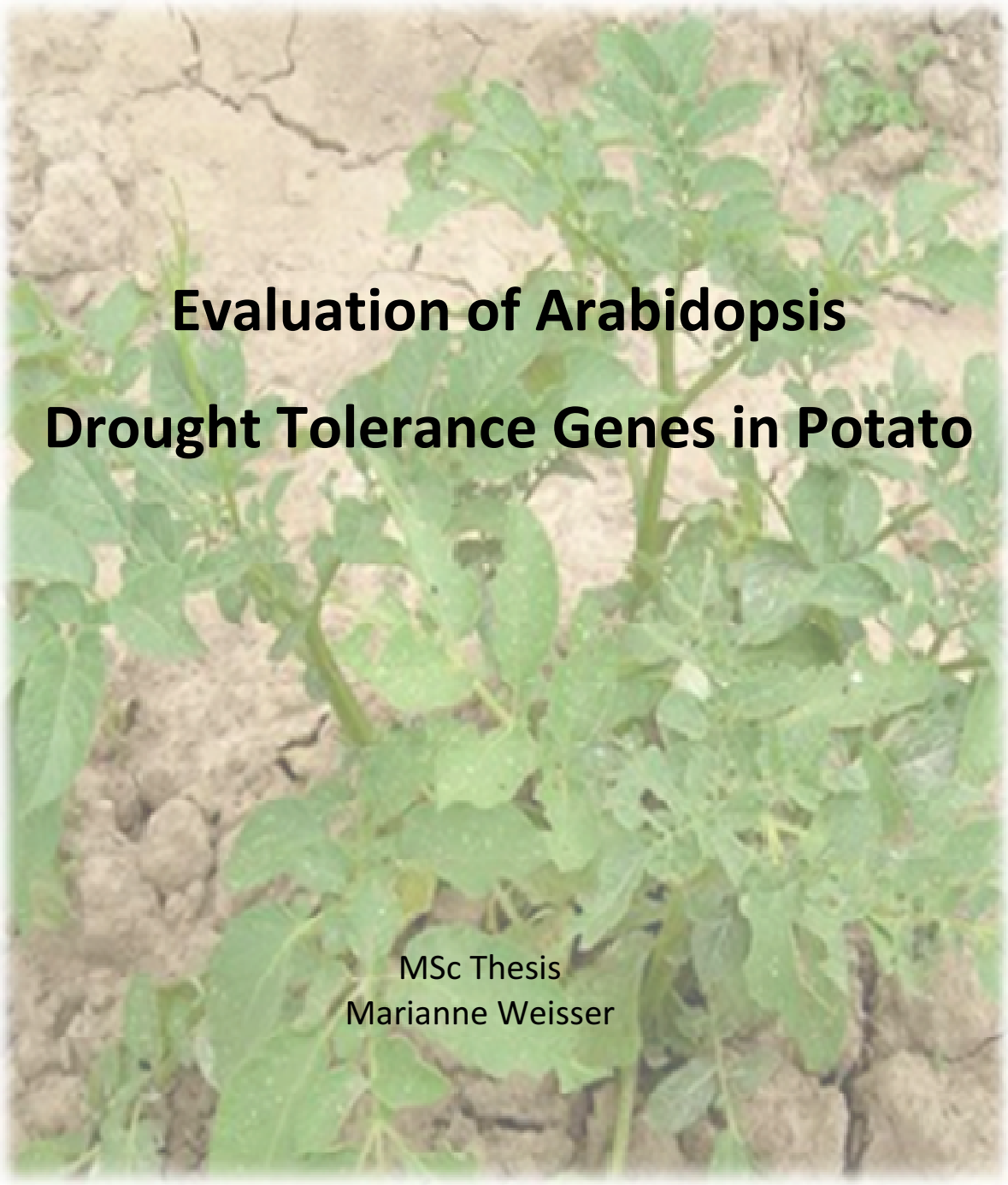


Evaluation of Arabidopsis Drought Tolerance Genes in Potato

MSc Thesis

Marianne Weisser

Evaluation of Arabidopsis Drought Tolerance Genes in Potato

MSc THESIS

Marianne Weisser

Marianne Weisser

Student Plant Biotechnology

Registration number 780214937060

Marianne.weisser@wur.nl

Supervisors:

Dr Gerard van der Linden

Anitha Kumari

Examiners:

Plant Breeding departament

Wageningen University

The Netherlands

July 2010

ACKNOWLEDGMENTS

I would like to offer my sincerest gratitude to Dr. Gerard van der Linden who has supported me throughout my thesis with his knowledge and encouragement. Thank you for giving me the opportunity to work in this project and showing me the scientific world. Also, special thanks to Anitha Kumari for her advice and guidance and for giving me the tools and allowing me the room to work in my own way. I would further like to thank to the many technicians and phD students from the Molecular Biology and Tissue Culture labs, specially to Hanneke van der Shoot and Marjan Bergervoet van Deelen for their constant and unconditional help with any problems that I had and made my work in the lab a pleasure.

My deepest appreciation goes to my family especially to my parents for their constant love and support throughout my life. Also to God for guiding me and take care at all times. And, to my love Trevor, I wish to give him the greatest acknowledgment by dedicating this thesis to him, for all of the long nights that he was with me by my side from the beginning to the end of this Thesis and most importantly, for leaving everything and moving to the other side of the world just for love.

SUMMARY

The potato (*Solanum tuberosum*) is the fourth largest food crop in the world. More than half of the global potato output comes from developing countries. However, the majority of the crop production land in developing countries is under extreme drought conditions, obtaining great harvest losses. In the next two decades, the world's population will grow by more than a hundred million people a year and most of this growth will come in the developing countries. Therefore, there is a necessity of developing potato varieties that can withstand these harsh conditions. Conventional breeding approaches to improve water scarcity in potato have had limiting results. Gene transfer technology, on the other side, can offer a useful tool in the development of drought stress tolerance in potato varieties.

Several genes have been identified and transformed into crops obtaining abiotic tolerance with successful results. Some of them are Hardy (HRD) and Shine 1 (SHN1), two *Arabidopsis* transcription factors that in nature are expressed in the inflorescence, however ectopic overexpression of these TFs can trigger drought stress tolerance. In order to obtain drought resistant potato plants, HRD and SHN1 were inserted into the potato genome by *Argobacterium* Mediated Plant Transformation technique. The constitutive promoter of Cauliflower Mosaic Virus (CaMV35s) was utilized to drive expression of both genes in the vector. Due to the high expression of HRD in the root and SHN1 in green tissue, the root inducible promoter AKT1 and the light inducible promoter SsuAra were also used respectively in the vectors. In addition, the drought inducible promoter RD29A was used to observe the presence or absence of expression of the genes under water scarcity treatments.

The transformation technique showed an efficiency of 100% of transformed plants tested by a DNA analysis of leaf tissue. Moreover, an RNA analysis of the expression of HRD and SHN1 in the transgenic potato lines revealed that they were expressed in all the leaf and/or root samples of both genes under the control of all different promoters. Also, the control potato plants containing the RD29A promoter without inducing drought stress condition showed expression of both TF genes.

Engineering stress tolerant potatoes containing the Hardy and Shine 1 Arabidopsis transcription factors could aid the growth and sustainability of the globes food supply in a world with increasing water scarcity. Therefore, further investigation needs to be done to evaluate if the genetically modified potato can not only express the TFs genes, but also develop a phenotype of drought stress tolerance in the potato plants.

TABLE OF CONTENT

1. INTRODUCTION

1.1. Plant Abiotic Stress and water scarcity as a major worldwide problem

Environmental stresses, such as drought, salinity, extreme temperatures and radiation represent the most limiting factors on the growth of plants and agricultural production. The set of mentioned stresses, termed as Abiotic Stress, is the main cause of crop loss worldwide (Rodriguez et al.2005). Every year up to 82% of annual crops yield is lost due to abiotic stress and the amount of available, productive arable land is continuously decreasing, forcing the agricultural production to move to areas where the potential for abiotic stress is even greater (Skinner 2005). Among the abiotic factors, drought is one of the major problems in crop production, preventing plants from realizing their full genetic potential (Boyer, 1982). Drought severity depends on different factors, such as moisture storing capacity of soils, evaporative demands and quantity and distribution of rainfall (Wery et al. 1994).

The worldwide population is growing exponentially and the demand for water is increasing at an alarming rate, therefore the availability of water is becoming an extremely scarce resource. Globally, there is a vast number of countries (around 80) living with extreme drought conditions, which makes up close to 40% of the world population (Hamdy et al, 2003). Around 15% of the worlds irrigated lands produce nearly 30% of the globes food. Due to the rapid growth of the population, the search is on to find new land to be cultivated, however the most favourable land and resources have already been exploited (Munns 2002). Therefore, it is necessary to generate crop plants that could withstand such harsh conditions.

1.2. Drought effects on plants

The effects of drought range from morphological biochemical and physiological levels and are evident at all phenological stages of plant growth at whatever stage the water shortage takes place. Photosynthesis is one of the major metabolic processes that are directly affected by drought. A reduction in photosynthesis, determine a decrease in leaf expansion, stomata closure, impaired photosynthetic machinery, enhance formation of reactive oxygen species (ROS), premature leaf senescence, decrease in assimilates translocation and associated reduction in crop production (Farooq et al. 2009a). In addition, the stress imposed by drought conditions affect the water

relation, such as water use efficiency, relative water content, leaf water potential, stomatal resistance, rate of transpiration leaf and canopy temperature (Farooq et al. 2009b).

1.3. Drought resistance mechanisms on plants

Due to the drought effects on plants, they respond by the induction of several morphological, physiological and molecular mechanisms that enable the plant to withstand the stress. Drought resistance mechanisms can be grouped in to three categories, i.e. drought escape, drought avoidance and drought stress tolerance.

Drought escape indicates that plants have adapted by having rapid growth, maturation, flowering/fruiting and senescence, permitting them to reproduce before the environment becomes dry. This keeps tissues from being excessively exposed to dehydration (Price et al. 2002).

Drought stress avoidance consists of mechanisms that reduce water loss from plants and improve the water uptake. Reduction of water loss is performed by reducing epidermal (stomatal and lenticular) conductance, thickening of the cuticle (cutin and cuticular waxes) and epicuticular waxes, decreasing absorption of radiation by leaf rolling or folding and reducing evaporation surface (leaf area). Water uptake is improved by maintenance of turgor through an extensive and efficient (deep and thick) root system with large active surface area and an increase in hydraulic conductance. Plants under drought condition survive by managing a balancing act between maintenance of turgor and reduction of water loss (Mitra 2001).

Drought tolerance is defined as the ability to grow, flower and display economic yield under suboptimal water supply (Farooq et al. 2009a). Plants are tolerant to desiccation to some extent, and that moderate short –term disturbances of plant water balance do not immediately affect yield (Schafleitner 2009). The mechanism of the plant to tolerate the drought stress consists of the maintenance of cellular stability and turgor through osmotic adjustment, compatible solutes, antioxidation and a scavenging defence system (Madhava et al. 2006).

1.4. Drought stress signalling and regulatory pathways in plants

The plant response to drought stress involves the activation of a wide array of genes and biochemical-molecular mechanisms, which together with constitutive traits determine whether a plant is more tolerant or susceptible to drought (Farooq et al. 2009a; Schafleitner. 2009).

1.4.1. Signalling cascades and transcriptional control

Currently, it is not clearly defined on how plants perceive drought stress. However, a general model describing the responses of plants to water stresses can be illustrated in the following: starting with the perception of signals from the environment. The molecules that perceive the initial stress signal are called sensors or receptors, like hystidine kinases. These receptors will initiate or suppress a phosphorilate cascade to transduce and amplify the signal information by activating the mitogen-activated protein kinase (MAPK) cascade or triggered second messengers such as ligand-sensitive Calcium channels, inositol phosphates, phospholipids, hormones and ROS. Then ending with the synthesis of functional protein responses, like osmolytes, ROS scavengers or membrane protectors (Rodriguez et al. 2005).

1.4.2. Regulation of gene expression

Previous studies of the expression pattern of genes induced by drought have revealed a broad variation in the timing of their induction and differences in their responsiveness to the phytohormone absicic acid (ABA). These observations indicated that both, ABA-dependent and ABA-independent regulatory systems are involved in drought stress responsive gene expression (Shinozaki and Yamaguchi-Shinozaki 2000; Yamaguchi-Shinozaki and Shinozaki 2005). There are at least five regulatory systems for gene expression, three are ABA-dependent and the other two are ABA-independent (Shinozaki and Yamaguchi-Shinozaki. 2007).

In genes regulated by ABA there are three main groups of transcription factors involved. One of them is AREB/ABF that corresponds to a two basic leucine zipper (bZIP). This transcription factor can bind to ABRE (ABA responsive element), which is the major cis-acting element, and activate ABA dependent gene expression. The other group consist of MYC and MYB transcription factors that bind the cis-elements MYCRS and MYBRS respectively, and activate RD22 gene promoter. And the last one is RD26 NAC transcription factor that activate the Gly gene (Shinozaki and Yamaguchi-Shinozaki. 2007; Rodriguez et al.2005).

