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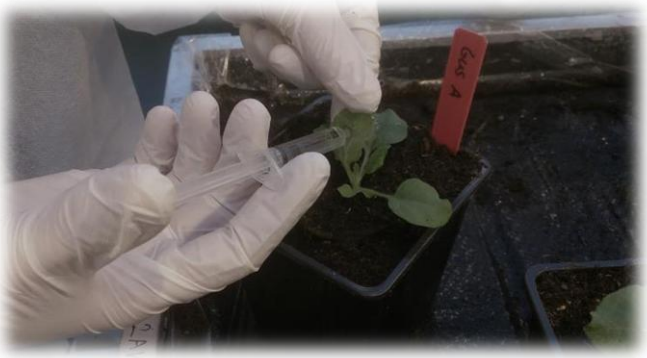

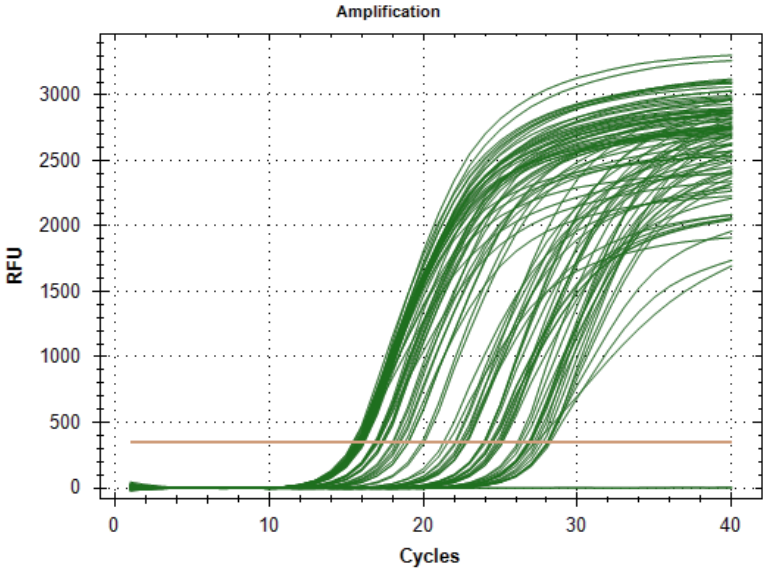

USING MOLECULAR TECHNIQUES TOWARDS DROUGHT TOLERANT POTATO

Providing tools for functional characterization of drought response and tolerance genes in potato

Cover page illustrations (clockwise, starting from the top):
-Agroinfiltration of Nicotiana benthamiana plants to achieve Virus Induced Gene Silencing (VIGS).
-Farmer from Uzbekistan showing potatoes from a drought tolerant potato breeding program (cipotato.org).
-Screenshot showing a fluorescence chart of a qPCR.

Using Molecular Techniques Towards Drought Tolerant Potato

Providing tools for functional characterization of drought response and tolerance genes in potato

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This thesis would never have been realized without the help of other people. Therefore I would first like to thank Gerard van der Linden, who supervised me and enabled me to do research on this topic. I feel that this thesis will be a major milestone in my professional career, since it shaped my vision for the future. Furthermore it taught me a lot about plant breeding in general, it strengthened my molecular biology skills and it demanded a certain assertiveness that I now know is very important. So thank you Gerard, for providing me with this fantastic opportunity.

The next person I would like to thank has helped me to really leave something of worth behind after finishing my thesis. Marian Oortwijn, thank you for helping me with facing my challenges in the lab, where not everything went as smoothly as I would have hoped. You motivated me to go on when there were disappointing results, you rewarded me with butter cake when after many tries, the desired result finally was there. Furthermore, you adopted the laborious stable constructs when time did not allow me to finish them myself, and managed to finish the major part. So a warm thank you is certainly not superfluous.

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Last but not least, thank you Dennis van Muijen. It was you that recommended me to do this thesis, which would not have been there, had you not paved the path. Your excellent results did provide so much opportunities that multiple master theses would be needed to digest these.

