in mature leaves rather than in young leaves. Additionally, overexpressing LHT1 *Arabidopsis* plants were found to grow bigger than wild types under restricted nutrient conditions. These findings supported that overexpression of LHT1 genes can maintain plant growth under limited nutrient conditions (Hirner *et al.,* 2006). According to Tegeder & Ward (2012), LHT gene expression was also found in sepals and petals of *Arabidopsis*. However, no expression was detected in siliques and seeds highlighting the proposed role of LHT1 on the uptake of amino acids from soil to mesophyll (Hirner *et al.,* 2006). LHT genes showing influence in sexual reproductive parts are usually related to seed plants, whereas LHT genes with expression in non-reproductive tissue have been found in algae suggesting high specificity of these genes (Tegeder & Ward, 2012).

LHT genes encode proteins related to the transport of amino acids and transmembrane interaction domains (EMBL-EBI, 2016). Including a vesicular amino butyric acid (GABA) transporter predicted to have other transmembrane domains. Other proteins with this domain include proline transporters and other amino acid transporters (Spud DB, 2016). Since LHT genes have been found to be expressed in a great variety of tissues in *Arabidopsis*, it is possible that the effect of LHT genes in potato responds and function in a similar fashion. LHT genes have been described to be active not only in the transport of amino acids but also playing important roles under restricted nutrient conditions. A second role of LHT1 was proposed in relation to cell uptake in leaves by an increased amino acid content in the apoplasm where the amino acid composition was affected causing an increase in total protein content of *Arabidopsis* leaves (Hirner *et al.,* 2006). Higher accumulation of amino acids may enhance the total protein formation in source tissues. Moreover, due to the natural function of LHT genes, it might be possible to consider a major role in the mobilization of amino acids from sources to sink tissues in potato. Furthermore, LHT genes were shown to be important in reproductive organs rather than in sink organs. Reproduction in potato is slightly different compared to other flowering plant species since the production of seeds in potato is not as important as in *Arabidopsis*. Therefore, differences in the function of LTH1 genes can be expected. Nevertheless, the accumulation of amino acids in potato leaves might be as impacting as in *Arabidopsis*. Conversely, the LHT4 gene mainly expressed in the roots and stems might play an important role in potato since high expression of LHT4 may enhance a higher accumulation of amino acids (Winter *et al.,* 2007). Together, LHT genes reported to be expressed through the whole plant are interesting candidate genes to elucidate the mechanisms involved in the formation and transport of proteins in potato.

1.2.5 Hexose carrier protein (HCP).

Hexose carrier protein genes (HCP) have been identified in big families of sugar transporter genes in different crops. Among crops, it has been shown that HCP genes are expressed differentially in sink and source tissues. HCP genes in potato are likely expressed in the following plant parts: flowers, leaves, petioles, roots, shoot apex, stamens, stems, stolons, tuber cortex, tuber peel, tuber pith, in vitro plants and young tubers (Spud DB, 2016). In tomato (*S. lycopersicum*), the sugar

transporters gene family is composed by 18 members including HCP genes (Reuscher *et al.,* 2014). Three hexose transporter genes (LeHT1, LeHT2 and LeTH3) were characterized and studied by Gear *et al.,* (2000). LeHT2 was able to encode a functional transporter of glucose as found in functional analyses by complementation tests in a hexose transport-deficient yeast strain. Moreover, expression of LeTH3 and LeHT1 was found in relatively high levels in young fruits (Gear *et al.,* 2000). Similarly, expression analysis of hexose transporter genes in grape (*Vitis vinifera*) were carried out using a construct of the VvHT1 in tobacco (*N. tabacum*). GUS and VvTH1 promoters were used to generate transgenic plants. GUS activity was found in adult and young leaves, stems and roots but none of the sink organs were spotted. Transgenic plants with the VvHT1 promoter were found with sink organs such as roots, stems and young leaves (Atanassova *et al.,* 2003).

Molecular studies performed by McCurdy *et al.,* 2010 aimed to expand the knowledge of the LeHT1, LeHT2 and LeTH3 hexose transporter genes. The three cDNA sequences were cloned from young fruits and flowers of tomato plants into a yeast strain able to grow in maltose but not in sucrose or fructose medium (McCurdy *et al.,* 2010). LeHT1 showed a restored growth under glucose or fructose medium while LeHT3 was unable to rescue the yeast growing under fructose or glucose suggesting a low-affinity transporter role of LeHT3. Interestingly, yeast colonies containing the LeHT1 or LeHT2 were found in the plasma membrane of yeast by GFP expression. Moreover, LeHT1 and LeHT2 yeast colonies were able to take up more glucose or fructose than the untransformed colonies which are in agreement with the findings of Gear *et al.,* (2000) on that LeHT1 is able to transport glucose. Hexose accumulation was decreased in tomato fruits by RNAi LeHT3 lines suggesting a role of HCP genes in plant performance (McCurdy *et al.,* 2010). Similarly, in cucumber (*Cucumis sativus*) complementary tests in yeast unable to grow in monosaccharides were performed. CsHT1 was reported to have high affinity for glucose and lower preference for galactose. CsHT1 expression levels was observed to be higher in anthers and pollen than in petals or sepals suggesting a role of hexose transporters in seed development (Cheng *et al.,* 2015). Hexose carrier proteins have been also reported to be involved in phloem loading in several plants. In potato, this might be important since the availability of sugars as energy source for different biological process in planta are essential. Enhanced transport of sugars in the phloem sap may influence the synthesis of elaborates including amino acids in potato. Additionally, the biomass might be effected by higher availability of sugars leading subsequently to higher accumulation of proteins.

1.2.6 Amino acid permease (AAP).

Genes from the amino acid permease family (AAP) are known to show a broad selectivity for amino acids (Fischer *et al.,* 2002; Tegeder & Ward, 2012; Tegeder *et al.,* 2013). Studies related to AAP genes have been performed in some legumes and *Arabidopsis*. AAP proteins have been found in non-vascular plants (*Physcomitrella patens*), non-seed vascular plants (*Selaginella moellendorffii*) and seed plants including *Arabidopsis*, *O. sativa* and *Medicago truncatula* (Tegeder & Ward, 2012). An interesting study in *Arabidopsis* aiming to evaluate the aphid's (*Myzus persicae*) performance after knocking out the AAP6 gene provided valuable information on AAP genes. The mutant plants were found to be significantly lower in amino acid content in sinks and through the vascular system. The mutation in the AAP6 gene also affected the phenotype by increasing the rosette diameter (measured at flowering time) and the number of cauline leaves in mutated plants (Hunt *et al.,* 2009). It is also known that AAP6 genes are responsible for allocation of amino

acids to seeds, although the number of seeds was not affected, the size and protein content of seeds was recorded to be higher in mutated plants (Hunt *et al.,* 2009). AAP transporters are commonly found in the leaf phloem of legumes which supports the function of these genes in phloem loading. Zhang *et al.,* (2010) showed that AAPs are important genes involved in phloem loading in vascular plants and it is consistent with the study by Hunt *et al.,* (2009), which suggested that AAPs genes play a role in the regulation of amino acid composition of sink and sieve sap elements. In *Arabidopsis*, the AAP genes are often localized in phloem in roots, stem and major veins from leaves where the main function is the transfer of amino acids from xylem to phloem (Zhang *et al.,* 2010).

Studies in pea (*P. sativum*) by overexpression of the AAP1 gene carrying the promoter from *Arabidopsis* showed similar amino acid content as the model plant. Zhang *et al.,* (2015) developed two transgenic pea lines overexpressing the AAp1 genes. Total free amino acid content was tremendously increased to 232% in pea. Individual amino acids content was analysed showing increments above 100% for all the measured amino acids. Furthermore, comparison between the pod phloem and leaf sap exudates revealed a constant increase in free amino acids content higher than 190%. It was demonstrated that AAP1 transgenic pea plants had an enhanced phloem loading capacity suggesting a role in transfer and delivery of amino acids to seeds by AAP1 genes. Additionally, seed protein content was also tested resulting in an increment of up to 8% in total N content and soluble protein was also improved in overexpressing AAP1 pea plants (Zhang *et al.,* 2015). AAP genes have been described to influence the protein content in pea at a great extent. Potato might be also affected in a similar fashion as in pea since the accumulation of free amino acids in the whole pea plants was increased. Differences in the allocation of proteins in seeds of pea and tubers in potato may assist to address the impact of AAP genes in potato. Although the results obtained from overexpressing AAP genes in pea provided great insight on the main roles and function of AAP, similar approaches would serve to clarify the roles of AAP genes in potato. Lastly, enhanced accumulation of amino acids in stems and leaves can be expected to happen in potato. Of course, the influence of AAP genes in potato should not be anticipated nor considered to be as influencing as reported in pea.

2 Materials and Methods

cDNA from potato roots and tubers of cultivar Kardal were used for amplification and cloning of the candidate genes through the Polymerase Chain Reaction (PCR). cDNA synthesis was performed. Specific primers were design for different purposes such as amplification of the candidate genes and colony PCR. High fidelity enzymes were used to amplify the genes from the different cDNA sources. The cloning strategy relies on the Gateway® system from Thermo Fisher Scientific which consists of a donor and destination vectors (pENTR™ TOPO® vector and destination vector PK7GW2.0). The destination vector was later cloned into *Agrobacterium* AGL1 strain cells to complete *Agrobacterium*-mediated transformation. DNA recovery from gel electrophoresis was performed to clean and purify DNA required for the different cloning steps (DNA Clean & Concentrator kit from QIAGEN, Zymoclean™ Gel DNA Recovery Kit from ZYMORESEARCH). Antibiotics were added for selection of bacterial colonies from the different cloning approaches (Appendix 8.3). The different PCR reactions were performed using GeneAmp™ PCR System 9700, GeneAmp™ PCR System 2700 and Veriti® 96-Well Thermal Cycler machines from Thermo Fisher Scientific. Gel electrophoresis was performed with 1%, 1.5% or 2% Agarose concentration in TAE buffer, depending on the size of the expected gene fragment to be amplified. The larger the size of the gene fragment, the lower Agarose concentration used for electrophoresis. Potato plants of cultivar Kardal were multiplied and grown for *Agrobacterium*-mediated transformation aiming the creation of transgenic lines.