In the ABA-independent pathway, the transcription factors belonging to the ERF/AP2 family, termed DREB2 (dehydration responsive element binding proteins) specifically interacts with the DRE/CRT (dehydration responsive element C-repeat) sequence of the RD29A promoter and activate the transcription of drought stress inducible genes. The other group correspond to NAC and HD-ZIP transcription factors that bind to the cis-acting elements of the ERD1 (Early response to dehydration) promoter and start with the gene expression. (Shinozaki and Yamaguchi-Shinozaki. 2007) (Rodriguez et al.2005) (Yamaguchi-Shinozaki and Shinozaki 2005).

Several drought inducible genes are not only induced by drought stress, but also by high salinity, low temperature or injury responses. This suggests that there is an intertwined network between the stress-signalling pathways, implying the interaction of biochemical process functions (figure 1) (Shinozaki and Yamaguchi-Shinozaki. 2007).

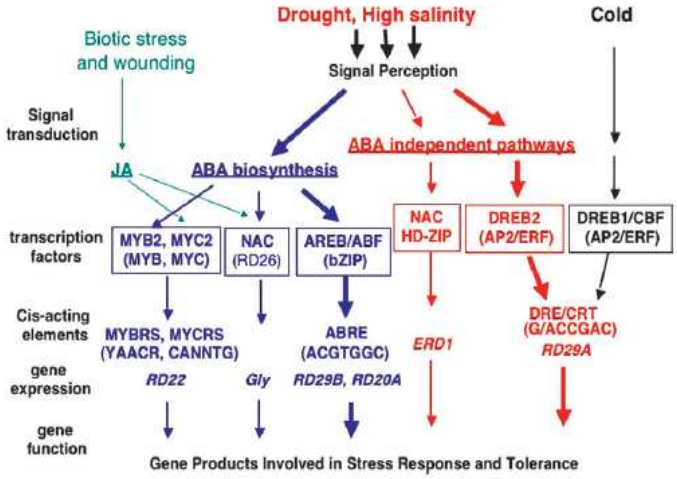


Figure 1. Transcriptional regulatory networks of drought stress signals, gene expression and a cross-talk between the stress signal transduction pathways. (Shinozaki and Yamaguchi-Shinozaki. 2007).

1.5. Genetic engineering for drought tolerance

1.5.1. Genetically modified crop plants

To cope with drought stresses, plants alter their metabolic pathways to adjust to changed environments. The metabolic pathways become more active to keep the plant survive under stress conditions. However, the initiation and efficiency of these pathways differ from species to species or genotype to genotype to a great extent. These genetically complex responses to drought stress conditions are very difficult to control (Madhava et al. 2006). Most of the major crops are sensitive

to drought stress; therefore sophisticated approaches for the molecular breeding of drought-tolerant crops are needed. Therefore, present engineering strategies rely on the transfer of one or several genes that directly confer the function of plant cells to resist water stress, such as osmotic regulatory protein, enzymes for osmolytes biosynthesis (e.g. betaine or proline) or detoxification enzymes. As well as, other types of genes, whose coding products play a role in regulating gene expression and signal transduction, such as protein kinases, enzymes in phospholipids metabolism and transcription factors (Yamaguchi-Shinozaki and Shinozaki 2005).One of the most widely utilized techniques of introducing the genes into the plant genome is *Agrobacterium*-Mediated Plant Transformation (Slater et al. 2008).

1.5.2. Engineering drought stress tolerance of transgenics by overexpressing transcription factors

Transcription factors (TFs) are small molecules that attach to specific sites on a DNA molecule called promoter, which is adjacent to the genes that they regulate, in order to activate or deactivate the expression of those genes (Rodriguez et al.2005). These DNA molecules contain a cis-element, which is the site where the TF binds to it. TFs and promoter genes have been found among the drought stress inducible genes, suggesting that various transcriptional regulatory mechanisms function in the drought stress signal transduction pathways.

The Hardy (HRD) gene, a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family, was identified in *Arabidopsis,* which is usually expressed in inflorescence tissue, such as mature seeds, petals and pollen*. Ectopic expression in Arapidopsis* by a gain-of-function mutant *hrd-D* (D denoting dominant effect), exhibits roots with enhanced strength, branching, and cortical cells, as well as, thicker leaves with more chloroplast-bearing mesophyll cells. These results showed drought resistance and salt tolerance in *Arabidopsis*. Experiments, of transformation of HRD *Arabidopsis* gene in rice, demonstrated improvements in drought resistance and water use efficiency by an increment in photosynthesis assimilation, efficiency and transpiration reduction, accompanied by an increase in plant biomass and bundle sheath cells (Karaba et al. 2007).

Shine (SHN) *Arabidopsis* genes, encodes a member of the ERF (ethylene response factor) subfamily B-6 of ERF/AP2 transcription factor family. Their normal expression in Arabidopsis plants is located mostly in flowers, fruits and seeds. Previous studies on SHN1 TFs revealed that when overexpressed in *Arabidopsis*, resulted in glossy leaf phenotype with curled structure and an alteration in the cuticle permeability, increasing drought tolerance (Broun et al. 2004; Kannangara et al. 2007). In tomato, constitutive overexpression of the *Arabidopsis* SHN1 gene led to a significant enhanced epicuticular

wax, affecting transpiration, stomatal conductance, photosynthetic efficiency and water use efficiency. SHN2 under normal expression in Arabidopsis has a highly specificity in flowering in Transgenic rice plants overexpressing the SHN2 *Arabidopsis* gene also show the characteristic phenotype of the *Arabidopsis* SHN1 overexpressor (Karaba 2007; Aharoni et al. 2004).

1.5.3. Role of promoters in transgenic drought tolerance plants

Depending on the objective of when (temporary), where (spatial) and the level of expression of the foreign genes in transgenic plants, it will determined the type of promoter that will be used (De Almeida et al. 1989).

Currently, the most widely used promoter is the constitutive 35s gene promoter of Cauliflower Mosaic Virus (CaMV35s), utilized to drive expression of genes in plant transformation vectors. This strong promoter is considered to be expressed in all tissues of transgenic plants, making it ideal for driving the expression of target genes, selectable markers and in some cases of reporter genes (Mitsuhara et al. 1996).

Nopaline synthase (NOS) gene, on the other hand, corresponds to an *Agrobacterium* gene. The NOS promoter has been frequently used for construction of plant-selectable markers, since the *nos* gene has been thought to be constitutive in all plant tissues (Mitra and An 1989). However, it has been observed that the NOS promoter is inducible by mechanical wounding and the wound response is further enhanced by the phytohormone auxin (An et al. 1990; Sanders et al. 1987).

Inducible promoters are a very powerful tool in genetic engineering, because the expression of genes linked to them can be turned on or off at certain stages of development of an organism or in a particular tissue. In Arabidopsis, the stress inducible gene responsive desiccation 29 A (RD29A) is responsible for dehydration, high salt, and low temperature and is triggered by ABA-independent pathway. A cis-acting element was identified in the promoter region containing the sequence TACCGACAT named the dehydration-responsive element (DRE). This sequence is also found in the promoter regions of others dehydration and low temperature stress inducible genes (Sakuma et al. 2002; Yamaguchi-Shinozaki and Shinozaki 1993; Wu et al. 2008).

Another inducible promoter is the Arabidopsis K^+ Transporter 1 (AKT1), which correspond to genes that encode plant potassium transporters. (Fox and Guerinot 1998). It is primarily expressed in root tissue, however has been also localized in leaf hydathodes and also in differentiated leaf primordia

by a promoter activity analysis (Lagarde et al. 1996; Dennison et al. 2001; Basset et al. 1995; Golldack et al.2003).

Furthermore, the rbcS gene family of Arabidopsis thaliana drives a tissue specific expression. It consists of four members, of which the ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit (rbcS) ats1A gene promoter (pSsuAra) appears to be the most highly expressed (Kumar and Timko 2004).The expression of rbcS genes is regulated by light and is under phytochrome control (Krebbers et al. 1988).

1.6. Potato plant and drought stress: future perspectives

The potato (*Solanum tuberosum*) is a tuberous crop that belongs to the Solanaceae family. It is native to the Andes Mountains in Chile, Peru and Bolivia and has been cultivated for at least 2.400 years. Potato is an easy to grow plant and can provide more nutritious food faster and on less land than any other food crop (Mullins et al. 2006).

The potato is the world's fourth largest food crop (Chakraborty et al. 2000). On a global scale, since the 1960s the potato production has remained relatively static, with a steadily rising (Mullins et al. 2006). Slight decreases in potato consumption in developed countries in North America and Europe are overcompensated by strong increases in developing countries, such as the one from Asia, Africa and Central and South America, where potato production has tripled between 1960 and 2000 and will need to continuously rise to satisfy the constant demand. There is an impetuous need for an increase and stable potato production to meet increasing demands for food from human population growth during a period of climate change and water scarcity increments (Hijmans 2003; Ghosh et al. 2001).

The majority of the world potato production areas are in developing countries, which have extreme drought conditions contributing to great harvest losses. Even though, these regions still yield around 30% of the global production. Therefore, if potato varieties are engineered to drought tolerance, the increase of the yield would be significant (Schafleitner 2009).

Potato has a major influence on the socioeconomic world market; therefore there is an impetuous necessity of potato producing countries to remain competitive and to increase the development of water use efficiency technologies (Condon et al. 2004).

Experiments to increase drought stress tolerance in potato by using conventional breeding methods have faced significant failures due to the genetic complexity of potatoes. In addition, new developed varieties require not only an enhanced water tolerance trait, but also high yield and quality, which in turn increase the difficulty of the breeding approach. Gene transfer technology, on the other side, offer a useful tool in the development of drought stress tolerance in potato varieties, which are already accepted in the market (Waterer et al. 2010)

1.7 Scope of the thesis

Abiotic stress, such as drought can reduce yield and quality to potato crop. In the present study, the Gene transfer technology approach was used to evaluate the feasibility of obtaining transgenic potato plants conferring drought resistance. Hardy and Shine1 are two Arabidopsis transcription factors, which in nature under normal conditions are present in the inflorescence; however when they are overexpressed can trigger drought tolerance in Arabidopsis. Therefore, in order to obtain drought resistance in potato plants, Hardy and Shine1 were inserted into the potato genome by *Agrobacterium* Mediated Plant Transformation technique. Both transcription factors were driven by the Cauliflower mosaic virus (CaMV35s) constitutive promoter and the stress inducible gene responsive desiccation promoter (RD29A). Furthermore, SHN1 was carried by a ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit (rbcS) ats1, a light inducible promoter SsuAra and HRD by a Arabidopsis K^+ Transporter 1 root inducible AKT1 promoter. All the plantlets tested after transformation by a RNA analysis showed expression of both transcription factors. These results suggest that the transformation technique used in the present experiment was highly efficient and that it was feasible to transform HRD and SHN1 TFs into potato plant.

2. MATERIALS AND METHODS

2.1. Plant material and growth conditions

Solanum tuberosum L. plants variety Desiree was used in this experiment. The plants were propagated in vitro in plastic containers (9X8 cm) containing MS 20 medium (4.4 g/l Murashige and Skoog (MS) with vitamins, and 20g/l saccharose). The medium was adjusted to pH 5.8, 8 g/l agar was added and then autoclaved for 15 minutes at 120°C. Potato plantlets were provided by Marjan Bergervoet van Deelen (Tissue Culture laboratory of Plant Breeding departament, Wageningen University). Explants of approximately 2 cm were incised from the apical area of the plantlets, transferred to the containers and cultured in a growth chamber with a photoperiod of 16 hours light at 21°C for a period of 5 weeks, prior to the transformation procedure.

2.2. Constructs

The binary vectors pBINplus-DEST (figure 2) containing the gene cassettes of promoters and transcription factor genes: CaMV35S::HRD, RD29a::HRD, AKT1::HRD, CaMV35S::SHN1, RD29a-SHN1 and SsuAra::SHN1 were kindly provided by Hanneke van der Shoot (Plant Breeding department, Wageningen University, The Netherlands). The different combination of the promoters and transcription factors with their corresponding sizes inserted in the pBINplus-DEST vector are shown in table 1.

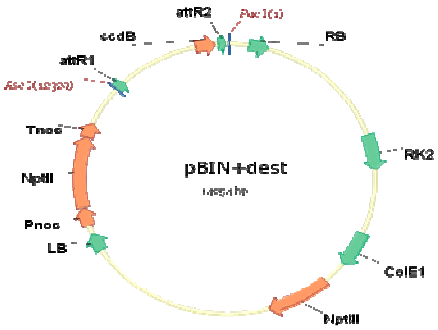


Figure 2. Binary vector pBINplus-DEST. nptII: neomycin phosphotransferase type II; nptIII: neomycin phosphotransferase type III; Asc I and Pac I: restriction sites; Pnos: promoter of nopaline synthase; t- nos: terminator of nopaline synthase; attR1 and attR2-sites: promoter recombination sites; ColE1 ori: origin of replication in *E. coli*; RK2: origin of replication in *Agrobacterium*; ccdB: negative selectable marker.

Table 1. Promoters, transcription factors, vector and genes cassettes with their corresponding fragment sizes.