Abstract

Abiotic stress is the main reason for yield loss all over the world. The main abiotic stress that reduces yield, drought, is also estimated to affect the world more and more, both through climate change and population growth. Furthermore, the increased welfare of people demand more resources. It is of vital importance that breeding companies, researchers and farmers anticipate timely on this development. *Solanum tuberosum* is a crop that has potential in feeding the world, since it exhibits an excellent water use efficiency, unmatched by any other staple crop. Unfortunately, the potato is poorly drought tolerant. Breeding for drought tolerance is difficult, due to the polygeny and lack of drought tolerance traits. Unravelling the molecular mechanism behind the drought response might provide information on how to improve the drought tolerance of potato. The *StNF-YC4* gene, encoding a subunit of the trimeric transcription factor NF-Y, was found to be down regulated under severe drought conditions. No effect was seen when the gene was transiently silenced in *Nicotiana benthamiana,* but it is speculated that the gene acts in streamlining the drought response. The *StTAS14* gene was found to be upregulated under both severe and mild drought stresses. Under severe stress the expression level of the gene increased when the drought persisted. Since the gene has been associated with protecting the membrane against the detrimental effects of dehydration, it is logical that it is formed in increasing amounts as the drought continues to stress the plant. For *StER24* there was great upregulation shortly following the initiation of severe drought, whereas the expression returned to control levels when the drought endured. It putatively acts in regulating the heat shock response, which is also active in protecting the cell under drought. For all three genes constructs have been developed aimed at the formation of transgenic plants, so that characterization of the genes in potato can be performed.

Key words: NF-Y; Drought stress; Drought tolerance; dehydrin; heat shock response; heat shock transcription factor; heat shock protein; Natural Antisense Transcript; Virus Induced Gene Silencing

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1. Introduction to the subject

1.1 Drought threatens farmers worldwide

Abiotic stresses such as drought, salinity, heat, radiation, hail and floods are the major causes for yield loss all over the world. For instance for potato, the average percentage of yield loss through abiotic stress is 55% (Boyer, 1986). This yield loss not only leads to less turnover than could potentially be achieved, it also creates a certain unpredictability and unreliability. For small farmers a few years of low yield through abiotic stress can be disastrous. One of the major abiotic stresses, drought, is expected to impact more and more people dependent on irrigated lands. The amount of people dependent on lands with severe water limitation is expected to increase from 2 billion in 1985 to more than 3 billion in 2025 both through reduced water availability and population growth (Vorosmarty, 2000; Dai, 2011). Furthermore, the demand for food is ever growing, because of population growth and increased welfare. The population is estimated to stabilize around 2050 at 9 billion people (Godfray et al., 2010). Increased welfare leads to increased consumption and a greater demand for food that has greater impact on the environment such as meat, fish and dairy. To keep feeding the world, measures have to be taken such as changing the diet of people and reducing waste of valuable food products (Godfray et al., 2010). What plant breeders focus on is another measure, closing the yield gap. To be able to produce enough food that meets the demand, the discrepancy between optimal and realized yield has to be minimized. Realizing that there are plenty factors underlying the yield gap, attempts to increase the drought tolerance of crops can lead to significant gains in reduction of the yield gap.

1.1.1 Drought tolerant potato has great potential, but to get there is difficult

Solanum tuberosum is a plant that has great potential in feeding the world. The potato plant is able to produce 9.52 kilograms of yield or 5626 of kcal per m^3 of water, which makes potato the crop with the highest water use efficiency by far (Renault, 2000). Unfortunately, the potato plant is not very drought tolerant. One of the possible explanations for this is that 85% of its root length is concentrated in the upper 30 centimetres of the soil (Opena, 1999). Since the potato is mainly planted in ridges, this top layer of the soil dries out very fast. When there is no rain or proper irrigation, it does not take long before the plant suffers from drought stress. This inhibits the potato to reach its full potential in many cases. Drought tolerance of the potato can be improved with different irrigation techniques (Liu et al., 2006), pre-stressing the plants to achieve an offspring that is more drought tolerant (Watkinson et al., 2006) and breeding for a deeper root system (Lahlou and Ledent, 2005). Breeding for drought tolerance traits has not been put to use extensively, since these traits often are not obvious. Conventionally breeders and farmers focussed on characteristics increasing the maximum yield, by selecting for big tubers, heavy and abundant seed, and so forth. A trait that contributes to drought tolerance can be unfavourable under well watered conditions, which might result in breeders overlooking such traits when selecting under well-watered conditions. Selection in areas which suffer from drought stress has not led to the development of highly drought tolerant cultivars, due to low heritability and interactions with the environment (Cattivelli et al., 2008). Next to that, the severity and the duration of the drought can elicit different responses in the potato (Jefferies, 1995). Mechanisms which are advantageous for surviving severe drought, might reduce the yield under mild drought. Furthermore wild relatives of the potato have been identified that are drought tolerant, but linkage drag, incompatibility and different photoperiod requirements have hampered the introgression of the drought tolerance traits (Monneveux et al., 2013). Molecular techniques might pose a solution to the problem by identifying the drought tolerance genes or QTLs and subsequent introgression of these traits by Marker Assisted Selection or genetic engineering.