2.1 cDNA synthesis

The cDNA used to amplify the genes was obtained by cDNA synthesis using RNA from different potato plant parts such as leaves, stems or tubers from cultivar Kardal. DNAse treatment was required for the RNA to avoid any DNA contamination during the process before performing cDNA synthesis. The DNAse treatment was done by adding 1 µg of RNA, 1 µl of a 10X DNAse I reaction buffer, 1 µl of DNAse I and milli-Q water up to a final volume of 10 µl. The mixture was kept at room temperature (~20 °C) for 15 minutes. Next, 1 µl of EDTA (25 Mm) was added to the mixture and placed at 65 °C for 10 minutes. cDNA synthesis was performed according the protocol iScript™ cDNA Synthesis Kit from BIO-RAD: 11 µl RNA (DNAse treated) followed by 4 µl 5x iScript™ reaction Mix, 1 µl iScript™ reverse transcriptase and finally 4 µl RNAse free water to a final volume of 20 µl. The mixture was placed in a PCR machine programmed as follows: 5 minutes at 25 °C, followed by 30 minutes at 42 °C, 5 minutes at 85 °C, 5 minutes at 4 °C, 5 minutes at 85 °C and finally the reaction was held at 10 °C. The cDNA was tested using Dream Taq polymerase PCR.

For cloning purposes, it was aimed to amplify the full length of the gene of interest or the Open Reading Frame (ORF). Specific primers were developed and tested beforehand. Amplification of the gene fragments was achieved by using high fidelity enzymes for PCR (Phusion® or Advantage® 2 PCR kits were used). Amplified gene fragments were confirmed in gel electrophoresis from the PCR and purification of the DNA was performed. Two approaches for DNA recovering and purification were used: a) DNA recovery from gel (MinElute® Gel Extraction Kit from QIAGEN or Zymoclean™ Gel DNA Recovery Kit from ZYMORESEARCH); b) DNA purification directly from PCR (DNA clean & concentrator kit ZYMORESEARCH). Both procedures aimed to remove traces of the primers used for amplification and any other kind of

contamination. Finally, pure DNA was verified by measuring its concentration with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). DNA concentration lower than 15 ng/µl were not used for cloning into the pENTR™ TOPO® vector.

2.2 Primers design and testing

Most of the primers used during the project were developed by Dianka Dees at an earlier stage of the process. Design of the primers was done using available online platforms from NCBI designing primers tool, Primer3 (version 4.0) and PrimerPlus software. The transcript sequence of each gene was used within these platforms to first identify the ORF of each gene. The different primers combination was selected and tested in silico for primer pair dimers formation, hairpins structure and the interaction between the forward and reverse primers using DNASTAR® Lasergene 13 software. A minimum of 20 nucleotides per primer was stablished as selectable for both Forward and Reverse primers. Primers contained at least 50% of GC nucleotides, however, some primers were selected when containing less than 50% GC but higher than 40% GC content. The melting temperature was set at 55°C as minimum and preferably higher than 60°C. Higher melting temperatures aimed to allow specificity of the Advantage® 2 PCR and Phusion® enzymes used for amplification of the genes. Additionally, a CACC nucleotide sequence was added at the end of the 5' end of the forward primers intended to amplify the genes for cloning in the pENTR™ TOPO® vector (Table 1). The primers were synthetized by Biolegio B.V. (The Netherlands) and tested afterwards with PCR. The best primer pair working combination were then selected for the following steps.

2.3 Gateway® cloning system

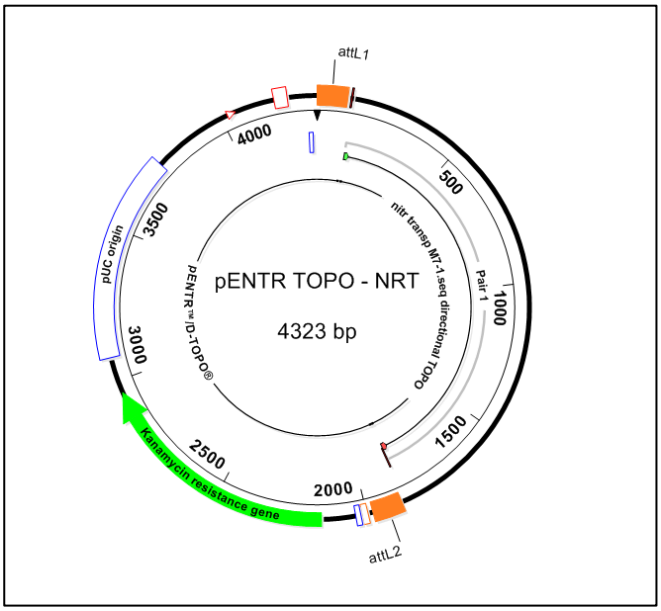

Figure 1. in-silico pENTR™ TOPO® vector containing the NRT gene built up with the DNASTAR® Lasergene 13 software.

The Gateway® cloning system uses *Escherichia coli* plasmids for recombination and transformation of the genes of interest. Two type of bacterial cells can be used for cloning: a) Chemically Competent *E. coli* cells and; b) Electrocompetent *E. coli* cells. Cloning and transformation protocols are different depending on what kind of cells were used for transformation. The system relies on recombination of attachment sites from a donor pENTR™ TOPO® vector (*att*L1 and *att*L2) with the attachment sites from a destination vector PK7GW2.0 (*att*R1 and *att*R2), mediated by the LR Clonase™ II enzyme mix from Invitrogen®. First, the gene of interest is cloned in between the attachments sites in the pENTR™ TOPO® vector (e.g. NRT gene, Figure 1) whilst a ccdB gene is contained in between the

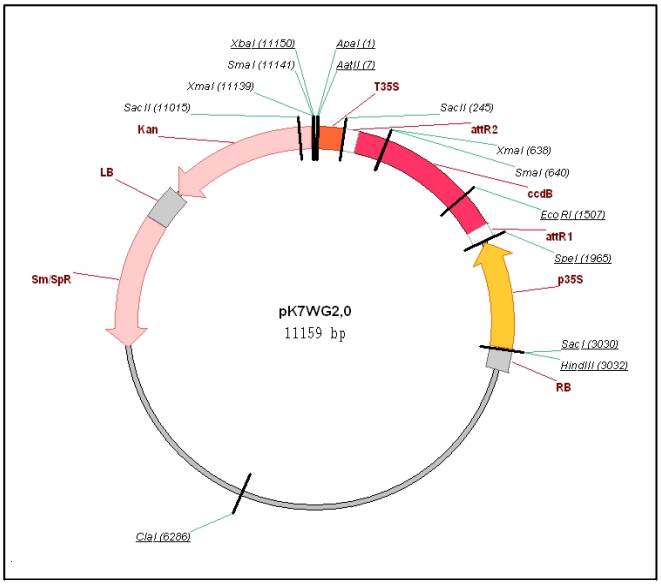

Figure 2. PK7WG2.0 binary vector Gateway® System for *Agrobacterium*-mediated plant transformation (Karimi, M. *et al*, 2002).

attachment sites of the destination vector PK7GW2.0 (Figure 2). Selection after cloning in the pENTR™ TOPO® vector was performed under plates containing Kanamycin. The Gateway® system is specific and allows recombination only of *att*L1 with *att*R1 and only *att*L2 with *att*R2 (known as "LR cloning"). Therefore, the gene of interest was integrated into the destination vector PK7GW2.0 by exchange of the ccdB gene and the gene of interest. Selection after LR cloning and transformation procedure was done under plates containing Spectinomycine. Only the bacterial plasmids containing the gene of interest in the destination vector were able to grow under Spectinomycine. Cloning and transformation procedures were carried out according the manufacturer's protocols with some modifications. Manufacturer's originals and modified protocols for cloning into the pENTR™ TOPO® vector, LR cloning and transformation procedures are detailed in Appendix 8.4. The colonies grown from the LR reaction were confirmed by colony PCR. Furthermore, positive colonies from colony PCR were subjected to pDNA isolation used to verify the insertion of the gene by sequencing. Confirmed colonies from the destination vector were cultured and used for transformation to *A. tumefaciens*.

2.4 *Agrobacterium tumefaciens*

A. tumefaciens is a Gram-negative bacterium that has the ability to transfer and integrate a small part of its own DNA to a limited number of plants. The transferred DNA from *A. tumefaciens* is known as Ti-DNA because its excessive cellular growth inducing capacity which causes tumour formations to infected plants. This is a natural occurring event being used by scientist for research purposes at a great extent. It has been possible to replace this Ti-DNA with potentially any gene and genetics studies have been speeded up since then. Currently, Agro-infiltration is a common practice to scientists studying the effects of genes all around the globe and its procedure has been standardized. Due to the relatively accurate and easy procedure for *A. tumefaciens* infection, we have selected this method to create transgenic plants containing our genes of interest.

2.4.1 *Agrobacterium tumefaciens* AGL1 strain

A. tumefaciens strain AGL1 (Lazo, *et al.*, 1991) was used for cloning the destination vector from the Gateway® cloning system containing only one gene of interest per transformation. AGL1 strain competent cells lacks the Ti-DNA fragment of natural *Agrobacterium* cells, however it carries on a resistance gene to Carbenicilin which is meant for selection after cloning. Spectinomycine was incorporated to the medium which allowed selection of the plasmids containing the genes of interest from the destination vector. Additionally, Chloramphenicol was used to prevent undesired colonies to grow from *Agrobacterium* plasmids carrying *vir* genes which are used to

check the viability of the AGL1 *Agrobacterium* plasmids. The expression vector was cloned to *Agrobacterium* by electroporation. Once the expression vector was cloned to *Agrobacterium*, the bacterial plasmids were cultured and selected in LB medium containing Carbenicilin, Spectinomycine and Chloramphenicol.

2.4.2 Electroporation procedure

Once the gene of interest has been cloned and transformed in the PK7WG2.0 destination vector, cloning into *A. tumefaciens* can be performed. pDNA of the destination vector is mixed to 30 µl AGL1 *Agrobacterium* competent cells. Next, via electroporation the circular DNA from both the destination vector and of *Agrobacterium* are linearized with a Gene Pulser® II Electroporation System (BIO-RAD) set at 1.4 kV with a resistance of 200 Ohms and a capacitance of 25 µF during 4 to 5 seconds. Immediately, 1 ml of LB medium was incorporated and mixed by pipetting up and down. The mixture was transferred to 2 ml cap tubes and put to incubation during 4 hours at 28°C. After incubation, the mixture was cultured in LB agar plates with antibiotics for selection of the positive transformants. The plates were kept at 28°C for 2 days to allow transformed *A. tumefaciens* colonies growth. Culturing colonies and verification by PCR: standard LB medium was used for culturing the colonies plus the addition of accordingly antibiotics. Some of the bacterial cells were mixed with 80% glycerol and stored at -80°C and some were used for transformation of potato *in-vitro* plants. 1 ml of fresh cultured colony plus addition of 250 µl of 80% glycerol were mixed for long term storage.