PROMOTER	SIZE (bp)	TF GENE	SIZE (bp)	VECTOR	SIZE (bp)	GENE CASSETES	SIZE (bp)*
CaMV35S	541	HRD	853	pBINplus-DEST	14254	CaMV35S-HRD	1414
RD29a	934	SHN1	891			RD29a-HRD	1807
AKT1	2031	SHN2	861			AKT1-HRD	2904
SsuAra	1731					CaMV35S-SHN1	1452
NOS	307					RD29a-SHN1	1845
						SsuAra-SHN1	2642
						NOS-SHN2	1188
						CaMV35S-SHN2	1427
						RD29a-SHN2	1815
						AKT1-SHN2	2912
						SsuAra-SHN2	2612

*20bp were included, due to the attR2 recombination site located between the promoter and gene.

2.3. Agrobacterium tumefaciens transformation

2.3.1. Bacterial strain

Agrobacterium tumefaciens strain COR308 and AGL1-virG were used for potato transformation. COR308 holds a helper plasmid carrying a selectable marker gene conferring resistance to tetracycline. AGL1-virG holds a helper plasmid carrying a carbenicillin resistance gene and an extra plasmid containing the virulence gene G which increases the virulence gene expression and carrying a cloramphenicol resistance gene.

2.3.2. Introduction of vector into Agrobacterium

pBINplus-Dest vector was introduced into *A. tumefaciens* strain COR308 and AGL1-virG by electroporation using the Electroporator Gene Pulser II (Bio-Rad Laboratories, The Netherlands) at 1.4KV according to the manufacturer's instructions. Bacterial cells were placed on LB agar plates containing 4 mg/l of tetracycline (bacteria selection) and 50 mg/l of kanamycin (vector selection) for COR308 strain and 100mg/l of carbenicillin (bacteria selection), 50 mg/l of cloramphenicol (vir-G selection) and 50 mg/l of kanamycin (vector selection) for AGL-virG strain. They were incubated for two days at 30°C in a shaker at 200 rpm.

Selections of colonies from each bacterial plate were grown for two days at 30°C in liquid Luria Bertani (LB) medium containing their respective selective antibiotics. For plasmid isolation a QIAprep Miniprep kit (Qiagen Benelux B.V., The Netherlands) was used as described by the manufacturer. To

verify the presence of the plasmid, an agarose gel 1% was run by loading 2 µl of the samples. The colony cultures were then stored in glycerol at -80°C.

For the confirmation of the presence of the gene cassettes in the vectors, a Polymerase Chain Reaction (PCR) was performed. The promoters with their corresponding TF gene were tested in each vector. The primer sets used for PCR were: 5'-AGTCAAAGATTCAAATAGAGGAC-3' (forward) and 5'-TGGTCAAGAGTCCCCCGTGTTCTCT-3' (reverse) for pCaMV35S, 5'-GAGGAGAGAGGAGGTAAACA-3' (forward) and 5'- CAAACGGCACATCCTTCTCA-3' (reverse) for pRD29A, 5'-GCGACATAGCAATCAAACCT-3' (forward) and 5'-GTTCATCATCCCTAGATCGT-3' (reverse) for pAKT1, 5'-AAACAATGAGTTGGTGCATG-3' (forward) and 5'-CCTACATCACTTCCCTATCG-3' (reverse) for pSsuAra, 5'-ATGCAAGGAACCTCCAAAGA-3' (forward) and 5'-CTAGAGCTGCCACGTCATAG-3' (reverse) for HRD and 5'-GGTACAGACGAAGAAGTTCA-3' (forward) and 5'-TATGGGAGCTGGCTGTGTCA-3' (reverse) for SHN1.

PCR was carried out on a Veriti thermocycler (Applied Biosystems, USA). The temperature protocol for PCR amplifications was as follows: initial denaturation at 94°C for 5 min, followed by 30 amplification cycles of 30 s at 94°C, 30 s at 55°C and 2 min at 72°C, followed by final extension at 72°C for 10 min. The PCR reaction mixture contained 2µl 10X PCR buffer Supertaq (HT Biotechnology, UK), 2 µl dntp's (1mm), 2 µl of each primer, forward and reverse (2 µm), 0.1 µl Supertaq polymerase (HT Biotechnology, UK), 9.9 µl sterile mQ water and 2 µl of the template in a final volume of 20µl. PCR products were separated on a 1% agarose gel and stained with ethidium bromide.

For reconfirmation of the previous PCR results, a restriction enzyme digestion analysis was carried out. The T-DNA of the binary vector pBINplus-DEST has specific flanking restriction sites that alowed the cleavage of the section containing only the promoter and gene (Figure 1). AScI and PacI were the restriction enzymes (Fermentas, Germany) used for the digestion reaction. Digestion of pBINplus-DEST was performed by incubation of 2µl of enzyme with 4µl of plasmid in 2µl of Buffer 4 and 12µl of mQ water for 2 hours at 37°C. The products of digestion were analysed by electrophoresis on 1% agarose gel.

2.4. Plant transformation via Agrobacterium tumefaciens

For Desiree transformation, internode explants 0.5 cm long approximately, excised from 5 weeks old plantlets in vitro cultures were used. The explants were placed horizontally on filter papers in 9 cm Petri-dishes containing REB medium (100 explants approximately per Petri- dish). Two layers of filter paper were placed on the top of the Petri-dish containing R3B medium (4.4 g/l MS, 30 g/l saccharose, 8 g/l agar, 2mg/l NAA and 1 mg/l BAP with a pH 5.8 before autoclaving). In order to moisture the filter paper, 1.5 ml of liquid PACM (4.4 g/l MS, 2 g/l caseine hydrolysate, 30 g/l saccharose 1 mg/l 2.4 D, 0.5 mg/l kinetin and pH 6.5) was added to the paper on each dish.

The transformed *A. tumefaciens* strains COR308 and AGL1-virG were prepared by inoculating a loop of the *Agrobacterium* glycerol stock in 5 ml liquid medium of LB containing the corresponding antibiotics and grown for overnight at 28°C in a shaker at 150 rpm. After 24 hours the 5 ml liquid culture were transferred to 95 ml liquid LB medium containing antibiotics to further grow overnight at the same previous temperature and shaking revolutions.

Approximately 50 ml of *A. tumefaciens* culture was poured in a 9 cm Petri dish. The outer layer of filter paper containing the explants was removed from the Petri dish and placed upside down facing the culture. Afterwards, the filter paper was removed from the dish and only the culture and explants remained together for an incubation period of 5 minutes. Then, the explants were separated from the culture by a sieve and placed on new filter paper in order to eliminate the excess of culture. Subsequently, the explants were placed back on their previous Petri dishes, on the second remaining filter paper layer. Explants were cultured under controlled conditions at 24°C and 16 hours light photoperiod for two days.

After co-cultivation, explants were transferred to new Petri-dishes (9 cm) with selection medium ZCVK, containing MS 20, 1mg/l zeatin, 100 mg/l cefotaxin, 200 mg/l vancomycin and 100 mg/l kanamycin (the hormone and antibiotics were filter-sterilized). Explants were maintained under culture room conditions (as above) for three weeks. A number of 20 internodal explants per Petri dish were used and placed in a horizontal position as before. Three controls used in this transformation experiment consisted on: non-inoculated explants without kanamycin selection medium, non-inoculated explants with kanamycin selection medium and inoculated explants without kanamycin selection medium. After 3 weeks, the explants were transferred to new fresh Petri dishes containing the same medium as above. This procedure was repeated every three weeks

until the explants developed shoot formation and were used for further plant regeneration. Two batches of each, *Agrobacterium* containing Hardy and Shine constructs with their corresponding promoters, were transformed into potato internodal explants (approximately 300 explants per construct).

Shoots longer than 2 cm, grown from the putative transformed explants were excised and transferred to plastic containers (8x9 cm) containing MS 20 with antibiotic selective medium (100 mg/l cefotaxin and 100 mg/l kanamycin). A maximum of ten shoots were placed in each container. The explants that did not present the desirable shoot size, were transferred to fresh ZCVK medium in new Petri dishes. After four weeks, the plantlets (well grown and rooted) were multiplied and placed in plastic containers (8x9 cm). Internodal explants were placed with the cut face in contact with the medium containing MS 20 with 100 mg/l cefotaxin and grown at 24°C and 16 hours light photoperiod.

2.5. Genomic DNA extraction and PCR analysis

Genomic DNA was extracted from approximately 100 mg of leaf tissues of putative transformants as well as from wild-type control. For plantlets containing pAKT1::HRD construct, root samples were also harvest. The DNA was obtained by using the DNeasy Plant kit (Qiagen Benelux B.V., The Netherlands) as described by the manufacturer. PCR was carried out by using the same PCR cycling parameters as described in experiments above. PCR results were reconfirmed by a restriction enzyme analysis. The restriction enzymes containing a unique cleavage site for the promoters and TF genes were; MspI for pCaMV35s, AluI for pRD29a, BcuI for SsuAra, EcoR1 for pAKT1, MspI for HRD and TAqI for SHN1 (Fermentas, Germany). Tango was the compatible buffer used for the digestion reaction (Fermentas, Germany). The vectors containing the corresponding construct were used as positive control. Digestions of the previous PCR products were carried out by incubation of 2µl of enzyme with 4 µl of template in 2µl of Buffer Tango and 12µl of mQ water for 2 hours at 37°C. The products of digestion were analysed by electrophoresis on 1% agarose gel.

2.6. Drought and light induction assay

For drought induction, 3 transgenic lines of each of the construct pRD29a::HRD and pRD29a::SHN1 construct were analysed. For each line a number of 6 explants (replicates) were used, with a total of 18 explants per construct. The tranformants were multiplied and transferred to glass tubes containing 15 ml of MS 20 medium. Internodal explants were placed (one per tube) with the cut face in contact with the medium and cultured them under controlled conditions at 24°C and 16 hours light photoperiod for ten days. Subsequently, the plantlets were transferred to new MS 20 medium glass tubes containing (PEG) solution (2.2 MS with vitamins, 20 g/l succhrose and 1.2 g/l MES buffer, pH 5.8, autoclaving and 400 g/l PEG 8000 afterwards). The preparation of the medium containing PEG solution was done as follows: to a 15 ml MS 20 medium tube, 20 ml of PEG solution was added and left it for 4 days to allow the contact between the medium and PEG. Then, the excess of PEG solution was removed from the tube and was ready to be used. The plantlets were cultured under the same controlled conditions as before. After 7 days, leaf samples (30 mg approximately) of the drought induced plantlets were harvest in 1.5 ml tubes, frozen in liquid nitrogen and stored in -80°C for further analysis.

For the plantlets containing the light inducible promoter pSsuAra, the induction was by receiving the standard 8 hours dark of photoperiod during all the stages of culturing, as well as the rest of the plantlets from the experiment.

2.7. RNA extraction and reverse transcription PCR analysis

Total RNA was extracted from leaf tissue of transgenic plantlets containing the cassettes: CaMV35S::HRD, RD29a::HRD, AKT1::HRD, CaMV35S::SHN1, RD29a-SHN1 and SsuAra::SHN1, as well as the plantlets carrying the pRD29a::HRD and pRD29a::SHN1 constructs with PEG treatment. For pAKT1::HRD, root tissue was also analysed.

RNA was extracted by using the RNeasy Plant mini kit (Qiagen Benelux B.V., The Netherlands) according to the manufacturer's instructions. The RNA was then treated with DNase, by using the Deoxyribonuclease I Amplification Grade kit (Invitrogen, USA) as described by the manufacturer, to eliminate false positives from DNA contamination of the RNA.

For cDNA synthesis the iScript cDNA synthesis kit (Bio-Rad Laboratories, The Netherlands) was used, as described by the supplier. Consecutively a reverse transcription polymerase chain reaction (RT-PCR) analysis was carried out. The primers combination for the HRD and SHN1 genes are the same as described previously. Specific oligonucleotides ef1αF1 (5'-ATTGGAAACGGATATGCTCCA-3') and ef1αR1 (5'-TCCTTACCTGAACGCCTGTCA-3') for the Elongation factor-1-alpha, a housekeeping gene, was used as a positive RNA control. The same cycling parameters were done as described in the previous PCR performance. The PCR products were then separated by a 1% agarose gel electrophoresis.

2.8. Comparative sequence analysis

A comparison analysis of the genes encoding Hardy (at2g36450) and Shine 1 (at1g15360) in *Solanum tuberosum* genome was performed by using the following databases: NCBI (www.nbci.nlm.nih.gov), PotatEST DB (http://biosrv.cab.unina.it/potatestdb), Computational Biology and functional genomics Laboratory (http://compbio.dfci.harvard.edu/), SOL Genomic Network (http://solgenomics.net/) and Potato Genome Sequencing Consortium (http://www.potatogenome.net/index.php). For each data bank program alignments were done, searching in the *Solanum tuberosum*, *Solanum* family and nucleotide collections databases. Basic Local Alignment Search Tool BLASTN, BLASTX, and TBLASTX programs were used to find the targeted regions of sequence homology. In addition, a multiple alignment by using the program Molecular Evolutionary Genetic Analysis (MEGA 4) was done with selected sequences containing the highest level of similarity to the Arabidopsis HRD and SHN1 genes.