1.1.2 Breeding for drought tolerance

The first step in the endeavour for drought tolerant varieties is identification of the drought tolerant traits that are available. Due to the complexity of the drought response this is not an easy task, as opposed to relatively easy monogenic traits such as resistance to certain pathogens or pests. With the help of molecular markers an attempt can be made to identify drought tolerance QTLs. However, since the traits often are polygenic and dependent on each other, it is difficult to introduce these and significantly increase the drought tolerance of the crop (Cattivelli et al., 2008). Therefore steps have been taken to understand the molecular mechanisms that underlie the drought tolerance traits and the general drought response. Drought triggers a response in the plant that is similar to the response that is observed upon salt or heat stress (figure 1.1). This is not surprising, since the effect of the different stresses is manifested in a comparable way in the plants by osmotic and oxidative stress (Xiong, 2002). Understanding elements of the pathway opens up the possibility to intervene in order to increase the drought tolerance. Targeting genes in this pathway by molecular engineering has been done on several levels already (Cattivelli et al., 2008). Interventions at the transcription factor level (Oh et al., 2005) as well as at the stress responsive gene level (McKersie, 1996), lead to increased tolerance to drought. When intervention occurs higher in the pathway, the possibility of detrimental side effects is higher, such as lower yield and stunted growth. Further characterisation of gene function and stress induced expression instead of overexpression might help to overcome this problem.

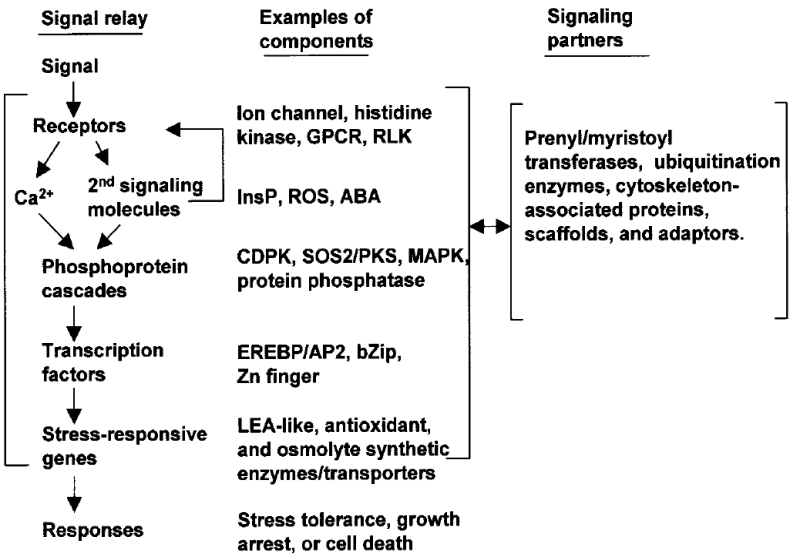


Figure 1.1 The figure depicts the general signalling pathway that is activated when the plant is subjected to different kinds of abiotic stresses such as drought, heat and salinity. The pathway consists of perception of the signal, often through osmotic and oxidative stress. The signal is transduced via Ca^{2+} and other secondary signalling molecules, which amplify the cascade by a feedback mechanism indicated by the backward arrow. Phosphorylating protein signalling cascades are activated, ultimately leading to regulation of either stress responsive genes or transcription factors that further fine-tune the stress response. The signalling partners are essential for the functioning of the signalling pathway, but are not part of it. (Figure adapted from Xiong et alumni, 2002.)

1.2 Genetic dissection of drought tolerance in potato

To get a better understanding of the traits that contribute to drought tolerance in potato and the genes involved, experiments have been performed at Wageningen University and Research Centre as part of the PhD thesis by Anithakumari (Anithakumari, 2011). The CxE diploid mapping population was used to study the expression profiles of drought stressed plants and link these as so called eQTLs to the genome, similar to conventional QTL mapping. The CxE population is the result of a cross between C (USW5337.3; (Hanneman, 1967)) and E (77.2102.37 (CxVH34211) (Jacobsen, 1980)). 94 genotypes of the CxE population were subjected to severe drought in the greenhouse, withholding water for 3 weeks. The experiments by Anithakumari were further analysed by Dennis van Muijen using bioinformatics and statistical tools. This analysis led to the identification of three

genes involved in the drought response, which are the main focus of this thesis: TAS14 (section 1.3), ER24 (section 1.4) and NF-YC4 (section 1.5) (van Muijen, 2013).