2.4.3 *Agrobacterium*-mediated transformation

A. tumefaciens generally enters the plant via wounds caused by abiotic factors such as wind or mechanical damage. Biotic factors such as insects feeding in the leaves or the stems damages the plant tissue which can also be a way for *Agrobacterium* infection. In laboratory conditions, vegetal material such as leaf disks and 2 -5 mm stems from the internodes of *in-vitro* plants are commonly used for Agro-infiltration. The selected colony to be used for Agro-infiltration was started two days in advance of scheduled procedure for Agro-infiltration. 24 h before the transformation, the explants (internodal stems of 2 to 5 mm length) were cut from *in-vitro* plants and placed in R3B regeneration medium containing sterile paper and 1.5 ml of PACM medium. On the day of the transformation, the bacterial culture previously started was adjusted to a OD_{600} value of 0.5 by diluting in LB without antibiotics. 50 ml of LB with the bacteria were divided in three Petri dishes and the explants were submerged into the bacterial suspension. The explants were kept for about 5 to 10 minutes, dried on sterile filter paper and placed back to the R3B and PACM medium. Three controls were taken: a) No bacteria, no selection antibiotic; b) With bacteria, no selection antibiotic; c) No bacteria, with selection antibiotic. The Petri dishes were sealed and stored in a climate chamber for two more days. After two days, the Agro-infiltrated explants were transferred to ZCVK selection medium to promote callus growing. Sixteen days later the explants were transferred to fresh ZCVK selection medium and this procedure was repeated twenty-one days later. Five weeks later, small shoots started to grow from callus which were transferred to individual CK medium containers to stimulate rooting and growing of each plant. Individual plants represent potentially a new transgenic line.

2.4.4 Verification of transgenic plants

For verification of transgenic plants recovered from *Agrobacterium* transformation, genomic DNA can be extracted from young shoots. Appropriate primers targeting a known region within the

construct can be used for screening plants using the PCR. Dream Taq polymerases can be used to check the construct depending on the gene fragment we expect. Once a positive fragment is observed in gel, the assessed plants can be said to be effectively transformed and further experiments can be started. Experiments such as gene expression analysis, phenotyping and growing performance are to be followed. However, this thesis was limited to design the primers to verify the presence of the constructs.

2.5 Medium and standard procedures

2.5.1 Medium for culturing and selecting bacterium colonies

Growing and maintaining bacteria is essential for the different cloning steps since plasmid DNA from *E. coli* and *A. tumefaciens* were used within different stages of the cloning strategy. Lysogeny broth (LB) standard medium was used to culture both *E. coli* and *A. tumefaciens* from the different cloning steps. LB liquid medium was used to grow selected colonies in tubes to perform plasmid DNA isolation (pDNA). The LB liquid medium contained 20 grams of LB medium dissolved in 1 litre of demineralized water. LB agar solid medium was used to culture bacteria in plates after transformation. LB solid medium contained 20 grams of LB medium + 1.5% of Agar bacteriological dissolved in 1 litre of demineralized water. All the different medium was sterilized by autoclaving at 121°C during 18 minutes, cooled down and stored at room temperature for liquid medium and at 4°C for solid medium. Additionally, antibiotics were incorporated to the medium depending on the resistance gene of the pENTR™ TOPO® vector, the destination vector and the *A. tumefaciens* strain used for the cloning procedure and selection after transformation.

Murashige & Skoog (4405.19 mg/l) + Vitamins (4.4 g/l) known as MS20 medium was used to multiply the potato plants from *in-vitro* material to further use them in Agro-infiltration procedure. MS20 medium mixture contained the following reagents in 1 litre of demineralized water: 20 grams Sucrose, 8 grams Micro Agar (Duchefa Biochemie B. V.). The pH was stabilized at 5.8 by adding KOH when the pH was lower and HCl when it was higher than 5.8. For regeneration of potato plants after transformation by Agro-infiltration, specific medium was used. LB liquid medium with low concentration in salt was used to adjust the OD_{600} of the *A. tumefaciens* bacterial culture. This medium contained 20 grams of LB low salt in 1 litre of demineralized water. R3B medium was meant to stimulate the explants just after Agro-infiltration and contains in 1 litre of demineralized water: 4.4 grams MS + vitamins, 30 grams Sucrose, 8 grams Agar bacteriological, 2 milligrams NAA and 1 milligram BAP.

PACM liquid medium was also used during transformation procedure and contained the following: 4.4 grams MS + vitamins, 2 grams Caseine hydrolysate, 30 grams Sucrose, 200 μl2,4D (1 mg/l), 0.5 ml Kinetine (0.5 mg/l) and stabilized at pH 6.5. ZCVK medium was used for regeneration and selection after Agro-infiltration in order to promote the growth of callus from transformed explants. ZCVK medium contained 4.4 grams MS, Sucrose 20 grams, Agar bacteriological 8 grams, Zeatine 1 milligram, Cefotaxime 200 milligrams, Vancomycine 200 milligrams and Kanamycin 100 milligrams per 1 litre of demineralized water. Lastly, CK medium containing 4.4 grams MS, 20 grams Sucrose, 8 grams Agar bacteriological, Cefotaxime 200 milligrams and Kanamycin 100

milligrams was used to continue with individual shoots regenerated from callus after transformation.

2.5.2 Colony PCR

From the transformation procedures, a couple of colonies were picked up for analysis by colony PCR with Dream Taq polymerase. Specific primers were used to confirm the presence of the gene of interest in the colonies (Table 1). A toothpick was used to take a small portion of each colony and placed for a few seconds in the PCR wells. After a few seconds, the toothpick was transferred to 3 ml LB liquid with antibiotics and kept at 37°C with shaking overnight. Culturing the bacteria was done to obtain higher amounts of plasmid to further extract pDNA. The addition of antibiotics depended on the origin of the cultured bacteria.

2.5.3 Plasmid DNA isolation

After culturing the bacteria in LB liquid medium, pDNA isolation was performed to use the isolated pDNA in the following steps. Two methods were used to extract pDNA from bacterial cultures: a) QIAprep® Spin Miniprep kit from QIAGEN and, b) Plasmid isolation from *E. coli* protocol "*Eric's* modification of original Birnboim method" (Appendixes 8.4.4 and 8.4.5 respectively). Both methods aimed to obtain pure pDNA for the different cloning steps. The QIAprep® Spin Miniprep kit from QIAGEN provides clear instructions and supplies materials to carry out the pDNA extraction which ensures the pDNA to be pure. However, the concentration of DNA obtained with this method was usually low since high amounts of DNA are lost during the filtering steps. The "*Eric's* modification of original Birnboim method" in the other hand is more rudimentary and has the advantage of recovering considerably more pDNA compared to the QIAprep® Spin Miniprep kit.

2.5.4 Sequencing

After successful pDNA isolation, the samples containing the DNA both from the gene of interest and the pENTR vector were confirmed by sequencing before continuing with further steps. The Laboratory of Plant Breeding relies on the GATC BIOTECH sequencing service (https://www.gatc-biotech.com/en/index.html). Each pDNA sample sent for sequencing normally contained 200, 400 or 500 ng of pDNA, 2.5 µl of only one single primer and milli-Q water up to a final volume of 10 µl per sample. Once the results are ready, the DNA samples and a reference sequence belonging to each gene of interest were aligned and checked for quality using the DNASTAR® Lasergene 13 software. Sequencing does not always work as desired due to different factors despite the fact that the procedures are standardized. When the results from sequencing are not conclusive, other methods may lend a hand to verify that the gene of interest has been cloned into the vector.

2.5.5 Enzymatic digestion

Alongside DNA sequencing, verification for the presence of a gene can be done via enzymatic digestion. Software and tools such as DNASTAR® Lasergene 13 served to identify the enzymes that best suits our objectives. A set of different characteristics and features from DNASTAR® Lasergene 13 made possible to integrate our gene of interest with the vector which was used for cloning. An in-silico cloning approach containing the pENTR™ TOPO® vector and our genes of interest were used as template to obtain the restriction enzymes. Amongst the parameters facilitated by DNASTAR® Lasergene 13 software, unique cutting sites of the enzyme and six plus

cutters were considered for selection of the enzymes. *AscI* and *ClaI* enzymes were used for DNA digestion to validate the presence of the STR gene after several attempts to clone the gene into the pENTR™ TOPO® vector.

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

5' - 3' SEQUENCE[†]	CODE	Cloning	Amplification	Colony PCR	Gene presence	Sequencing
TCATTACATCATTCTACTTCCCCGTAGCAGA	Pr13R[1]	+	+			
caccACAGTAAATGGGCTGAAGACGAC	Pr27F[1]	+	+			
GGGCAAACCTGGCTCAAATC	Pr29R[1]	+	+			
GTGTTCTTAGCCCTGCTGTATCCT	Pr40F[1]			+	+	
CCCTGCCTTATAAACTCCACTCC	Pr41R[1]			+	+	
TTAACCTTCCTCTTCTTTGATGATAACTTCA	Pr09R[2]	+				
CCACACCCTGTCCATTTTCTCAATAG	Pr12R[2]	+				
caccGGCAGGGGGATTAGCAAATGA	Pr35F[2]	+				
caccATGGCAGGGGGATTAGCA	Pr36F[2]	+				
CCAAAACCTTCTCTTAACATCTTCT	Pr37R[2]	+				
caccTCATTTGCAAACATGGTTTCATCTTCTC	PR04F[3]	+	+			
TCAAATGTATTCTTCATGAATAGAACTCATAGG	Pr05R[3]	+	+			
TCATGAATAGAACTCATAGGAGGATGAATCAGC	Pr06R[3]	+	+			
ATTAGGATTGTTCCAAGCAGGTG	Pr23F[4]			+		+
TCAGGATCTTCAATCATACAGGCTCT	Pr24R[4]			+		+
caccTGTAAAAAAAATGGAAGAAAATAGAGTTATAGATA	PR18F[5]	+				
TCAAACATGTTCTGGTAGTTGATACTTTACAGT	Pr19R[5]	+				
AGAACCAAATAGAATTACAAAGCTTATACAATAGAA	Pr20R[5]	+				
CTGAAGGAATTGCAGTGGGGAGG	Pr21F[5]			+		+
AGGGATGTCGATAAGTCCAATAACAGC	Pr22R[5]			+		+
GTAAAACGACGGCCAG	M13F[6]					+
CAGGAAACAGCTATGAC	M13R[6]					+

[†]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]Ethylene receptor gene primers. [2]Hexose carrier protein gene primers. [3]Lysine/histidine transporter gene primers. [4]Nitrate transporter gene primers. [5]Sulfate transporter and exchanger gene primers. [6]Universal forward and reverse primers for sequencing from any vector containing the N-terminal coding sequence of the *lacZ* gene.