2.9. Construction of plant Expression Vector containing SHN2 gene

The promoters NOS, CaMV35s, RD29a, SsuAra and AKT1, each inserted into pENTR vectors (Entry Clone), were recombined into pBINplus-DEST vector (Destination Vector) carrying the Shine 2 transcription factor gene by using the LR Reaction of Gateway Cloning Technology (Invitrogen life technologies, UK) accordingly to the manufacturer's instruction (figure 3). All the pENTR and pBINplus-DEST vectors were kindly provided by Hanneke van der Shoot (Plant Breeding department, Wageningen University, The Netherlands).

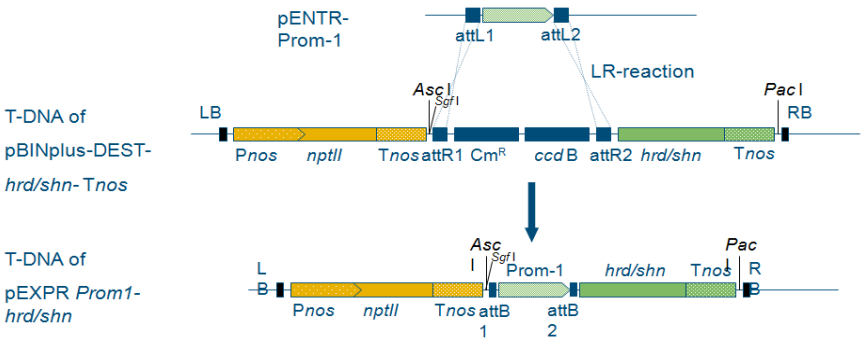


Figure 3. Representation of the recombination of a promoter present in a PENTR vector in pBINplus-DEST vector and forming into a pEXPR vector. PNOS: promoter of nopaline synthase; nptII: neomycin phosphotransferase type II; *Asc, AscI, Pac* and *PacI*: restriction sites; t- nos: terminator of nopaline synthase; attR1 and attR2-sites: promoter recombination sites; CmR: chloramphenicol resistance; ccB: negative selectable marker; LB and RB: left and right borders.

For E.Coli transformation, One Shot TOP10 Chemically Competent *E. coli* kit was used (Invitrogen life technologies, UK) as described by the manufacturer. E.Coli cells were placed on LB agar plates containing 50 mg/l of kanamycin (vector selection) and incubated overnight at 37°C. Colonies from the plates were selected and grow overnight in liquid LB containing 50 mg/l kanamycin selective antibiotics at 37°C shaking. For plasmid isolation a QIAprep Miniprep kit (Qiagen Benelux B.V., The Netherlands) was used, as described by the manufacturer. The rest of the colony cultures were stored in glycerol at -80°C.

To confirm the presence of the vectors carrying their corresponding gene cassettes, a PCR was done. A combination of SHN2 forward primer with pBINplus-DEST reverse primer was used in all vectors, as well as promoters NOS, 35s, RD29a, AKT1 and SsuAra forward primers with SHN2 reverse primers. As a negative control, the reverse primers of all five promoters and SHN2 were also used.

The primer sets used for PCR were: 5'- CTCTGCGTTAAGATCTCCCA-3' (forward) and 5'-GATGTGTGAGCTGTCGTTGT -3' (reverse) for SHN2, 5'- TACACAGCCAGTCTGCAGGT-3' (forward) and 5'- GTGGTTGGCATGCACATACA-3' (reverse) for pBINplus-DEST and 5'-GATCATGAGCGGAGAATTAAGGGAG-3' (forward) and 5'-AGATCCGGTGCAGATTATTTGGATTG-3' (reverse) for pNOS. For the rest of the promoters, the primer combinations were the same used as described in the previous experiments.

Sequencing of the region between the promoters (NOS, 35s, RD29a, AKT1 and SsuAra) and SHN2 was carried out. The targeted regions were amplified using only one oligonucleotide primer

orientation per vector: 5'-CTCCTCTCCAAATGAAATGAACTTC-'3 (forward) for pNOS, 5'-AGTCAAAGATTCAAATAGAGGAC-3' (forward) for p35s, 5'-GAGAAGGATGTGCCGTTTGT-3' (forward) for PRD29a, 5'-GCCGATAAGGGTCTCAACAC-3' (forward) for pSsuAra, 5'-AACTCTTCCTCTTCGGTCTA-3' (forward) for pAKT1 and 5'-GATGTGTGAGCTGTCGTTGT-3' (reverse) for SHN2. The amplification conditions were as follows: 25 cycles at 94°C for 20 s, 50°C for 15 s and 60°C for 1 min. PCR products were sent for sequencing to Greenomics (facility of the Wageningen University and Research Centre, The Netherlands).

3. RESULTS

3.1. Engineering of transgenic potato plants

As described in previous sections, overexpression of Hardy and Shine1 *Arabidopsis* transcription factors trigger drought tolerance not only in the plant itself, but also in other crops, such as rice for HRD and also tomato for SHN1. In order to verify if potato can also express drought tolerance by overexpressing those genes, a transformation was done.

In order to have a significant difference in drought tolerance, between a control and transformed potato, a desiccation susceptible cultivar was used as the plant model. Previous studies of Anitha Kumari (2008 and 2009) demonstrated that Desiree cultivar showed one of the lowest weights of dry biomass after drought stress induction and high relative reduction of the traits upon drought stress (data not published).

The constructs used in this experiment consisted of two drought tolerance transcription factors, Hardy and Shine 1, each one driven by three different promoters. In order to have a constitutive expression on both transcription factors, theCaMV35S promoter was used, and for a stress inducible expression, the RD29A promoter was utilized. An ectopic expression of Hardy in *Arabidopsis* exhibits a phenotype of enhancement in strength and branches on roots. Therefore, in order to drive a spatial expression, the root inducible promoter AKT1 was used. On the other side, Shine 1 led to a significant enhanced epicuticular wax in green tissue, therefore, the light inducible promoter SsuAra was used.

The binary vectors pBINplus-DEST containing the gene cassettes of CaMV35S::HRD, RD29A::HRD, AKT1::HRD, CaMV35S::SHN1, RD29A::SHN1 and SsuAra::SHN1 were successfully introduced into *Agrobacterium tumefaciens*, as confirmed by enzyme digestion and PCR analysis (appendix 1). For the promoters pCaMV35S, pRD29A, pAKT1, pSsuAra and TF genes HRD and SHN1 a PCR fragment of 530 bp, 305 bp, 418 bp 460 bp, 180 bp and 370 bp was amplified respectively.

Afterwards, the constructs were introduced into Desiree plants by *Agrobacterium*-Mediated Plant Transformation. Kanamycin selection medium was used as selection for transformants. Only the transgenic explants containing the antibiotic resistance to kanamycin were able to grow in the

medium containing the antibiotic. The controls of non-inoculated explants without selection medium and inoculated explants without selection medium showed a faster growth than the inoculated explants in kanamycin medium. On the other side, the non-inoculated explants with selection medium (control) did not present any growth. The controls of HRD and SHN1 showed the same pattern of growth as expected. The inoculated explants with the controls are illustrated in figure 4.

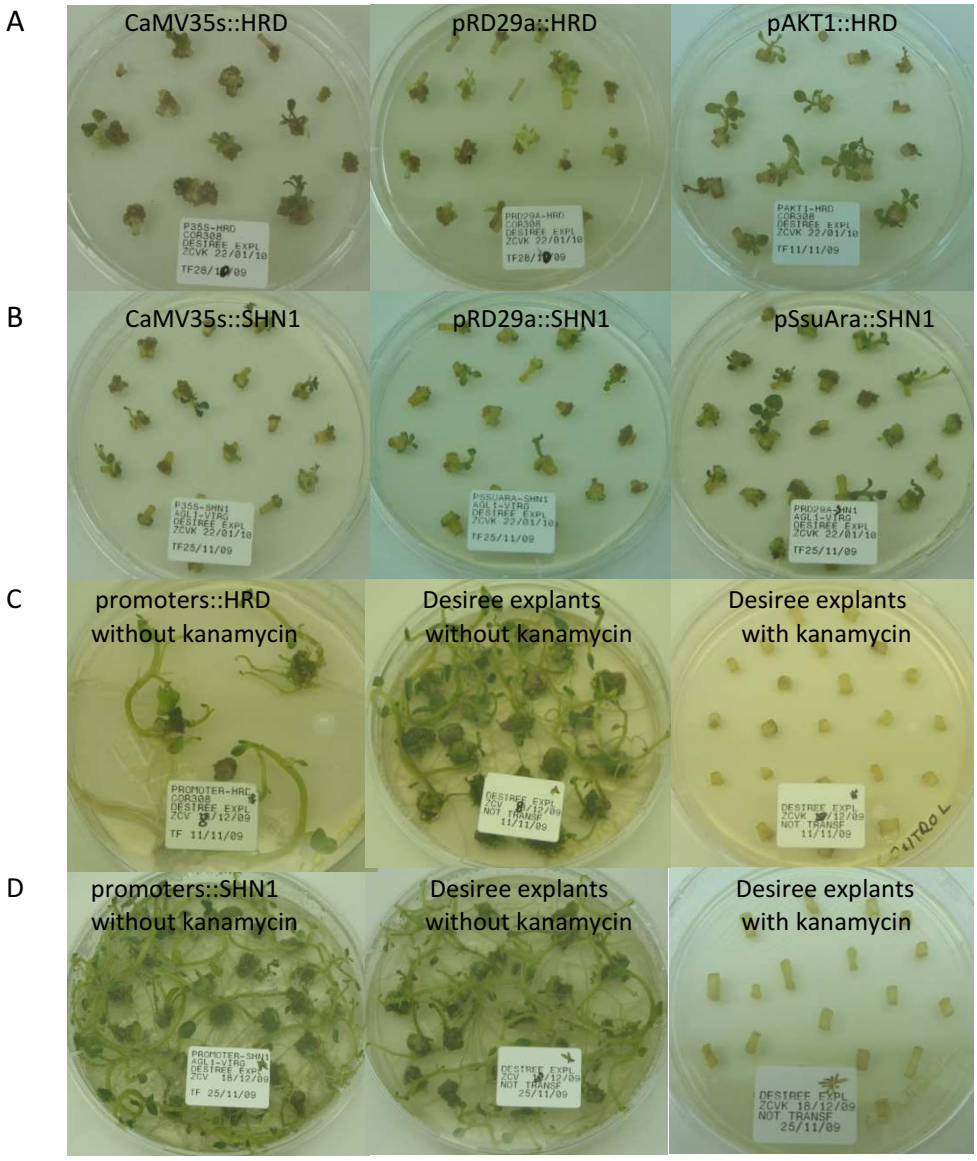


Figure 4. Putative transformed explants in selection medium containing kanamycin and the controls. From left to right: (A) explants containing the CaMV35S-HRD, RD29A-HRD and AKT1-HRD cassettes; (B) explants containing the CaMV35S-SHN1, RD29A-SHN1 and SsuAra-SHN1 cassette; (C and D) HRD and SHN1 controls of inoculated explants without selection medium, non-inoculated explants without selection medium and non-inoculated explants with selection medium.

From a total of 1,833 and 2,446 inoculated explants with the HRD and SHN1 construct respectively, a rate of 96% for HRD and 99% for SHN1 explants showed callus and shoot developed under kanamycin selection medium.

The putative transgenic stem segments, within the first four weeks showed callus formation and later shoot regeneration. The growth of shoots between the HRD and SHN1 constructs were significative dissimilar, as well as within the HRD explants. After 85 days of inoculation, only 3 putative transgenic explants containing the CaMV35S::HRD construct showed shoots of 2 cm or more of length, as well as the one containing the RD29A::HRD cassette. For stem segments inoculated with *Agrobacterium* containing the AKT1::HRD construct, a total of 22 shoots from 2 cm length were regenerated. A higher number of shoots showed the putative transgenic explants carrying the SHN1 cassettes. A number of 60, 64 and 63 shoots from 2 cm of length were regenerated 85 days after inoculation on explants containing the CaMV35S::SHN1, RD29A::SHN1 and SsuAra::SHN1 constructs respectively.

In order to avoid false positives from the antibiotic selection, putative transformed plantlets were tested for the presence of the gene cassettes by PCR analysis. Wild-type Desiree plantlets were also analysed in order to check for presence of possible *Arabidopsis* HRD and SHN1 gene homology in potato genome. The pBINplus-DEST vectors containing the cassettes were used as a positive control. The expected amplification fragments were the same size as the previous PCR performance. An example of the PCR analysis is illustrated in figure 5.