3 Results

3.1 Cloning and Transforming the candidate genes

Experiments were performed aiming to amplify and clone the candidate genes into the Gateway® cloning system which facilitates further cloning into *A. tumefaciens*. Four genes were subjected to most of the experiments. Results of the Nitrate Transporter (NRT), Sulfate/bicarbonate/oxalate exchanger and transporter (STR), Ethylene receptor (ETR) and Lysine/histidine transporter (LHT) genes are presented and discussed. The Hexose carrier protein (HCP) and Amino acid permease (AAP) genes were not amplified.

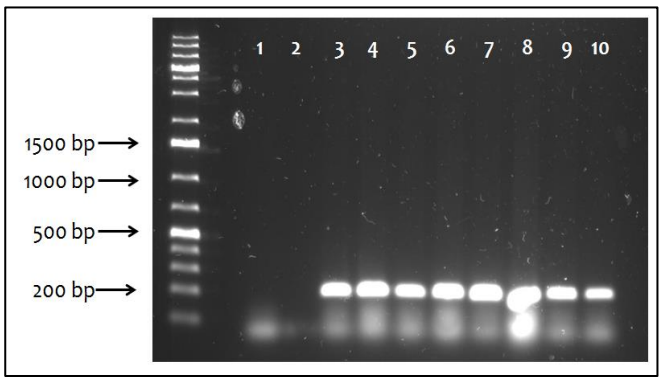

Figure 3. NRT Colony PCR with Dream Taq polymerase confirmation after transformation to *A. tumefaciens*. Primer pair Pr23F, Pr24R (163 bp). Numbers 1 to 10 are identifications for each selected colony.

The nitrate transporter gene (NRT) already cloned into the pENTR™ TOPO® vector was the starting point. pDNA isolation was performed resulting in two samples (97.9 ng/µl and 122.7 ng/µl) measured with a NanoDrop ND-1000 (NanoDrop Technologies) containing the pENTR vector. Alongside, pDNA isolation of the PK7GW2.0 binary vector was performed (B3: 259.9 ng/µl; ANN: 277.9 ng/µl). Together, the pENTR plasmids and the binary vector were used for the LR cloning reaction. The resulting bacterial colonies were cultured and selected in LB medium containing 200 µg/ml Spectinomycine. Primers pair Pr23 with Pr24 (163 bp) shown in Table 1 and Figure 3 were used to verify positive colonies by colony PCR after transformation. 12 colonies were then selected to perform the Forward sequencing reaction (6 colonies from B3, 6 colonies from ANN). The 4 best resulting sequences were then chosen to perform the Reverse sequencing reaction and Sanger alignment against the reference transcript sequence of the NRT gene was performed. Sequencing results for the selected colonies used for *A. tumefaciens* cloning showed a 100% match for the Forward sequencing reaction and 98% match for the Reverse sequencing reaction. Together, the Forward and Reverse sequencing reactions were able to cover the full transcript sequence of the NRT gene. pDNA from these colonies was isolated and used for cloning into *A. tumefaciens* AGL1 strain cells. Positive colonies from *Agrobacterium* cloning were able to grow in LB medium with 200 µg/ml Spectinomycin, 100 µg/ml Carbenicilin and 50 µg/ml Chloramphenicol as selection markers. Confirmation of the positive colonies was done by PCR. One single colony was selected to transform the potato plants via Agro-infiltration. Two weeks after Agro-infiltration, callus formation was observed in explants placed under ZCVK selection medium, suggesting that the transformation was successful. The controls were observed according to the expectations. Control explants placed under ZCVK selection medium without *A. tumefaciens* showed a brownish color and callus growth was not observed. Control explants inoculated with *A. tumefaciens* placed under ZCV medium (no selection) presented callus overgrowth compared to explants under ZCVK medium. Control explants placed under ZCV medium without inoculation of *A. tumefaciens* presented poor callus growth and a light green color was observed. After five weeks from Agro-infiltration, explants showing strong callus formation were kept and transferred to fresh selection

medium. 80 callus-growing explants were transferred to fresh ZCVK medium and 9 shoots were transferred to individual containers with CK medium. The remaining explants stayed to allow shoot growth aiming to recover a new potential plant. Each shoot was taken from a single callus formation and represents a new possibly transgenic plant. 9 plants growing in individual containers are ready to be checked for the presence of the NRT construct (Figure 7). Once the NRT construct is confirmed in the plants, multiplication of the material can be performed to continue with studies on the effects of expression of the gene.

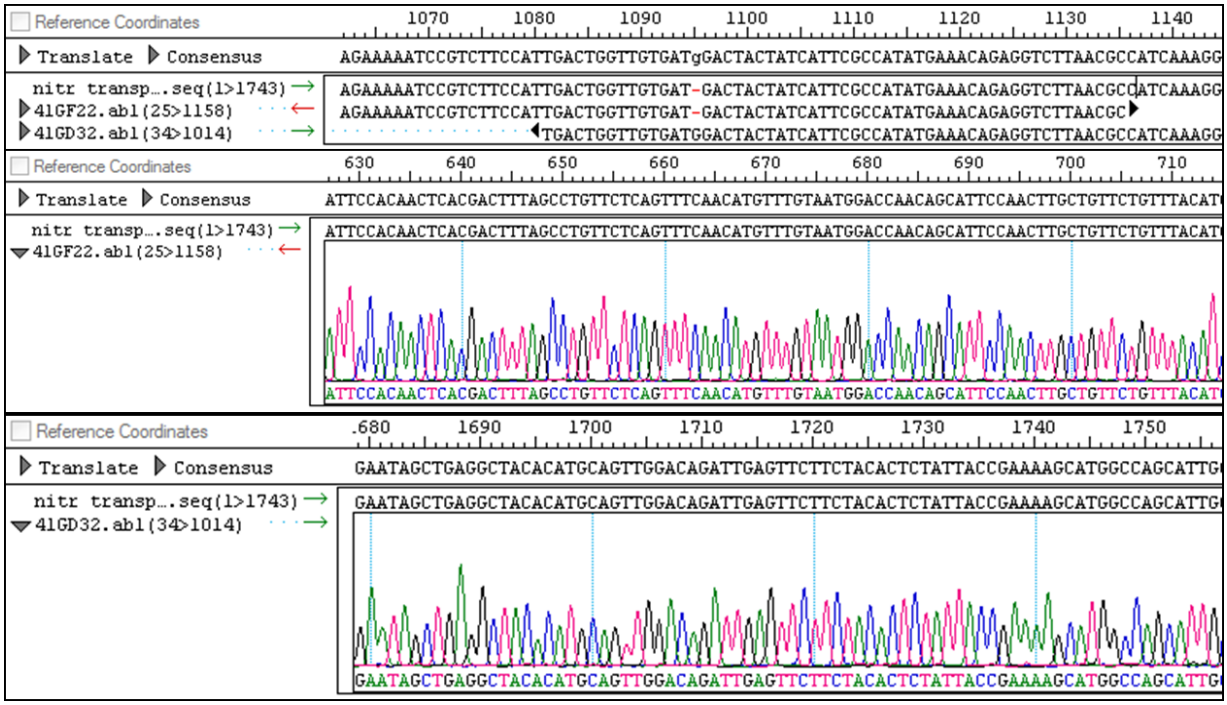

Figure 4. DNASTAR® Lasergene 13 partial output of the sequencing results from the NRT gene. The upper part in the figure shows the overlapping region of the two sequencing reactions in alignment with the reference transcript sequence of the NRT gene. Wavelength of the Forward and Reverse sequencing reactions give an indication of the quality of the sequence.

STR 1808 bp gene fragment was amplified for cloning into the pENTR™ TOPO® vector. First, a PCR using primer Pr18F with Pr20R (1990 bp) followed by a Nested PCR using primers Pr18F with Pr19R (1808 bp) were performed to obtain enough product to continue with cloning into the pENTR™ TOPO® vector. The transformation procedure after cloning in pENTR™ was carried out as described in Materials and Methods. Primers pair Pr21F with Pr22R (310 bp) were used to confirm the presence of the gene by colony PCR. The region covered by the primers used for colony PCR targeted the positions 1013 bp to 1323 bp of the transcript sequence indicating that the gene fragment indeed was present after cloning in the pENTR™ TOPO® vector. However, the number of colonies obtained was low suggesting a poor transformation efficiency according to the manufacturer. pDNA from three colonies was used for sequencing after the transformation procedure using the primers Pr21F, Pr22R and the M13F and M13R universal primers from Invitrogen®. 314 bp sequence resulted from pDNA samples with the M13F primer matching the pENTR™ TOPO® vector and the STR transcript sequence (Sanger alignment, DNASTAR® Lasergene 13). The first 86 bp belonged to the pENTR™ TOPO® vector (Figure 8). BLASTN analysis with the remaining 228 bp sequence showed homology with a predicted STR 3.3 gene in *S. tuberosum*, *S. lycopersicum* and other Solanaceae species. However, due to the short length of the

sequence it was not possible to confirm the presence of the gene from pDNA samples. Verification for the presence of the gene must be done before continuing with cloning into the destination vector.

Ethylene receptor (ETR) gene fragments of 2323 bp length were amplified from eight different cDNA sources. Five positive bands were observed in gel after performing Advantage® 2 PCR and isolating from the five positive samples. Two samples (5A: 35.1 ng/µl and 8A: 40.0 ng/µl, Figure 5) with the highest DNA concentration were used to perform the pENTR™ TOPO® cloning reaction. Colony PCR results after TOPO® transformation showed no presence of the gene in any of the cloning reactions. Several attempts to amplify the gene from PCR using Advantage® 2 and Phusion® PCR enzymes resulted in positive gene fragments. However, weak gene fragments were commonly found from the different PCR reactions. In addition, Advantage® 2 enzymes seemed to be more compatible with the ETR gene than Phusion® enzymes since the clearest gene fragments were found when Advantage® 2 enzymes were used. Aiming to obtain stronger gene fragments from the PCR, gradient PCR was performed using Advantage® 2 PCR with primers Pr27F and Pr13R (2323 bp). Weak gene fragments were observed in gel which led to increase the number of cycles in the annealing phase (increased from 30 to 35 and 40 cycles). This produced stronger bands (Figure 5) from five cDNA samples that were then recovered from gel using the Eric's modification method. After a couple of attempts to clone the gene in the pENTR™ TOPO® vector with no success, further attempts to clone the ETR gene were not continued.

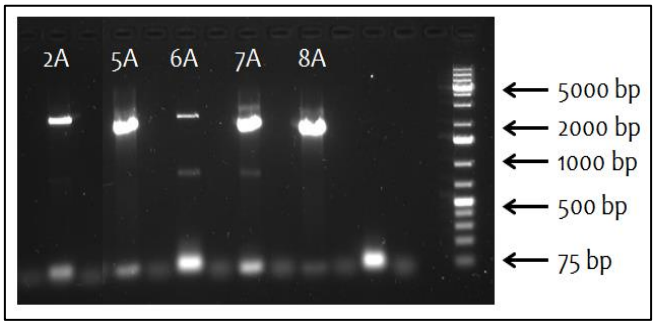

Figure 5. ETR cDNA amplification with Advantage® 2 PCR. Labels indicates the sample number and the primer pair producing a positive gene fragment. A = Pr27F, Pr13R. Samples 5A and 8A were recovered and purified to be used in the pENTR™ TOPO® cloning procedure.

Lysine/histidine transporter (LHT) gene fragments of 1351 bp were amplified from PCR using Advantage® 2 enzymes. The first attempt to retrieve the gene was resulted in a weak signal observed in gel after performing Advantage® 2 PCR reaction. Next, eight different cDNA sources were tested each one with two primers combination (Pro4F, Pro5R -1364 bp and Pro4F, Pro6R -1351 bp). Three gene fragments (Figure 6) were observed from primer pair Pro4F with Pro6R. second attempt with these gene fragments was performed. DNA was recovered from the second Advantage® 2 PCR reaction, however, the concentration of DNA from the three samples were below 4 ng/µl. Third and fourth attempts with Phusion® PCR and Advantage® 2 PCR respectively were carried out. Unfortunately, no gene fragments were observed after performing

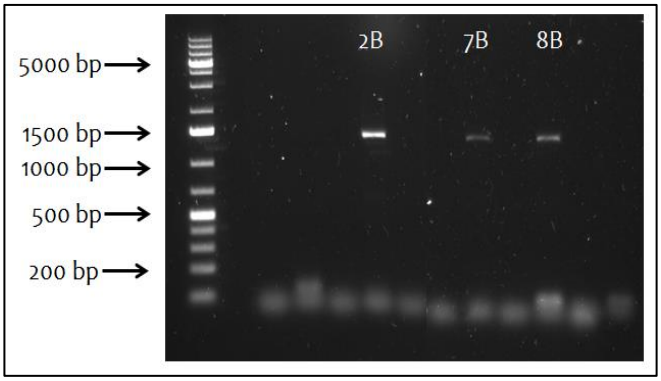

Figure 6. LHT cDNA amplification with Advantage® 2 PCR. Labels indicates the sample number and the primer pair producing a positive gene fragment. B = Pro4F, Pro6R. All three samples were recovered and used for cloning in the pENTR™ TOPO® vector.

these PCR reactions. Additionally, primers for cloning purposes and colony PCR to confirm the presence of the gene were developed. Internal primers were not tested since DNA concentration was not high enough to continue with cloning into the pENTR™ TOPO® vector.

Hexose carrier protein (HCP) gene is a small sized gene (237 bp based on transcript sequence from the Spud DB for potato) and primers were mainly developed for cloning purposes and no internal primers are available for this gene. First, Dream Taq polymerase PCR was used to amplify a gene fragment with primer combinations Pr36F with Pr37R (199 bp), Pr36F with Pr09R (236 bp), Pr35F with Pr37R (197 bp) and Pr36F with Pr12R (164 bp). Amplification of the gene fragment was not possible since no bands were observed from the PCR reactions. Next, Phusion® enzymes were used to attempt amplification of the gene fragments with the same primer combinations used for Dream Taq polymerase. However, no bands were observed in a 2% Agarose gel after the PCR reactions. Consequently, following steps within the cloning strategy were not performed. Development of internal primers still needs to be done and will be useful to check for the presence of the gene after TOPO® cloning and transformation procedures.

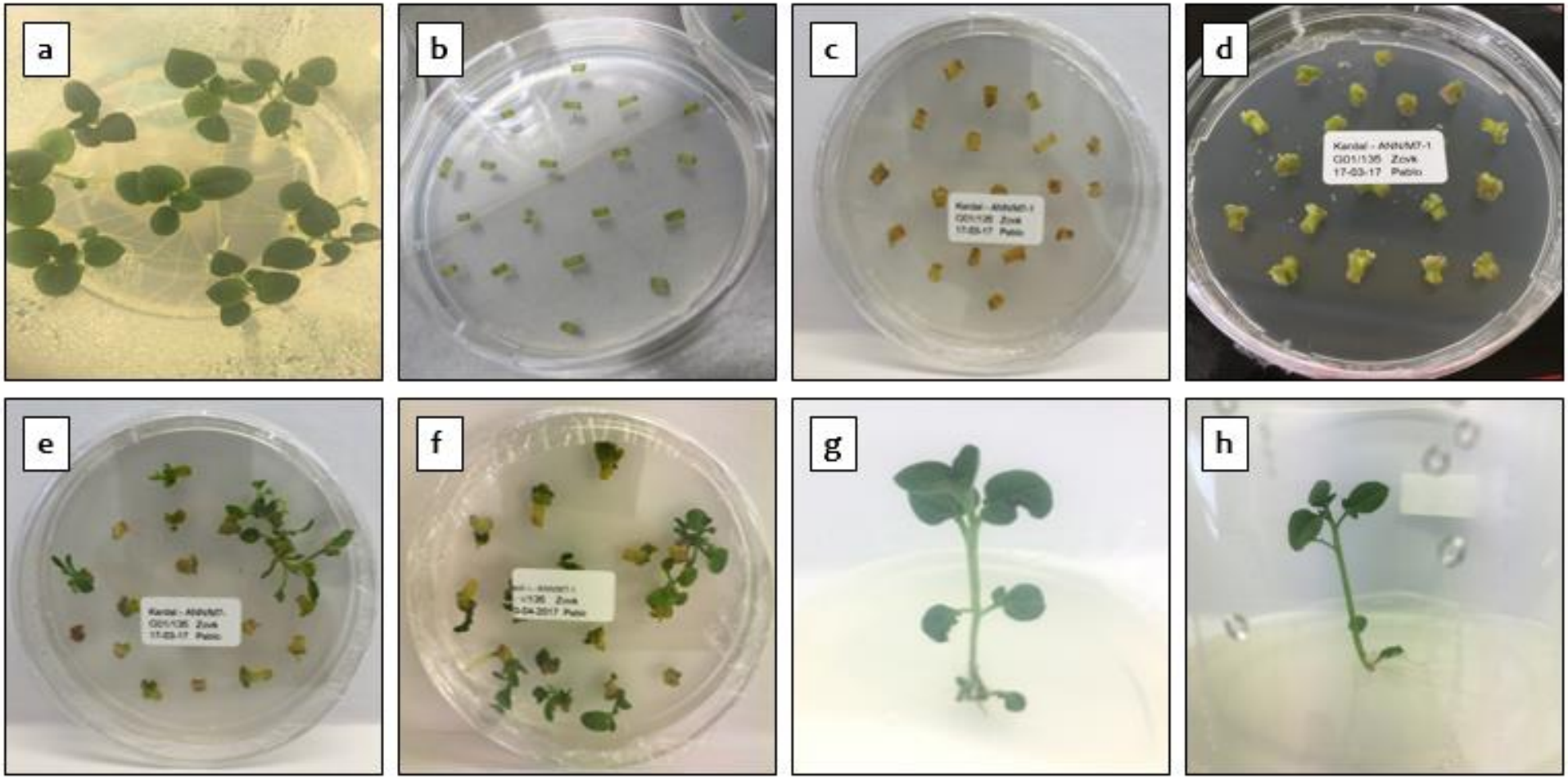

Figure 7. Chronological stages of *A. tumefaciens*-mediated transformation in potato. a) *in-vitro* plants used to obtain the explants for Agro-infiltration. b) Internodal stems 2 to 5 mm length inoculated with A. tumefaciens containing the NRT gene. c) Explants showing no callus formation after Agro-infiltration. d) Explants showing callus formation after Agro-infiltration. e) Five weeks after Agro-infiltration explants showing shoot growth. f) Seven weeks after Agro-infiltration shoots growing from callus ready for transferring to individual CK medium containers. g) & h) Small kanamycin resistant plants ready to be tested for the presence of the NRT construct.

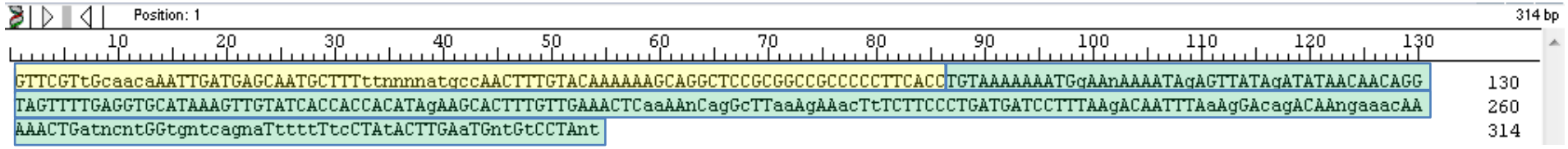

Figure 8. STR sequence results from pDNA after TOPO® transformation. The 314 bp sequence length was divided in the sequence from the pENTR™ TOPO® and from the STR gene. Highlighted in yellow: sequence of the pENTR™ TOPO® vector (86 bp). Highlighted in green: sequence of the STR transcript sequence (228 bp).