The number of plantlets analysed by PCR were as follows: 3 plants containing the CaMV35s::HRD construct, 3 plants containing the RD29a::HRD construct, 22 plants containing the AKT1::HRD construct, 10 plants were containing the CaMV35s::SHN1 construct, 14 plants containing the RD29a::SHN1 construct and 13 plants containing the SsuAra::SHN1 construct. All the plants were found to be positive for the promoters and transcription factors genes, as indicated by a PCR amplification showing the corresponding band sizes of 530 bp for CaMV35S, 305 bp for RD29A, 412 bp for AKT1, 460 bp for SsuAra, 180 bp for HRD and 370 bp for SHN1 genes. All the bands showed the expected results as depicted in figure 5. Wild-type potato genome amplified with Hardy primers did not show any band. However, Shine 1 primers, amplified a region of the genome of around 700 bp, being 330 bp longer than the target one (370 bp size of SHN1 primers amplification). The transformation results from PCR were reconfirming, by performing an enzyme digestion analysis (appendix 2).

In order to check if the transformants could exhibit different phenotypes due to the HRD and SHN1 genes in future experiments under greenhouse conditions, , transgenic lines were regenerated

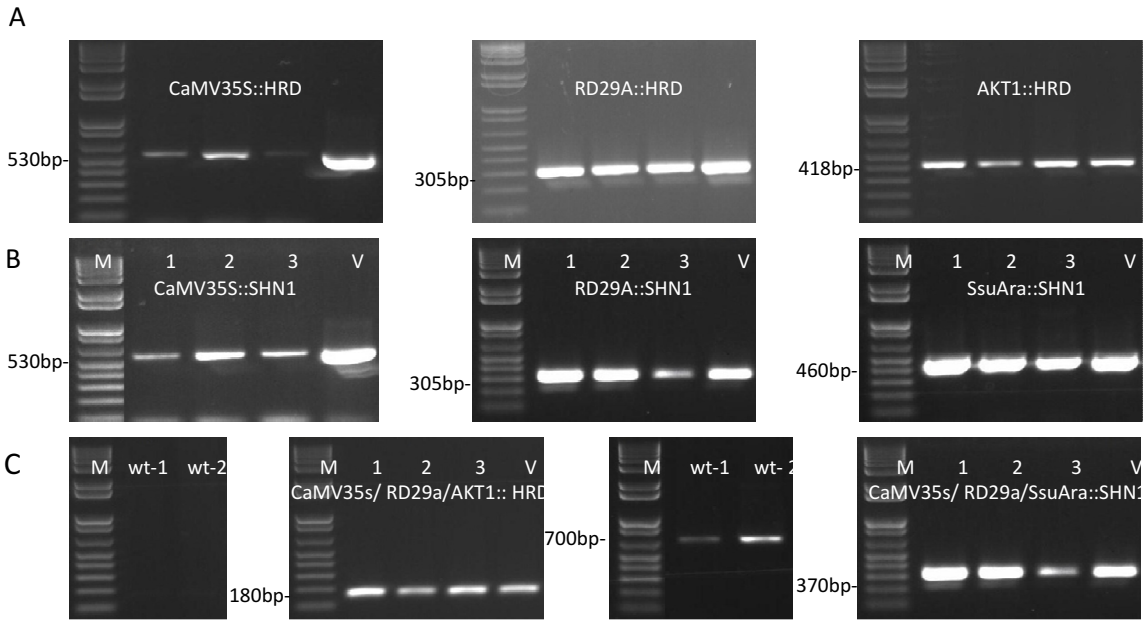


Figure 5. PCR analysis of DNA isolated from putative transgenic potato plantlets and wild-type, using primers for promoters and transcription factors. M= molecular weight DNA 1kb ladder; V= vector containing the constructs pBINplus-DEST/ CaMV35s::HRD, RD29a::HRD, AKT1::HRD, CaMV35s::SHN1, RD29a::SHN1 and SsuAra::SHN1 (positive control). (A) from left to right: Lanes 1-3= putative transgenic potato plants containing the cassette CaMV35s-HRD and using primers for pCaMV35S; Lanes 1-3= putative transgenic potato plants containing the RD29a::HRD cassette and using primers for pRD29a; Lanes 1-3= putative transgenic potato plants containing the AKT1::HRD cassette and using primers for pAKT1; (B) from left to right: Lanes 1-3= putative transgenic potato plants containing the cassette CaMV35s-SHN1 and using primers for pCaMV35S; Lanes 1-3= putative transgenic potato plants containing the RD29a-SHN1 cassette and using primers for pRD29a; Lanes 1-3= putative transgenic potato plants containing the SsuAra-SHN1 cassette and using primers for pSsuAra; (C) wt-1 and 2 lanes=wild-type potato plants using primers for HRD; Lanes 1-3= putative transgenic potato plants containing the cassette CaMV35s/ RD29a/AKT1:: HRD and using primers for HRD; wt-1 and 2 lanes=wild-type potato plants using primers for SHN 1; Lanes 1-3= putative transgenic potato plants containing the cassette CaMV35s/ RD29a/SsuAra:: SHN 1 and using primers for SHN 1.

3.2. Constitutive, spatial and temporal expression of HRD and SHN1 in transgenic plants

Transgenic plants tissue containing the constitutive induced promoter (p35s::HRD/SHN1), root inducible promoter (pAKT1::HRD), light inducible promoter (pSsuAra::SHN1) and drought stress inducible promoter (pRD29a::HRD/SHN1) were used for RNA isolation.

In order to induce the expression of the stress inducible desiccation response promoter pRD29a, an experiment to simulate low water potential (drought) stress using Polyethylene glycol 8000 (PEG) in

vitro was performed. After 7 days of treatment all the plantlets showed a decrease of growth, folded leaves and an increment of root length compared to the non PEG treated ones. Transgenic plantlets containing the light inducible promoter ribulose-1,5-bisphosphate carboxylase small subunit from Arabidopsis (pSsuAra), under the standard 8 hours of darkness of photoperiod during all the stages of culturing, did not show any difference in appearance, as the other plantlets from the experiment. The spatial promoter AKT1 driving the HRD gene in transgenic plantlets did not exhibit any phenotypic growth variation in the root, compared to the other plantlets. Transformants containing the constitutive promoter CaMV35s showed a lower growth compared to the ones containing the other promoters under the same growth conditions.

To determine the expression of the genes Hardy and Shine1 on the transgenic lines, a RT-PCR was performed with the cDNA of the RNA extracted from leaves and roots of transgenic lines. The results from the RT-PCR analysis showed expression of the *Arabidopsis* Hardy and Shine1 genes in all the transgenic lines, as indicated by amplification bands of 180 bp for HRD and 370bp for SHN1 in the samples and vector controls. All the plantlets, whose RNA was extracted from leaves containing the constructs CaMV35s-SHN1, RD29a-SHN1 (with and without PEG treatment) and SsuAra-SHN1, showed expression of the Shine1 gene. Expression of Hardy gene was shown in all the leaf tissues from plantlets containing the constructs CaMV35s-HRD and RD29a-HRD (with and without PEG treatment). Plantlets containing the cassette AKT1-HRD, expressed the Hardy gene in both, leaf and root tissue. No expression of the Hardy or Shine 1 transcription factors was present in the non transgenic Desiree plantlets.

In addition, EF1α primers detected successfully the presence of Elongation factor-1-alpha housekeeping genes in all the cDNA samples, as well as no amplification was registered in the samples without addition of Reverse Transcriptase for the cDNA synthesis. These controls can confirm a reliable PCR results. The results are depicted in figure 6.

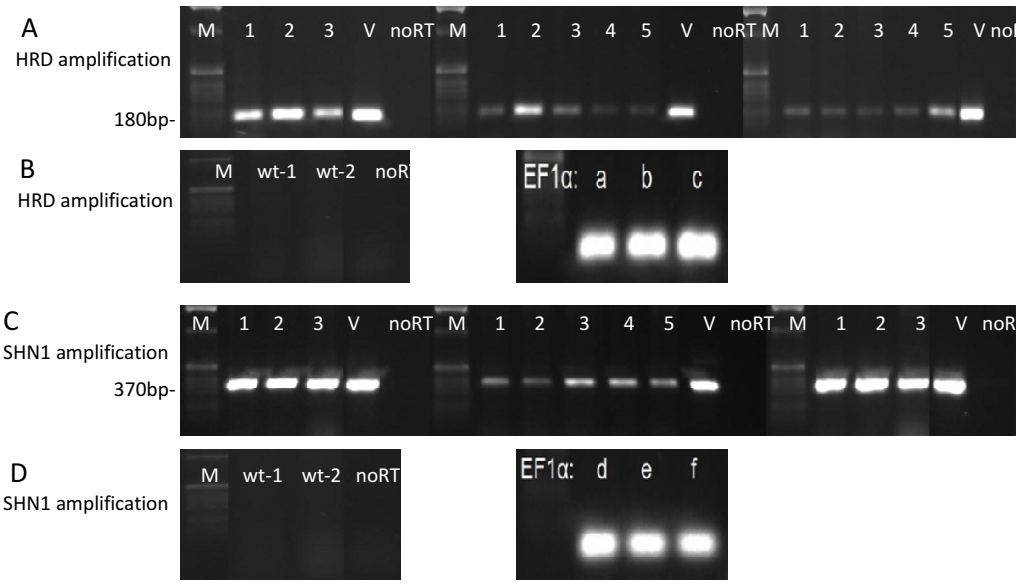


Figure 6. RT-PCR analysis of HRD and SHN1 gene expression in transgenic potato plantlets, using Hardy and Shine1 transcription factors primers. M= molecular weight DNA 1kb ladder; V= vector containing the constructs pBINplus-DEST/ CaMV35s::HRD, RD29A::HRD, AKT1::HRD, CaMV35s::SHN1, RD29a::SHN1 and SsuAra::SHN1 (positive control); noRT= sample without addition of Reverse Transcriptase to the cDNA synthesis (negative control); EF1α= Elongation factor-1-alpha, housekeeping gene (positive control); (A) from left to right, HRD primers used: Lanes 1-3= transgenic potato lines (leaf sample) containing the cassette CaMV35s::HRD; Lanes 1-2= transgenic potato lines (leaf sample) containing the RD29A::HRD cassette without PEG treatment and lanes 3-5= transgenic potato lines (leaf sample) containing the RD29A::HRD cassette and with PEG treatment; Lanes 1-3= transgenic potato lines (leaf sample) containing the AKT1::HRD cassette and lanes 4-5= root extract from transgenic potato lines containing the AKT1::HRD cassette. (B) from left to right, HRD primers used: wt-1 and wt-2 lanes= leaf extract of Desiree lines; Lanes a-c= EF1α housekeeping gene amplified in one line per construct of transgenic lines. (C) from left to right, SHN1 primers used: Lanes 1-3= transgenic potato lines (leaf sample) containing the cassette CaMV35s::SHN1; Lanes 1-2= transgenic potato lines (leaf sample) containing the RD29A::SHN1 cassette without PEG treatment and lanes 3-5= transgenic potato lines (leaf sample) containing the RD29A::SHN1 cassette and with PEG treatment; Lanes 1-3= transgenic potato lines (leaf sample) containing the SsuAra::SHN1 cassette and treated with standard photoperiod conditions. (B) From left to right, SHN1 primers used: wt-1 and wt-2 lanes= leaf extract of Desiree lines; Lanes d-f= EF1α housekeeping genes amplified in one line per construct of transgenic lines.

3.4. Comparative analysis of Arabidopsis Hardy and Shin1 genes with potato genome.

In order to find orthologous genes encoding HRD (at2g36450) and SHN1 (at1g15360) Arabidopsis transcription factors genes in *Solanum tuberosum* genome, a comparison analyses were performed. Based on all the database analysis in homology searches between *Solanum tuberosum* or *Solanum* family genome with Hardy and Shine1 *Arabidopsis* genes, no significant similarity were found. Using a cut-off E value of E<e−5 and a query coverage starting from 90%, no high similar sequences were obtained. However, by lowering the E- value and query coverage, it was possible to see significant

alignments between the two Arabidopsis genes and solanum family, but only in the AP2/ERF domain sequences.

3.5. Construction of plant Expression Vector expressing SHN2 gene

In order to continue with further investigations of Shine *Arabidopsis* transcription factor, five Expression Vectors were performed containing the SHN2 gene driven by different promoters. The Entry Vectors containing the promoters NOS, CaMV35s, RD29a, SsuAra and AKT1, were recombined into the Destination Vectors carrying the SHN2 transcription factor gene and checked by PCR and sequencing analysis.

The colony PCR analysis of the constructs NOS::SHN2, CaMV35s::SHN2, RD29A::SHN2, AKT1::SHN2 and SsuAra::SHN2 present in the pEXPR vector, showed de expected sizes of amplified fragments confirming the presence of the promoters and TFs and their correct orientation in the vector. The combination of SHN2 forward primer with pBINplus-DEST reverse primer showed a fragment size 445 bp and NOS, CaMV35s, RD29a, AKT1 and SsuAra forward primers with SHN2 reverse primers displayed a band size of 670 bp, 909 bp, 1077 bp, 1734 bp and 1675 bp respectively. For the negative control, no fragment was present on the gel as expected (appendix 3).

For the sequencing analysis, the primer used for amplification of the SHN2 target region was not 5'-GATGTGTGAGCTGTCGTTGT-3' (reverse), but 5'-CTCTGCGTTAAGATCTCCCA-'3 (forward), therefore both sequences (promoters and SHN 2) followed the same orientation. This problem did not affect the results, because it was still possible to show only 1 contig and see the overlap between the two sequences in CaMV35s::SHN2, RD29a::SHN2, AKT1::SHN2 and SsuAra::SHN2 constructs. Only NOS::SHN2 showed 2 contigs, however the results were also successful, because the NOS and SHN2 sequences were aligned with their corresponding templates and both showed identity. The results of the PCR and sequencing analysis are illustrated in appendix 4.