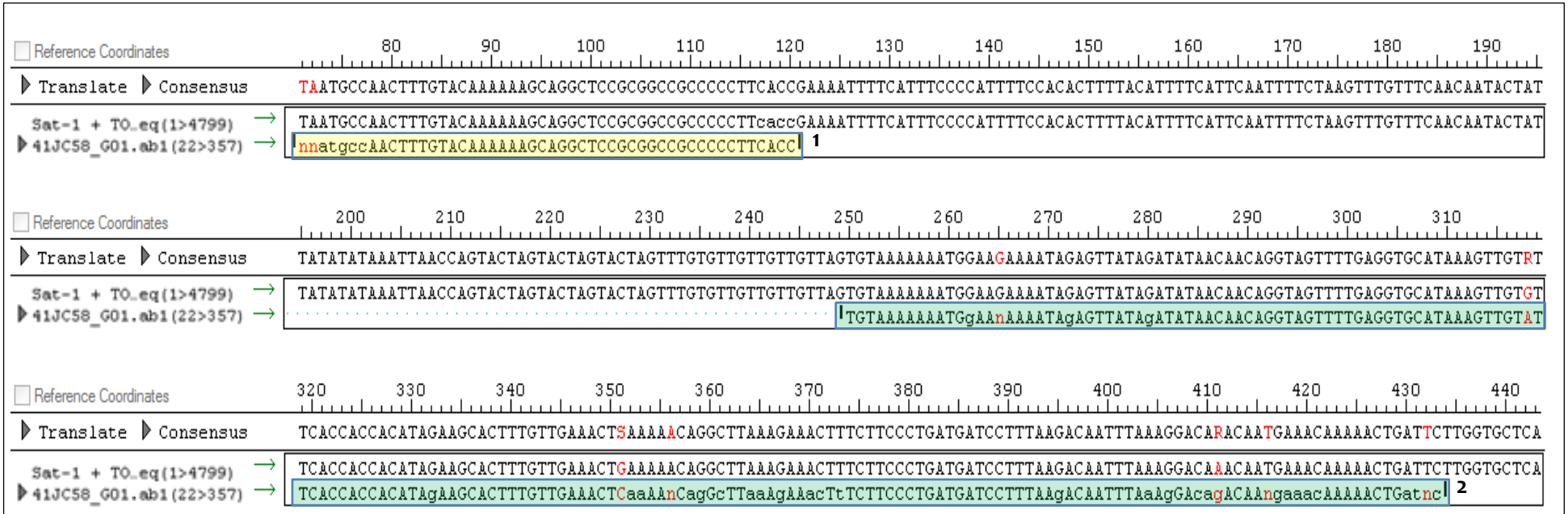

Figure 9. The STR sequence was split and aligned against the in-silico sequence of the STR gene cloned with the pENTR™ TOPO® vector. Minimum match percentage for assembly was 60%. **1.** Sample sequence of the pENTR™ TOPO® vector matches the end of the pENTR™ TOPO® vector (50 bp). **2.** STR sequence aligned against the in-silico sequence of the STR gene cloned with the pENTR™ TOPO® vector matches with the STR transcript sequence (184 bp). Primer M13F was used for sequencing the 41JC58_G01 STR sample.

4 Discussion

Cloning and transformation procedures for the NRT gene were carried out straight forward since each step ran efficiently. Amplification of the expected gene fragments from the different PCR setups was always possible. Maintenance of the bacteria carrying the NRT gene was an important step to perform the Agro-infiltration process and was executed without complications. This gene seemed to be very friendly for cloning and all further steps in colony PCR, pDNA isolation, sequencing and cloning to *Agrobacterium* were executed smoothly. As shown in Figure 4, the gap observed in the Reverse sequence and the NRT transcript and the addition of the nucleotide in the Forward sequence are contradictory. Usually, the quality and accuracy of the data tend to be lower at the beginning and the ends of the expected sequences causing misinterpretation of the software used to sequence. Lastly, Agro-infiltration and the generation of potential transgenic plants carrying the transformed NRT gene were successful. Positive plants were shown to be Kanamycin resistant since they were able to grow after Agro-infiltration under ZCVK selection medium. Small plants continue growing in a climate chamber to be confirmed for the presence of the NRT construct. Genomic DNA extracted from young leaves and specific primers to verify the presence of the construct remained to be performed. Afterwards, gene expression analysis studies can be started from positive transformants. Generally, all the procedures for the NRT gene worked out easily and according to schedule.

In contrast, the STR gene was difficult to clone since several attempts were required to obtain a partial confirmation into pENTR™ TOPO® vector. Amplification of the gene was successful and pDNA isolation resulted in enough DNA concentration for cloning. Despite that the cloning and transformation procedures were performed according the manufacturer's protocol, the results obtained were not as expected. Usually, the pUC19 DNA used as the control yielded less number of colonies than indicated by the manufacturer and low number of STR colonies were observed after transformation. A new purchased cloning kit from Invitrogen® was and used with the STR gene resulting in high number of colonies in the pUC19 DNA plates but low number of colonies from the STR gene, suggesting a problem in the cloning and transformation events. Whether the cloning step or the transformation procedure were the cause of the low number of colonies obtained after the process needs to be elucidated. Nevertheless, positive STR colonies confirmed by colony PCR were cultured for pDNA isolation were later sent for sequencing. Commonly, sequences obtained from STR pDNA samples resulted in short sequences or bad quality data. Additionally, the amount of pDNA sent for sequencing was varied (200, 400 and 500 ng) aiming to obtain better sequencing results. Finally, sequencing results from 200 ng of sample 41JC58_G01 (Figure 9) revealed the presence of the pENTR™ TOPO® vector and part of the STR gene. The sequence aligned with the STR gene showed homology with a predicted STR 3.3 gene from potato and other Solanaceae species. However, these results were not conclusive and verification for the presence of the STR gene in the pENTR™ TOPO® vector remains to be done.

Amplification of the gene fragments from the ETR and LHT genes was successful after several attempts. Nevertheless, complications were faced during the experiments such as weak fragments observed in gel for both ETR and LHT genes. First, the utilization of different PCR machines may have influenced the results from the PCR reactions, despite the fact that the reagents and procedure remained the same. Differences in the inner temperature of the PCR

machine may play a role in the different results obtained when trying to amplify any gene fragment. Additionally, storage of the genetic material may influence the results since DNA is stored at -20°C and goes through a thawing-freezing step as for many times as the material was being used. Regardless that DNA was always kept on ice after thawing, repeated thawing-freezing-thawing manipulation may have influenced the quality of the DNA and consequently the results. Furthermore, a test with internal primers used also for colony PCR was performed to check the viability of the cDNA. The gene fragments observed in gel were positive according the expected size, suggesting that DNA viability was good.

To contrast the results from DNA viability testing, cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit from BIO-RAD. Verification of the new cDNA was done by Advantage® 2 PCR with primers used for colony PCR from the STR gene (Table 1). However, amplification attempts of the STR, ETR and the LHT genes did not produce any of the expected gene fragments from PCR. One possible explanation for these results would be the time of storage of the RNA used for synthesis. When RNA is kept for a long period even under optimal storage conditions, it may happen that the RNA partially degrades and some genes still may be present. Therefore, some gene fragments can indeed be amplified after PCR. Alternatively, testing the RNA before performing cDNA synthesis would be useful to estimate the status of the material. In this way, several resources and time can be used more efficiently and in a more precise manner. Lastly, if the RNA source is not good to continue with cDNA synthesis, new RNA must be obtained from fresh potato plant parts such as leaves, stems, roots or tubers depending on the purpose of the research. The cDNA synthesis should work smoothly if the protocol is followed as recommended by the manufacturer. Verification by Dream Taq polymerase PCR as well as Advantage® 2 or Phusion® PCR should yield enough PCR product to continue with either DNA recovery from gel or DNA purification from PCR.

Finally, it would be advisable to keep the gene amplification and cloning processes using the same instruments. The same holds for DNA material used for amplification of the genes and cloning approaches. However, no real prove of the influence of performing the PCR reactions in different machines or different DNA material can be assessed within the experience of this work. Overcoming weak gene fragments from PCR can be addressed by testing different enzymes for PCR since Advantage® 2 and Phusion® enzymes were found to work different for each gene. Besides the type of enzymes used for amplification, the PCR setup also influenced the results observed for ETR and LHT genes. Increasing the number of cycles to 40 during the annealing phase resulted in stronger gene fragments of the ETR and LHT genes (Figure 5 and Figure 6 respectively). Additionally, Nested PCR was also useful to obtain stronger gene fragments from the STR and ETR gene. Moreover, cloning and transformation complications seemed to be improved when a ratio 3:1 (PCR ptoduct: pENTR™ TOPO® vector) was used for difficult genes such as the STR and ETR. Whereas cloning and transformation problems may be overcome, the purity of the pDNA sent for sequencing can also influence the results. In that sense, a combination of the two methods used to extract pDNA was performed from STR bacterial cultures to minimize the factors for the presented results. Higher amounts and highly pure pDNA were achieved from the combination of pDNA extraction methods. Nevertheless, cloning requires the integration of several steps and only a proper planning and the use of the same materials and methods may lead to elucidate whether the unexpected results would be the product of one or more factors effecting the experiments.

5 Conclusion

Cloning and transformation of the candidate genes was the main objective of this thesis which aimed to generate the starting material to study the protein content in potato. The cloning approach was based on the PCR with high fidelity enzymes and the Gateway® cloning system. Transformation of potato plants was performed by *Agrobacterium*-mediated transformation. Kanamycin resistant plants are available to be tested for the presence of the NRT construct by PCR using genomic DNA. New potential transgenic lines can be subjected to gene expression analysis to assess the effect of the NRT gene in these plants. Further research with the NRT gene will be determined depending on verification of the construct and gene expression analysis. The STR gene was partially confirmed in the pENTR™ TOPO® vector remaining to be verified before performing LR cloning and further steps. The ETR and LHT gene fragments were amplified from PCR staying in the first stage of the process after several attempts to amplify the respective gene fragments. HCP and AAP genes were not amplified from cDNA. Cloning genes from cDNA can be laborious and time consuming, therefore, proper planning and awareness of the measurements to be considered to achieve the objectives can speed up the process.