4. DISCUSSION

Hardy and Shine Arabidopsis genes encode a member of the Ethylene Transcription factor ERF/AP2 family. Under normal condition in the plant both genes are expressed in the inflorescence tissue. However, ectopic overexpression of these genes in Arabidopsis confers drought and salinity stress tolerance. Therefore, these genes may probably be involved in the protection against desiccation in the flower tissue (Karaba et al. 2007). In this study, the feasibility of obtaining transgenic potato plants containing the Arabidopsis drought tolerance genes was evaluated. The plants were transformed with HRD and SHN1 transcription factors under the control of a constitutive (CaMV35s), temporal (RD29a) and spatial (AKT1/SsuAra) promoter.

HRD and SHN1 transgenes were transformed into potato plants with successful results. The *Agrobacterium* Mediated Plant Transformation technique showed an efficiency of 100% by a DNA analysis. This high efficiency obtained may be supported by the use of the Desiree variety as a starting material, which it is highly used in *in vitro* culture as a model cultivar for transformation experiments (Beaujean et al. 1998). Additionally, transformation was highly effective with a 96 and 99% of survival rate, for explants containing HRD and SHN1 constructs respectively. However, the transgenic explants showed a slow initial growth of shoots, which can be attributed to the time needed to recuperate from the medium containing kanamycin or the transformation procedure. Studies on potato transformation techniques revealed that by using internodal explants the transformation efficiency increases, but it has an initial slow response to shoot regeneration (Visser et al. 1989).

Shoot regeneration on potato explants after the transformation containing the HRD gene showed a lower number of shoots compared to explants carrying the SHN1 gene. *Agrobacterium tumefaciens* strain COR308 and AGL1 virG were used for the transformation of HRD and SHN1 respectively. AGL1 contains the virulence gene virG, which is a transcription factor responsible for the induction of the virulence gene expression (Slater et al. 2008). Previous studies of virG revealed that this gene not only increased the transformation efficiency, but also the recovery of antibiotic-resistant plants (Chabaud et al. 2003). Therefore, the higher level of virulence of the AGL1 strain that was used in explants containing SHN1, could have directly contributed to a larger number transformants when compared to HRD transgenic lines.

RNA analysis of the expression of HRD and SHN1 in the transgenic potato lines revealed that they were expressed in all the leaf samples of both genes under the control of all different promoters.

Plantlets containing the HRD and SHN1 TF driven by the CaMV35s promoter showed expression in leaf tissue. According to Mitsuhara et al. (1996), the CaMV35s gene promoter confers a constitutive and strong expression in all tissue from transgenic plants, therefore HRD and SHN1 genes were expressed in the leaf samples of transformants as expected.

The transformed AKT1::HRD plantlets showed expression of the TF not only in the root, but also in leaf tissue. Experiments on the activity of AKT1 gene promoter by a Gus reporter analysis in transgenic Arabidopsis revealed an organ specific distribution of gene expression.The activity of the AKT1 promoter was observed preferentially in the root system, mainly in differentiated cell types specialized in K^+ uptake. However, the promoter displayed also activity in leaf hydathodes and also in differentiated leaf primordial (Lagarde et al. 2996). Therefore, due to the tissue-specific expression of AKT1 promoter, it was expected to obtain expression of HRD in both, root and leaf tissue.

Transgenic lines containing the SHN1 gene driven by the ribulose-1,5-bisphosphate carboxylase small subunit promoter from Arabidopsis (pSsuAra) showed gene expression in leaf samples. According to Kebbers et al. (1988) SsuAra promoter is induced by light, expressing the gene in green photosynthetic tissues. Therefore, expression of SHN1 transformants in leaf tissue under light was present as expected.

In order to mimic the effect of drought condition, a non–permeant osmotic agent Poly Ethylene Glycol (PEG) has been used in previous studies (Pospisilova 1977; Zhu et al. 2006; Gopal and Iwama 2007).Following the same technique, in the present experiment the potato transgenic plants were treated with PEG 8000. The analysis of the desiccation response promoter RD29A on potato transgenic leaf samples, showed expression of the HRD and SHN1 genes with and without PEG drought treatment induction. Studies on the expression of the RD29A promoter in transgenic *Arabidopsis* and tobacco plants showed induction at significant levels under drought, cold, saline conditions and ABA-independent pathway and were expressed in almost all organs and tissue of vegetative plants only during stress (Yamaguchi-Shinozaki and Shinozaki 1993; Wu et al. 2008). These results suggest that both, HRD and SHN1 should have been expressed only under drought

stress conditions. However, previous experiments from Sakuma et al. (2006) revealed expression of the DREB2A driven by the RD29A inducible promoter under both, stress and non-stress condition. Furthermore, according to Narusaka et al. (2003), the RD29A promoter shows both ABA-independent and ABA-responsive expression, meaning that it does not require ABA for the expression of dehydration and low temperature, but does respond to ABA, because its promoter region contain both cis-acting elements, drought responsive element (DRE) and ABA responsive elements (ABRE) (Yamaguchi-Shinozaki and Shinozaki 2005). Consequently, one possible reason for the induction under non drought stress condition may be caused by a cross-talk between genes that are regulated by different signalling pathways in response to the biotic and wound stress condition inducing the expression of the promoter RD29A.

The conventional *in vitro* environment is characterized for having a high relative humidity, no supplement CO_2 concentration, increase in ethylene, low photosynthetic photon flux density and high concentration of sucrose and nutrients containing in the medium. All these conditions often trigger a low photosynthetic efficiency and level of transpiration, malfunctioning of stomata, decrease in epicuticular wax and a reduced water, nutrient and CO_2 uptake. All of these features may have caused stress on the transgenic lines (Hazarika 2006). For instance, reductions in CO_2 form reactive oxygen species (ROS) that may induce an oxidative stress. Also, the incision of the explants or the antibiotic present in the medium could have triggered some signalling that activated the wound and/or biotic stress pathway respectively (Chandra et al. 2010; Desjardins 1995; Huang 2005).

Another possibility of the expression of the genes might be due to the higher level of ethylene gas accumulated in the vessels. Ethylene is a gaseous plant growth regulator that is involved in regulation of a wide range of physiological processes during plant growth and development and can act as a signal of damage or biotic stress, leading to the appropriate coordinated cellular response (Gonzalez et al. 1997). It is well known that plant tissues grown *in vitro* produce ethylene as a result of wounding during explanting or on subculture. The closed vessels, used in *in-vitro* culture to avoid contamination, can affect the gaseous exchange between the culture vessel and the outside atmosphere and thus increase the levels of ethylene present in the culture (Kumar et al. 1998). As well as HRD and SHN1, there are other members of the AP2/ethylene responsive element binding protein (EREBP) factor family regulate genes that are not only involved in response to drought, salinity, pathogens and cold, but also ethylene (Aharoni et al. 2004). An example of ethylene and drought response is Tiny gene. Sun et al (2007) found that Tiny, a transcription factor that belongs to

the DREB subfamily, may play a role in the cross-talk between DRE and ERE elements by binding to both cis-acting element and activating the downstream genes. DRE (Dehydration-responsive element-binding proteins) and ERE (ethylene responsive element) play a crucial role in the regulation of abiotic and biotic stress responses respectively. Therefore, it might be possible that the ethylene present in the vessel due to it being closed, could have initiated the signalling pathway, activating in the transformed potatoes an homolog of the *Arabidopsis* AP2/ EREBP family member, which could have bound not only to an ERE cis-acting element from a promoter involved in ethylene stress responses, but also to the DRE of the RD29A promoter, and consequently expressing the HRD and SHN1 TF.

It is important to remark that the level of expression of HRD and SHN1 in the transgenic lines was not analysed in this investigation, therefore it is not possible to identify if the RD29A promoter can induce a higher response under drought than in an unstressed condition.

On the other side, there was no expression of HRD and SHN1 in the wild type Desiree plantlets. These results suggest that although both, transgenic and wild type plantlets were under the same tissue culture condition, they did not expressed the TF genes Therefore, it seems that the Desiree wild type probably does not have the ortholog genes of HRD or SHN1 conferring drought stress resistance. Previous results of HRD and SHN1, supported by a DNA analysis, did not show amplification of Hardy gene in the wild type Desiree genome as expected. Nevertheless, SHN1 DNA analysis of the wild type amplified a region from the genome, although longer than the desired one. Therefore, due to the RNA analysis results, it appears that the SHN1 gene and the amplified region in the potato genome may have only similarity in some sequences, but does not have any SHN1 TF homolog present in the genome. Consequently, these observations suggest that neither HRD nor SHN1 transcription factor genes displayed expression on wild type Desiree.

It is important to remark, that the wild type Desiree control was cultured under the same conditions as the transgenic plantlets. However, the WTs were not transformed with an empty vector. This control may have given a more accurate result of the transformation process.

HRD transformants driven by CaMV35s, RD29a and AKT1 promoters showed differences in the amount of growth within the three constructs. Transgenic Lines containing the CaMV35s-HRD cassettes displayed the lowest number and a slow growth of regenerated plantlets. Previous studies on CaMV35s revealed that this promoter showed a high level of continual expression of transgenes

in monocot and dicot plants and this expression is often detrimental to the host cell because it creates an energy drain, thus diminishing essential host cell functions (Glick and Pasternak 2003; Kumar and Timko 2004). Therefore, this promoter could have induced a constant and strong expression of the HRD gene, affecting the normal plant development. RD29A-HRD transformants, however in a lower level, also showed a similar pattern of growth as CaMV35s-HRD transformants. This result might be attributed to the characteristic expression of RD29A promoter in almost all tissue when it is induced under stress (Yamaguchi-Shinozaki and Shinozaki 1993), revealing a possible constant HRD expression in these transgenic lines.

Despite the lower growth regeneration of transformants containing CaMV35s-HRD and RD29A-HRD constructs, all the transgenic lines appeared to have similar phenotype to those of the untransformed control (wild type Desiree). Suggesting that the random integration of the foreign gene did not disturb the normal expression of genes in the host genome by disrupting some genes encoding proteins involved in the plant growth regulation (Zabel 2008).

It is important to state that the previous results obtained were only a matter of observation found during the transformation process. However, in order to gain more clarity within the results a significative analysis has to be done.

The present knowledge of the abiotic stress metabolic pathway and their transcriptional regulatory mechanisms studied in Arabidopsis has been essential for biotechnological applications in potato plants. The engineering of the potato containing the Hardy and Shine1 transcription factors by *Agrobacterium* Mediated Plant Transformation technique was considered to be successful. From the total of plantlets analysed all of them confirmed to contain the constructs inserted in the genome. These results were endorsed by an expression analysis revealing the presence of HRD and SHN1 TF in all the transgenic plantlets tested. Even though, potato wild type showed DNA amplification of HRD, it did not reveal any expression in the transgenic plants. These results were confirmed by a database comparison analysis, where there were no satisfactory results. The RD29A stress inducible promoter expressed the TF genes in the transgenic plantlets, not only under drought induced treatment but also in the non induced ones, suggesting that it might be a crosstalk in the stress signalling pathway. The CaMV35s::HRD transformants showed a lower number and slow growth comparing with the other transgenic lines containing the inducible promoters, suggesting that the constitutive CaMV35s promoter may induce a lethal effect in transgenic plants when express the gene. On the other side, the survival of transgenic lines under inducible promoters for HRD, and both

inducible and constitutive promoters for SHN1, suggest that these transgenic lines may have potential for further investigations on the drought stress tolerance in potato.

Due to the early stage of development of the transgenic lines it is difficult to make any significant statement, therefore further analysis needs to be done. Thus, an abiotic stress trial could be conducted in a greenhouse. It would be interesting to evaluate the impact of not only drought, but also heat and salinity in the transformed lines by inducing those conditions in a controlled environmental trial. The level of expression of the HRD and SHN1 should be also analysed in the transformed plants. For the stress analysis different traits can be targeted, such as stomatal conductance, water use efficiency (WUE), Net Co_2 assimilation, chlorophyll fluorescence, root development and Na^+ homeostasis. An additional analysis of biotic stress may be interesting to evaluate in order to elucidate the cross-talk in the stress signalling pathway.

5. REFERENCES

Aharoni, A. Dixit, S. Jetter, R. Thoenes, E. Van Arkel, G.and Pereira, A. (2004) 'The SHINE Clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in arabidopsis', *The plant cell,* 16: 2463-2480.

An, G. Costa, M. and Ha, S. (1990) 'Nopaline Synthase Promoter is Wound Inducible and Auxin Inducible', *The plant cell*, 2: 225-233.

Basset, M. Conejero, G. Lepetit, M. Fourcroy, P. and Sentenac, H. (1995) 'Organization and expression of the gene coding for the potassium transport system AKT1 of Arabidopsis thaliana', Plant molecular biology, 29: 947-958.

Beaujean, A. Sangwan, R. Lecardonnel, A. Sangwan-Norreel, B. (1998) 'Agrobacterium-mediated transformation of three economically important potato cultivars using sliced intermodal explants: an efficient protocol of transformation', Journal of experimental botany, 49(326): 1589-1595.

Boyer, J. (1982) 'Plant productivity and environment (crop genetic improvement)', *Science,* 218 (4571): 443-448.

Brun, P. Poindexter, P. Osborne, E. Jiang, C. and Riechmann, J. (2004) 'WIN1, a transcriptional activator of epidermal wax accumulation in Arabidopsis', *PNAS*, 101 (13): 4706-4711.

Chabaud, M. De Carvlho-Niebel, F. Baker, D. (2003) 'Efficient transformation of Medicago truncatula cv. Jemalong using the hypervirulent Agrobacterium tumefaciens strain AGL1', Plant cell rep, 22: 46-51.

Chakraborty, S. Chakraborty, C. and Datta, A. (2000) 'Increased nutritive value of transgenic potato by expressing a nonallergenic seed albumin gene from Amaranthus hypochondriacus', *PNAS*, 97 (7): 73724–3729.

Chandra, S. Bandopadhyay, R. Kumar, V. Chandra, S. (2010) 'Acclimatization of tissue cultured plantlets: from laboratory to land', *Electronic Journal of Biotechnol Lett,* [Online] DOI: 10.1007/s10529-010-0290-0, (Accessed: 09 May 2010).

Condon, A. Richards, R. Rebetzke, G. Farquhar, G. (2004) 'Breeding for high water-use efficiency', Journal of experimental botany, 55(407): 2447-2460.

De Almeida, E. Gossele, V. Muller, C. Dockx, J. Reynaerts, A. Botterman, J. Krebbers, E. and Timko, M. (1989) 'Transgenic expression of two markers genes under control of an Arabidopsis rbcS promoter: Sequences encoding the Rubisco transit peptide increase expression levels'. *Mol Gen Genet. ,* 218: 78-86.

Dennison, K. Robertson, W. Lewis, B. Hirsch, R. Sussman, M. Spalding, E. (2001) 'Functions of AKT1 and AKT2 potassium channels determined by studies of single and double mutants of Arabidopsis', Plant physiology, 127: 1012-1019

Desjardins, Y. (1995) 'Photosynthesis in vitro – on the factors regulating Co2 assimilation in micropropagation systems', Environmental control in plants tissue culture, 393: 45-61.

Farooq, M. Wahid, A. Kobayashi, N. Fujita, D. Basra, S. 2009a 'Plant drought stress: effects, mechanisms and management', *Agron. Sustain*., 29: 185-212.

Farooq, M. Wahid, A. Lee, D-J. Ito, O. Siddique, K. 2009b 'Advances in Drought Resistance of Rice', *Critical Reviews in Plant Science*, 28: 199-217.

Fox, T. Guerinot, M. (1998) 'Molecular biology of cation transport in plants', Annu. Rev. Plant physiol. Plant mol. Biol, 49: 669-96.

Ghosh, S. Asanuma, K. Kusutani, A. and Toyota, M. (2001) 'Effect of salt stress on some chemical components and yield of potato Soil', Science and Plant Nutrition, 47 (3): 467-475.

Glick, B. Pasternak, J. (2003) *Molecular Biotechnology, Principles and applications of recombinant DNA*, 3rd ed., ASM press: Washington D C.

Golldack, D. Quigley, F. Michalowski, C. Kamasani, U. Bohnert, H. (2003) 'Salinity stress-tolerant and –sensitive rice (oryza sativa L.) regulate AKT1-type potassium channel transcripts differently', plant molecular biology, 51: 71-81.

Gonzalez, A. Arigita, L. Majada, J. and Sanchez, J. (1997) 'Ethylene involvement in *in vitro* organogenesis and plant growth of *Populus tremula* L.', *Plant Growth Regulation,* 22: 1–6.

Gopal, J. Iwama, K. (2007) 'In vitro screening of potato against water-stress mediated through sorbitol and polyethylene glycol', Plant cell rep, 26: 693-700.

Hamdy, A. Ragab, R. Scarascia-Mungnozza, E. (2003) 'Coping with water scarcity: Water saving and increasing water productivity', Irrigation and drainage, 52: 3-20.

Hazarika, B. (2006) 'Morpho-physiological disorders in in vitro culture of plants', Scientia Horticulturae, 108: 105-120.

Huang, C. Chen, C. (2005) 'Physical properties of culture vessels for plant tissue culture', Biosystems engineering, 91(4): 501-511.

Kannangara, R. Branigan, C. Liu,Y. Penfield, T. Rao, V. Mouille, G. Hofte, H. Pauly, M. Riechmann, J. and Broun, P. (2007) 'The transcription factor WIN1/SHN1 regulates cutin biosynthesis in *Arabidopsis thaliana', American Society of Plant Biologists,* 19: 1278-1294.

Karaba, A (2007) 'Improvement of Water Use Efficiency in Rice and Tomato using Arabidopsis Wax Biosynthetic Genes and Transcription Factors' *PhD thesis Wageningen University*, 156.

Karaba, A. Dixit, S. Greco, R. Aharoni, A. Trijatmiko, K. Marsch-Martinez, N. Krishnan, A. Nataraja, K. Udayakumar, M. Pereira, A. (2007) 'Improvement of water use efficiency in rice by expression of HARDY, an Arabidopsis drought and salt tolerance gene', *PNAS*, 104 (39):15270-15275.

Krebbers, E. seurink, J. Herdies, L. Cashmore, A. and Timko, M. (1988) 'Four genes in two diverged subfamilies encode the ribulose-1,5 bisphosphate carboxylase small subunit polypeptides of *Arabidopsisthaliana', Plant Molecular Biology,* 11: 145-759.

Kumar, P. Lakshmanan, P. and Thorpe, T. (1998) 'Regulation of morphogenesis in plant tissue culture by ethylene', *Society for In Vitro Biology*, 34:94-103.

Kumar, S. and Timko, M. (2004) 'Enhanced tissue-specific expression of the herbicide resistance bar gene in transgenic cotton (gossypium hirsutum L cv. Coker 310FR) using the Arabidopsis rbcS acts1A promoter', *Plant biotechnology*, 21 (4): 251-259.

Lagarde, D. Basset, M. Lepetit, M. Conejero, G. Gaymard, F. Astruc, S. and Grignon, C. (1996) 'Tissue-specific expression of Arabidopsis AKT1 gene is consistent with a role in K+ nutrition', *The plant Journal*, 9(2): 195-203.

Madhava Rao, K. Raghavendra, K. and Reddy, J. (2006) 'Physiology and molecular biology of stress tolerance in plants', *Sprinter*, 1-14.

Mitra, J. (2001) 'Genetics and genetic improvement of drought resistance in crop plants', *Current science*, 80 (6):758-763.

Mitra, A. Gynheung, A. (1989) 'Three distinct regulatory elements comprise the upstream promoter region of the nopaline synthase gene', Mol Gen Genet, 215: 294-299.

Mitsuhara, I. Ugaki, M. Hirochika, H. Oshima, M. Murakami, T. Gotoh, Y. Katayose, Y. Nakamura, S. Honkura, R. Nishimiya, H. Otsuki, Y. and Ohashi, Y. (1996) 'Efficient promoter cassetes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants', *Plant Cell Physiol.* , 37: 49-59.

Mullins, E. Milbourne, D. Petti, C. Doyle-Prestwich, B. and Meade, C. (2006) 'Potato in the age of biotechnology', *Trends in Plant Science*, 11 (5): 254-260.

Munns, R. (2002) 'Comparative physiology of salt and water stress', Plant, Cell and Environment, 25: 239-250.

Narusaka, Y. Nakashima, K. Shinwari, K. Sakuma, Y. Furihata, T. Abe, H. Narusaka, M. Shinozaki, K.Yamaguchi-shinozaki, K. (2003) 'Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses', *The plant journal,* 34: 137-148.

Pospisilova, J. (1977) 'Water relations in primary leaves of bean plants treated with polyethylene glycol solution', *Biologia Plantarum*, 19 (4): 316-319.

Price, A. Cairns, J. Horton, P. Jones, H. and Griffiths, H. (2002) 'Linking drought-resistance mechanisms to drought avoidance in upland rice using a QTL approach: progress and new opportunities to integrate stomatal and medophyll responses', *Journal of Experimental Botany*, 53 (371): 989-1004.

Rodriguez, M. Canales, E. and Borras-Hidalgo, O. (2005) 'Molecular aspects of abiotic stress in plants', *Biotecnologia Aplicada,* 22:1-10.

Sakuma, Y. Liu, Q. Dubouzet, J. Abe, H. Shinozaki, K. Yamaguchi-Shinozaki, K. (2002) 'DNA-Binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, Transcription factors involved in dehydration- and cold –inducible gene expression', *Biochemical and Biophysical research communications*, 290: 998-1009.

Sakuma, Y. Maruyama, K. Osakabe, Y. Qin, F. Seki, M. Shinozaki, K. Yamaguchi-Shinozaki, K. (2006) 'Functional analysis of an Arabidopsis transcription factor, DREB2A, involved in drought-responsive gene expression', *The plant cell*, 18: 1292-1309.

Sanders, P. Winter, J. Barnason, A. Rogers, S. Fraley, R. (1987) 'Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants', *Nucleic acids research*, 15 (4): 1543-1558.

Schachtman, D. (2000) 'Molecular insights into the structure and function of plant K+ transport mechanisms', *biochimica et biophysica acta*, 1465: 127-139.

Schafleitner, R. (2009) 'Growing more potatoes with less water', *Electronic Journal of Tropical plant Biol.*, [Online] Doi 10.1007/s12042-009-9033-6 (Accessed: 3 August 2009).

Shinozaki, K. Yamaguchi-Shinozaki, K. (2000) 'Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signalling pathways', *Current Opinion in Plant Biology*, 3:217-223.

Shinozaki, K. Yamaguchi- Shinozaki, K. (2007) 'Gene network involved in drought stress response and tolerance', *Journal of Experimental Botany*, 58 (2): 221-227.

Skinner, D. (2005*) Plant Abiotic Stress,* State Avenue: Ames.

Slater, A. Scott, N. and Fowler, M. (2008) Plant *Biotechnology the genetic manipulation of plants*, 2nd ed., Oxford University Press: New York.

Sun, S. Yu, J. Zhao, T. Fang, X. Li, Y. Sui, S. (2008) 'TINY, a dehydration-responsive element (DRE)-binding protein-like transcription factor connecting DRE- and ethylene-responsive elements-mediated signalling pathways in Arabidopsis', *Journal of biological chemistry*, 283(10): 6261-6271.

Visser, R. Jacobsen, E. Hesseling-Meinders, A. Schans, M. Witholt, B. Feenstra, W. (1989) 'Transformation of homozygous diploid potato with an *Agrobacterium tumefaciens* binary vector system by adventitious shoot regeneration on leaf and stem segments', *Plant Molecular Biology*, 12: 329-337.

Waterer, D. Benning, N. Wu, G. Luo, X. Liu, X. Gusta, M. McHughen, A. Gusta, L. (2010) 'Evaluation of abiotic stress tolerance of genetically modified potatoes (Solanum tuberosum cv. Desiree)', *Mol Breeding*, 25: 527-540.

Wery, J. SIlim, S. Knights, E. Malhotra, R. Cousin, R. (1994) 'Screening techniques and sources tolerance to extremes of moisture and air temperature In cool season food legumes', *Euphytica*, 73: 73-83.

Wu, Y. Zhou, H. Que, Y. Chen, R. Zhang, M. (2008) 'Cloning and identification of promoter Prd29A and its application in sugarcane drought resistance', *Sugar tech*, 10 (1): 36-41.