6 Recommendations

The study of homolog genes in other crops may be helpful to understand the role of the genes in potato. Various genes have several functions in other crops such as NRT genes in wheat and *Arabidopsis* functioning not only in the transport of nitrate but also in signalling functions as described previously. Similarly, ETR genes in plants have been described in relation to signalling roles during interaction with pathogens and have been also described to be important in the maturity and senescence of plants. It would be particularly interesting to study the effect of the AAP genes in potato, since long distance mobilization of amino acids from source to sink tissue may play a key role in the allocation of proteins in potato. Difficult genes to clone such as the STR may be feasible to study by looking to alternatives such as the *Arabidopsis* homologs. The STR gene from *Arabidopsis* are cloned into the pENTR™ TOPO® vector and they are available for research purposes (Michiel Klaassen, personal communication). The process might be speeded up by using the genes from the model plant and the first functional studies can be performed. Similar approaches may be available for other genes and other options still need to be considered.

Although all the procedures are standardized in molecular cloning, a more precise way to work in the laboratory is advisable. For example, limit to the use of one PCR machine for amplification of the gene fragments. Long-time stored kits may have reduced its cloning and transformation efficiency. Differences were noticed when a new cloning kit was purchased from Invitrogen®, as the control plates contained considerably larger amounts of colonies from the pUC19 DNA than in previous experiments, suggesting that storage time may affect the efficiency of the reagents. Since the costs of the different kits and reagents used in the laboratory are expensive, it is recommended to particularly test the primers for amplification of the gene fragments beforehand. Finally, the different steps to clone and transform the genes are relatively easy to follow, nonetheless, adaptations need to be done depending on laboratory conditions.

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8 Appendix

8.1 High fidelity enzymes used for PCR and setup of the reaction

The different PCR procedures were carried out as follows with the different enzymes: Phusion® PCR, Advantage® 2 PCR and Dream Taq polymerase. PCR setup for each of the enzymes are described below.

8.1.1 Phusion® PCR

Phusion® PCR reaction		PCR setup		
- Phusion polymerase	0.5 µl	98°	30”	
- cDNA	2.0 µl	98°	10”	5X
- 5X phusion buffer	10.0 µl	60°	30”	
- Forward primer	2.5 µl	72°	3’	
- Reverse primer	2.5 µl	98°	10”	28X
- dNTPs	2.0 µl	62°	30”	
- Milli Q	30.5 µl	72°	3’	
Total volume	**50 µl**	72°	10’	
		10°	∞	

8.1.2 Advantage® 2 PCR

Advantage® 2 PCR reaction		PCR setup		
- 50X Advantage polymerase mix	1.0 µl	94°	30”	
- cDNA	2.0 µl	94°	30”	5X
- 10X Advantage buffer	5.0 µl	60°	30”	
- Forward primer	1.0 µl	72°	3’	
- Reverse primer	1.0 µl	94°	30”	30X
- dNTPs	5.0 µl	62°	30”	
- Milli Q	35.0 µl	72°	3’	
Total volume	**50 µl**	72°	10’	
		10°	∞	

8.1.3 Dream taq polymerase PCR

deTaq polymerase PCR reaction		PCR setup		
- Dream Taq polymerase	0.05 µl	94°	5’	
- colony	1 colony	94°	30”	35X
- 10X deTaq buffer	2.5 µl	55°	30”	
- Forward primer	0.5 µl	72°	2’	
- Reverse primer	0.5 µl	72°	10’	
- dNTPs	1.0 µl	10°	∞	
- Milli Q	20.5 µl			
Total volume	**25 µl**			

8.2 Antibiotics

Table 2. List of antibiotics used for selection after transformation from different cloning steps and regeneration of plants after *Agrobacterium*-mediated transformation.

Culturing bacteria	Stock solution (mg/ml)	Working dilution
Ampicillin	50	1/500
Carbenicilin	50	1/500
Chloramphenicol	50	1/1000
Kanamycin	50	1/1000
Spectinomycine	100	1/500
Regeneration of plants	**Final concentration (mg/l)**	**Added to medium (ml/l)**
Cefotaxime	100	2
Kanamycin	50	2
Vancomycine	100	2

8.3 Plasmid DNA isolation and DNA purification

8.3.1 Zymoclean™ Gel DNA Recovery Kit - ZYMORESEARCH

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

Step 1 Excise the DNA fragment1 from the agarose gel using a razor blade, scalpel or other device and transfer it into a 1.5 ml microcentrifuge tube.

Step 2 Add 3 volumes of ADB to each volume of agarose excised from the gel (e.g. for 100 µl (mg) of agarose gel slice add 300 µl of ADB).

Step 3 Incubate at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved2.

For DNA fragments > 8 kb, following the incubation step, add one additional volume (equal to that of the gel slice) of water to the mixture for better DNA recovery (e.g., 100 µl agarose, 300 µl ADB, and 100 µl water).

Step 4 Transfer the melted agarose solution to a Zymo-Spin™ Column in a Collection Tube.

Step 5 Centrifuge for 30-60 seconds. Discard the flow-through3.

Step 6 Add 200 µl of DNA Wash Buffer to the column and centrifuge for 30 seconds. Discard the flow-through. Repeat the wash step.

Step 7 Add ≥ 6 µl DNA Elution Buffer4 or water5 directly to the column matrix. Place column into a 1.5 ml tube and centrifuge for 30-60 seconds to elute DNA.

Ultra-pure DNA is now ready for use.

8.3.2 DNA Clean & Concentrator™-5 - ZYMORESEARCH

Buffer Preparation. Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml DNA Wash Buffer concentrate. Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml DNA Wash Buffer concentrate.

Protocol

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

1. In a 1.5 ml microcentrifuge tube, add 2-7 volumes of DNA Binding Buffer to each volume of DNA sample (see table below). Mix briefly by vortexing.
2. Transfer mixture to a provided Zymo-Spin™ Column2 in a Collection Tube.

Application	DNA Binding Buffer : Sample	Example
Plasmid, genomic DNA (>2 kb)	2 : 1	200 µl : 100 µl
PCR product, DNA fragment	5 : 1	500 µl : 100 µl
ssDNA (*e.g.* cDNA, M13 phage)	7 : 1	700 µl : 100 µl

3. Centrifuge for 30 seconds. Discard the flow-through.
4. Add 200 µl DNA Wash Buffer to the column. Centrifuge for 30 seconds. Repeat the wash step.
5. Add ≥ 6 µl DNA Elution Buffer3 or water4 directly to the column matrix and incubate at room temperature for one minute. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge for 30 seconds to elute the DNA.

Ultra-pure DNA is now ready for use.

8.3.3 QIAGEN® MinElute® gel extraction kit

Notes before starting

This protocol is for clean-up of up to 5 µg DNA fragments (70 bp to 4 kb).

All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature (15–25°C).

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg gel ~ 100 µl). The maximum amount of gel slice per spin column is 400 mg. For >2% agarose gels, add 6 volumes Buffer QG.
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min during incubation to help dissolve the gel.
4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

5. Add 1 gel volume of isopropanol to the sample and mix by inverting.
6. Place a MinElute spin column in a provided 2 ml collection tube.
7. Apply sample to the MinElute column and centrifuge for 1 min. Discard flow-through and place the MinElute column back into the same collection tube. For sample volumes of more than 800 µl, simply load and spin again.
8. Add 500 µl Buffer QG to the MinElute column and centrifuge for 1 min. Discard flow-through and place the MinElute column back into the same collection tube.
9. Add 750 µl Buffer PE to MinElute column and centrifuge for 1 min. Discard flow-through and place the MinElute column back into the same collection tube.
10. Centrifuge the column in a 2 ml collection tube (provided) for 1 min. Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
11. Place each MinElute column into a clean 1.5 ml microcentrifuge tube. To elute DNA, add 10 µl Buffer EB (10 mM Tris-Cl, pH 8.5) or water to the center of the MinElute membrane. (Ensure that the elution buffer is dispensed directly onto the membrane for complete elution of bound DNA.) Let the column stand for 1 min, and then centrifuge the column for 1 min.
12. If purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA, and mix by pipetting up and down before loading the gel.

8.3.4 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. Re-suspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a micro-centrifuge 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.
4. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 4–6 times.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) 4°C in a table-top microcentrifuge.
6. Apply 800 µl supernatant from step 5 to the QIAprep 2.0 spin column by pipetting. Centrifuge for 60 s and discard the flow-through.
7. Recommended: Wash the QIAprep 2.0 spin column by adding 0.5 ml (500 µl) Buffer PB. Centrifuge for 60 s and discard the flow-through.
8. Wash the QIAprep 2.0 spin column by adding 0.75 ml Buffer PE. Centrifuge for 60 s and discard the flow-through. Transfer the QIAprep 2.0 spin column to the collection tube.
9. Centrifuge for 1 min to remove residual wash buffer.
10. Place the QIAprep 2.0 column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB (10 mM TrisCl, pH 8.5) or water to the center of the QIAprep 2.0 spin column, let stand for 1 min, and centrifuge for 1 min.
11. If the extracted DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

8.3.5 Plasmid isolation from *E. coli* (Eric's modification of original Birnboim method)

1. Take 1.5 ml of overnight *E. coli* culture in LB and spin at 12000 rpm for 3 minutes.
2. Discard the supernatant and add 200 µl TGE (solution I – P1 buffer).
3. Re-suspend the cells by vortexing and incubate 5 minutes on ice.
4. Add 400 µl lysis mix (solution II – P2 buffer), mix by inverting the tube and incubate 5 minutes on ice.
5. Add 300 µl KAc (solution III – P3 buffer), shake well and keep on ice for 9 minutes.
6. Centrifuge at 12000 rpm at 4°C for 10 minutes.
7. Take 800 µl of supernatant and add 450 µl isopropanol, place them in new tubes, mix by vortexing and keep for 5 minutes at room temperature.
8. Spin at 12000 rpm for 10 minutes and wash the pellet 2 times with 70% ethanol.
9. Dry (air or SpeedVac) the pellet of DNA and dissolve in 30 µl of milli-Q water or TE buffer.

8.4 Gateway cloning protocols

8.4.1 pENTR™ TOPO® cloning protocol

Use the following procedure to perform the TOPO® Cloning reaction. Set up the TOPO® Cloning reaction depending on whether you plan to transform chemically competent *E. coli* or electrocompetent *E. coli*. 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*	Electrocompetent *E. coli*
Fresh PCR product	0.5 - 4 µl	0.5 - 4 µl
Salt Solution	1 µl	-
Dilute Salt Solution (1:4)	-	1 µl
Sterile Water	add to a final volume of 5 µl	add to a final volume of 5 µl
TOPO® vector	1 µl	1 µl
Final volume	6 µl	6 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*, page 13.

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™-T1^R. Chemically Competent *E. coli* (Box 2) are included with the kit to facilitate transformation, however, you may also transform electrocompetent cells (see page 22 for ordering information). Protocols to transform chemically competent or electrocompetent *E. coli* are provided in this section.

Required materials

Components required but not supplied:

- TOPO® Cloning reaction (from step 2, page 12)
- 15-ml sterile, snap-cap plastic culture tubes (for electroporation only)
- LB plates containing 50 µg/ml kanamycin (2 for each transformation)
- LB plates containing 100 µg/ml ampicillin (if transforming pUC19 control)
- 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 Mach1TM-T1R chemically competent *E. coli* (Box 2)
- S. O.C. Medium (Box 2)
- Optional: pUC19 positive control (Box 2)

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 (for chemical transformation) or set up your electroporator if you are using electrocompetent *E. coli*.
- Warm the vial of S.O.C. Medium from Box 2 to room temperature.
- Warm selective plates at 37°C for 30 minutes.
- Thaw on ice 1 vial of One Shot® cells from Box 2 for each transformation.

One Shot® chemical transformation protocol

Use the following protocol to transform One Shot® TOP10 or Mach1™-T1^R chemically competent *E. coli*.

1. Add 2 µl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, step 2, page 12 into a vial of One Shot® Chemically Competent *E. coli* and mix gently. Do not mix by pipetting up and down.

Note: If you are transforming the pUC19 control plasmid, use 10 pg (1 µl).

2. Incubate on ice for 5–30 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.

Transformation by electroporation

Use ONLY electrocompetent cells for electroporation to avoid arcing. Do not use the One Shot® TOP10 or Mach1™-T1^{R} chemically competent cells for electroporation.

1. Add 2 µl of the TOPO® Cloning reaction from Performing the TOPO® Cloning Reaction, step 2, page 12 into a sterile microcentrifuge tube containing 50 µl of electrocompetent *E. coli* and mix gently. Do not mix by pipetting up and down. Avoid formation of bubbles. Transfer the cells to a 0.1-cm cuvette.

2. Electroporate your samples using your own protocol and your electroporator.

3. Immediately add 250 µl of room temperature S.O.C. Medium.

4. Transfer the solution to a 15-ml snap-cap tube (i.e. Falcon) and shake for at least 1 hour at 37°C to allow expression of the kanamycin resistance gene.

5. Spread 20–100 µl from each transformation on a prewarmed selective plate and incubate overnight at 37°C. To ensure even spreading of small volumes, add 20 µl of S.O.C. Medium. We recommend that you plate 2 different volumes to ensure that at least 1 plate will have well-spaced colonies.

6. An efficient TOPO® Cloning reaction may produce several hundred colonies. Pick 5–10 colonies for analysis.

To prevent arcing of your samples during electroporation, the volume of cells should be between 50–80 µl (0.1-cm cuvettes) or 100–200 µl (0.2-cm cuvettes). If you experience arcing during transformation, try one of the following suggestions:

- Reduce the voltage normally used to charge your electroporator by 10%
- Reduce the pulse length by reducing the load resistance to 100 ohms
- Ethanol precipitate the TOPO® Cloning reaction and resuspend in water prior to electroporation

8.4.2 LR Clonase™ II Enzyme Mix cloning protocol

Prepare LR Reaction

LR Clonase™ II enzyme mix is supplied as a 5X solution. If you wish to scale the reaction volume, make sure the LR Clonase™ II enzyme mix is at a final concentration of 1X. For a positive control, use 100 ng (2 µl) of pENTR™-gus.

1. Add the following components to a 1.5-ml microcentrifuge tube at room temperature and mix:

- 1–7 µl entry clone (50–150 ng)
- 1 µl destination vector (150 ng/µl)
- TE buffer pH 8.0, to 8 µl

2. Thaw on ice the LR Clonase™ II enzyme mix for about 2 minutes. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).

3. To each sample (step 1), add 2 µl of LR Clonase™ II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly

4. Return LR Clonase™ II enzyme mix to –20°C or –80°C storage.

5. Incubate reactions at 25°C for 1 hour.

6. Add 1 µl of the Proteinase K solution to each sample to terminate the reaction.

Vortex briefly Incubate samples at 37°C for 10 minutes.

Transformation

1. Transform 1 µl of each LR reaction into 50 µl of One Shot™ OmniMAX™ 2 T1 Phage-Resistant Cells (Cat. no. C8540-03). Incubate on ice for 30 minutes. Heatshock cells by incubating at 42°C for 30 seconds. Add 250 µl of S.O.C. Medium and incubate at 37°C for 1 hour with shaking. Plate 20 µl and 100 µl of each transformation onto selective plates.

Note: Any competent cells with a transformation efficiency of >1.0 × 108 transformants/µg may be used.

2. Transform 1 µl of pUC19 DNA (10 ng/ml) into 50 µl of One Shot™ OmniMAX™ 2 T1 Phage-Resistant Cells as described above. Plate 20 µl and 100 µl on LB plates containing 100 µg/ml ampicillin.

Expected results

An efficient LR recombination reaction will produce >5000 colonies if the entire LR reaction is transformed and plated.

8.5 Modified Gateway® cloning and transformation procedures

8.5.1 pENTR™ TOPO® cloning

Depending on what kind of *E. coli* cells were used for the transformation, the ligation process was performed different. Two type of bacterial cells were used for cloning: a) Chemically Competent *E. coli* cells and; b) Electrocompetent *E. coli* cells. According the manufacturer's instructions, a 0.5:1 to 2:1 molar ratio of PCR product:Topo® vector are recommended for successful cloning and transformation of the gene of interest. However, a 3:1 ratio was also performed during the cloning processes. The cloning procedure is similar for Chemically and Electrocompetent *E. coli* cells, except for the Electrocompetent *E. coli* cells which must be used with a diluted 1:4 salt solution for electroporation. The cloning mixtures consist of the DNA (~40 ng/µl) from the PCR with the gene of interest, Salt solution, milli-Q water and the Topo® vector (15-20 ng/µl) to a final volume of 6 µl. The mixture is kept at 22°C for 30 minutes before the transformation procedure. The transformation protocol can be performed immediately after the ligation procedure or can be stored at -20°C.

8.5.2 LR cloning

After successful extraction of the pDNA from both, the pENTR™ TOPO® vector containing the gene of interest and the destination vector (PK7GW2.0), LR cloning was performed. Performing the reaction required 40 ng of the pENTR clone, 75 ng of the destination vector, TE buffer and 5X LR Clonase® II mix reaction buffer and the LR Clonase® II enzyme. The reaction was carried out in a final volume of 10 µl containing the pENTR clone and the destination vector, 2 µl of 5X LR Clonase® II mix reaction buffer, 2 µl of LR Clonase® II enzyme and TE buffer up to a final volume of 10 µl. The mixture is placed in a PCR machine set at 25°C preferably running overnight. 1 µl of proteinase K was added to the reaction and put at 37°C for 10 minutes. After completion of the process, the transformation procedure was performed.

8.5.3 pENTR™ TOPO® and LR transformation procedure

The transformation protocol was adapted from the original manufacturers protocol and the quantity of competent cells and ligation product from the pENTR™ TOPO® and LR cloning reactions were reduced. From the transformation procedure, a couple of colonies were picked up for analysis by colony PCR with Dream Taq polymerase. Some colonies were further cultured in LB liquid containing antibiotics for selection to obtain higher amounts of bacterial cells to perform plasmid DNA isolation for the following steps. The description below details how the transformation protocol was carried out during the project (see also Appendix). a) Chemically

competent *E. coli* cells transformed by heat-shock; b) Electrocompetent *E. coli* cells transformed by electroporation.

a) 2 µl from the pENTR™ TOPO® and 1 µl from the LR cloning were mixed independently with 25 µl of Library Efficiency® DH5α™ Competent or One Shot® TOP10 Chemically Competent cells and incubated on ice for 30 minutes. For control of the transformation efficiency, pUC19 DNA (10 ng/µl) is provided with the Library Efficiency® DH5α™ Competent *E. coli* cells and was performed along to check the efficiency of the transformation. Next, the cells were heat-shocked for 30 seconds in a water bath at 42°C and placed back to ice for 2 more minutes. 225 µl of room temperature S.O.C. medium was added to each cloning reaction and incubated at 37°C with shaking for 1 hour. The transformed cells were then plated in Petri dishes containing 50 µg/ml of Kanamycin (pENTR™ TOPO® cloning), 200 µg/ml Spectynomicin (LR cloning - PK7GW2.0 destination vector) and 100 µg/ml Ampicillin (pUC19 DNA) for selection and incubated at 37°C overnight. Both, the transformation of the genes of interest and the controls were plated in two different volumes (75 µl and 175 µl) to allow one of the plates to grow well spaced colonies. The transformed colonies were later used for verification by colony PCR and culturing colonies for plasmid DNA isolation.

b) 2 µl from the pENTR™ TOPO® reaction were added to 50 µl One Shot® TOP10 Electrocom™ *E. coli* cells and mixed. The cells were then transferred to a pre-chilled electroporation cuvette. The electroporation took place using a Gene Pulser® II Electroporation System (BIO-RAD) set at 2.0 kV with a resistance of 200 Ohms and a capacitance of 25 µF during 4 to 5 seconds. Immediately 250 µl of S.O.C. medium was incorporated to the cells and transferred to 1.5 ml tubes. The tubes were placed at 37°C with shaking for 1 hour. The transformed cells and the control pUC19 were then plated in Petri dishes containing 50 µg/ml of Kanamycin and 100 µg/ml Ampicillin respectively. Incubation at 37°C overnight and the following steps were performed as mentioned for Chemically Competent *E. coli* cells.