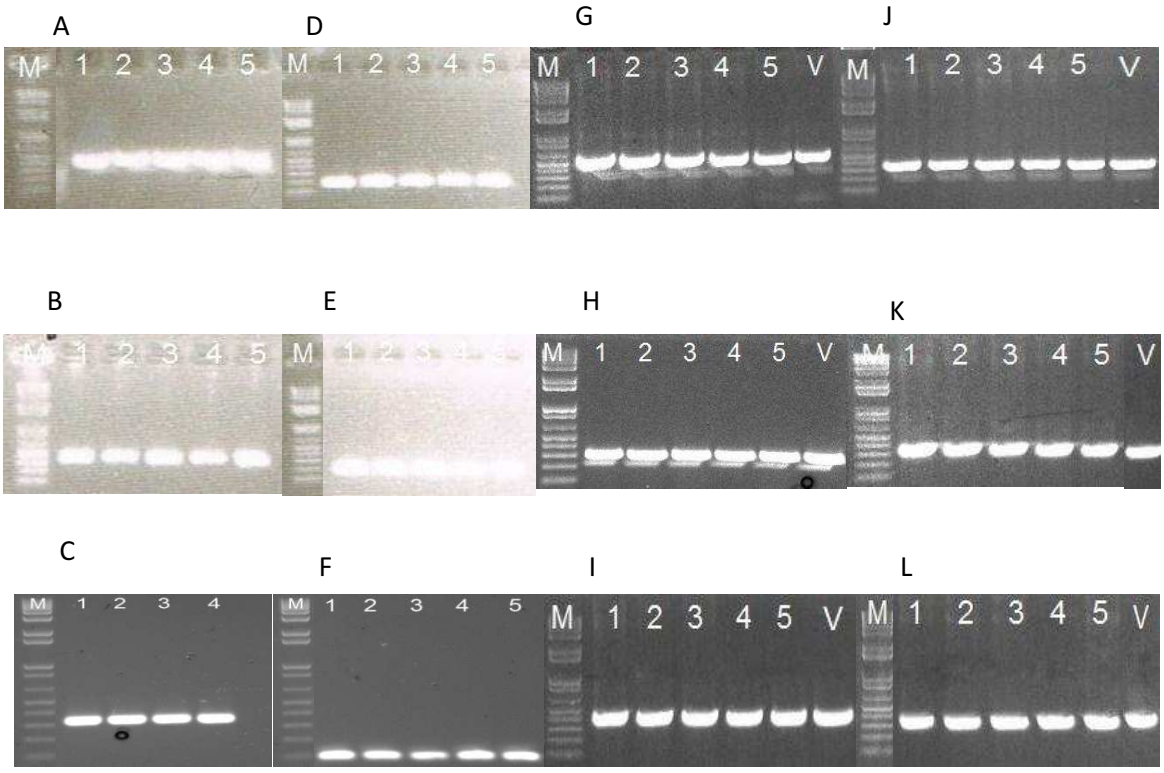
Yamaguchi-Shinozaki, K. Shinozaki, K. (1993) 'Characterization of the expression of a desiccation-responsive rd29 gene of Arabidopsis thaliana and analysis of its promoter in transgenic plants', *Mol gen genet*, 236: 331-340.

Yamaguchi-Shinozaki, K. Shinozaki, K. (2005) 'Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters', *Trends in Plant Science*, 10 (2): 88-94.

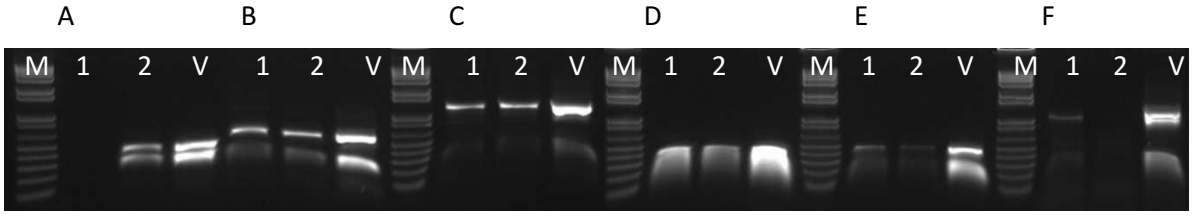
Zabel, P (2008) Gene Technology, 2007/2008 ed., Wageningen University: Wageningen.

Zhu, J. Kang, H. Tan, H. Xu, M. (2006) 'Effects of drought stresses induced by polyethylene glycol on germination of pinus sylvestris var. Mongolica seeds from natural and plantation forests on sandy land', *The Japanese forest society and springer*, 11: 319-328.

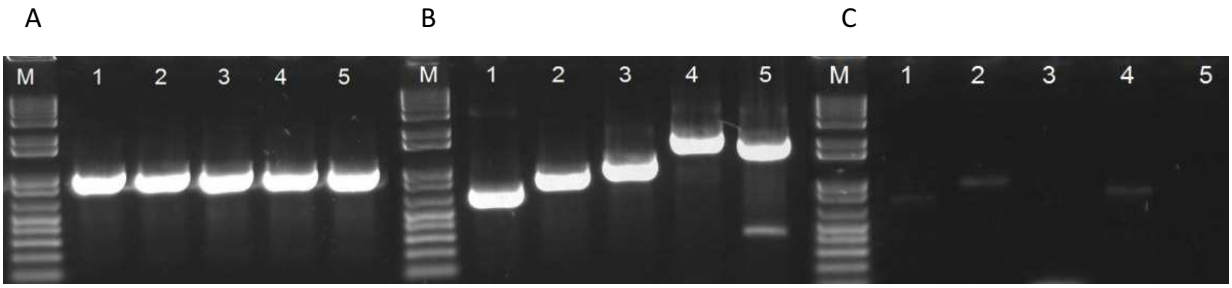
APPENDIX



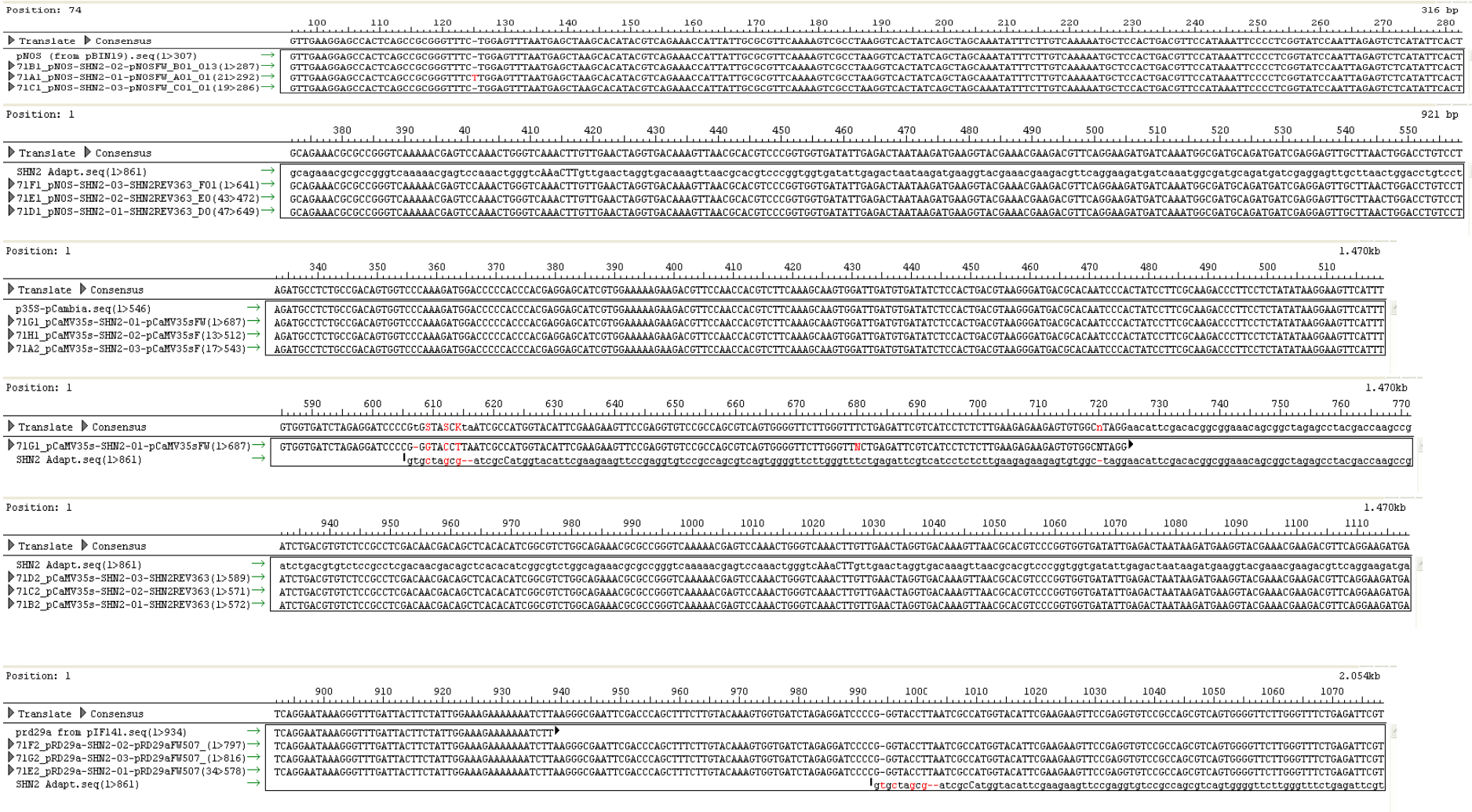
Appendix 1. PCR analysis of the pBINplus-DEST vector containing the target gene cassettes transformed into *Agrobacterium*. Primer pairs of the promoters CaMV35S, RD29A, AKT1, SsuAra *and TF* HRD, SHN1 were used. M= molecular weight DNA 1 kb ladder; V= pBINplus-DEST vector containing the respective gene cassettes (positive control); CaMV35s primer pair: (A) lanes 1-5= vector containing the CaMV35s::HRD cassette; (G) lanes 1-5= vector containing the CaMV35s::SHN1 cassette. RD29A primer pair: (B) lanes 1-5= vector containing the RD29a::HRD cassette; (H) lanes 1-5= vector containing the RD29A::SHN1cassette. AKT1 primer pair: (C) lanes 1-4= vector containing the AKT1::HRD cassette. HRD primer pair: (D) lanes 1-5= vector containing the CaMV35s::HRD cassette; (E) lanes 1-5= vector containing the RD29a::HRD cassette; (F) lines 1-5= vector containing the AKT1::HRD cassette. SsuAra primer pair: (I) lanes 1-5= vector containing the SsuAra::SHN1 cassette. SHN1 primer pair(J) lanes 1-5= vector containing the CaMV35s::SHN1 cassette; (K) lanes 1-5= vector containing the RD29a::SHN1 cassette; (L) lines 1-5= vector containing the SsuAra::SHN1 cassette.



Appendix 2. Restriction enzyme analysis from a PCR amplification of DNA containing the constructs. Primer pairs of promoters (FW primer) with the TFs (REV primer) were used. V= pBINplus-DEST vector containing the respective gene cassettes and amplified with their corresponding primer pairs (positive control). M= molecular weight DNA 1 kb ladder. (A) lines 1-2= enzyme digestion of CaMV35s::HRD amplification; (B) lines 1-2= enzyme digestion of RD29a::HRD amplification; (C) lines 1-2= enzyme digestion of AKT1::HRD amplification; (D) lines 1-2= enzyme digestion of CaMV35s::SHN1 amplification; (E) lines 1-2= enzyme digestion of RD29A::SHN1 amplification; (F) lines 1-2= enzyme digestion of SsuAra::SHN1 amplification.



Appendix 3. PCR analysis of the constructs (1)NOS::SHN2, (2)CaMV35s::SHN2, (3)RD29a::SHN2, (4)AKT1::SHN2 and (5)SsuAra::SHN2 inserted in the pEXPR vectors. M= molecular weight DNA 1 kb ladder. (A) lines1-5= amplification using SHN2 forward with pEXPR reverse primers; (B) lines 1-5= amplification using NOS forward with SHN2 reverse primers, CaMV35s forward with SHN2 reverse primers, RD29a forward with SHN2 reverse primers, AKT1 forward with SHN2 reverse primers and SsuAra forward with SHN2 reverse primers; (C) lines 1-5= control using NOS reverse with SHN2 reverse primers, CaMV35s reverse with SHN2 reverse primers, RD29a reverse with SHN2 reverse primers, AKT1 reverse with SHN2 reverse primers and SsuAra reverse with SHN2 reverse primers.


Position: 74
316 bp
Translate
Consensus
pNOS (from pBIN19).seq(1>307)
71B1_pNOS-SHN2-02-pNOSFW_B01_013(1>287)
71A1_pNOS-SHN2-01-pNOSFW_A01_01(21>292)
71C1_pNOS-SHN2-03-pNOSFW_C01_01(19>286)
Position: 1
921 bp
SHN2 Adapt.seq(1>861)
71F1_pNOS-SHN2-03-SHN2REV363_F01(1>641)
71E1_pNOS-SHN2-02-SHN2REV363_E0(43>472)
71D1_pNOS-SHN2-01-SHN2REV363_D0(47>649)
Position: 1
1.470kb
p35S-pCambia.seq(1>546)
71G1_pCaMV35s-SHN2-01-pCaMV35sFW(1>687)
71H1_pCaMV35s-SHN2-02-pCaMV35sF(13>512)
71A2_pCaMV35s-SHN2-03-pCaMV35sF(17>543)
Position: 1
1.470kb
71G1_pCaMV35s-SHN2-01-pCaMV35sFW(1>687)
SHN2 Adapt.seq(1>861)
Position: 1
1.470kb
SHN2 Adapt.seq(1>861)
71D2_pCaMV35s-SHN2-03-SHN2REV363(1>589)
71C2_pCaMV35s-SHN2-02-SHN2REV363(1>571)
71B2_pCaMV35s-SHN2-01-SHN2REV363(1>572)
Position: 1
2.054kb
prd29a from pIF141.seq(1>934)
71F2_pRD29a-SHN2-02-pRD29aFW507_(1>797)
71G2_pRD29a-SHN2-03-pRD29aFW507_(1>816)
71E2_pRD29a-SHN2-01-pRD29aFW507(34>578)
SHN2 Adapt.seq(1>861)

Appendix 4. Sequencing analysis of the Shine 2 gene driven by the promoters NOS, CaMV35s, RD29A, SsuAra and AKT1.