1.3 Genotype clustering led to the identification of TAS14

The most appealing trait in terms of drought tolerance is the ability to recover after a period of drought. The CxE population showed high variability for the different drought tolerance traits. Especially for the recovery ability of the plants this was the case (figure 1.2). The expression profiles of the plants were used to predict the phenotype (van Muijen, 2013). Genotypes with a high correlation in the expression profile were clustered. In this way, a specific group of plants was clustered that showed high correlation in their expression profile (figure 1.3). The phenotypic traits of this group that were significantly different compared to those of the ungrouped genotypes were identified.

Figure 1.2 Variation in the recovering ability of the CxE plants. Some were not able to recover at all (left), while other were able to fully recover (right).

It appeared that the grouped genotypes were better capable of recovering after the drought treatment. Noteworthy is that the plants that were able to fully recover were also the plants that showed wilting signs earliest and had a lower relative water content. These are traits that are often associated with drought sensitivity. The average biomass that was generated by the genotypes in the cluster was 15% higher than that of other genotypes after stress (van Muijen, 2013). These observations could classify the grouped genotypes as being more drought tolerant than the ungrouped genotypes. To point out what makes the difference, differential expression analysis between the grouped and ungrouped genotypes was analyzed. The gene that was differentially expressed to the largest extend between the grouped genotypes and the ungrouped genotypes was the abscisic acid and environmental stress-inducible protein TAS14.

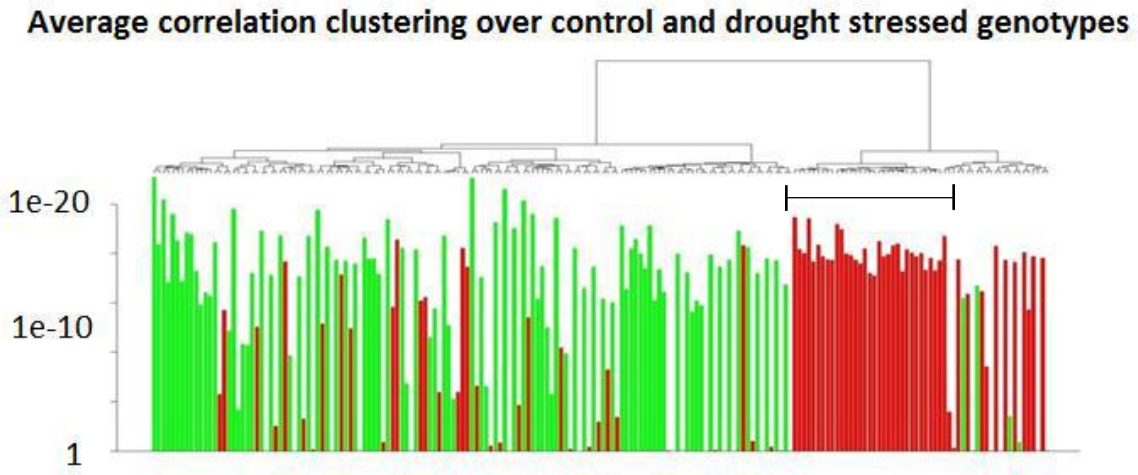


Figure 1.3 Clustering of the genotypes based on their expression profile. Red bars indicate the drought treated samples, whereas the green bars represent the control samples. A specific group of genotypes that shows substantial similarity in their expression profile is indicated with a bar.

1.3.1 TAS14 is a dehydrin

TAS14 is a gene that is known to be a dehydrin, which is a Late Embryogenesis Abundant (LEA) protein. LEA proteins were detected for the first time in the desiccation stage of a maturing seed (Dure, 1981). All known LEA proteins are found to be upregulated upon osmotic stress perception, which is an indication that the involvement in abiotic stress tolerance is widespread. The mechanism through which they act has not been fully elucidated, but several functions have been found. Dehydration assays have been performed in which it was demonstrated that LEA proteins from different organisms were able to protect the function of lactate dehydrogenase and malate dehydrogenase in vitro (Reyes, 2005). Furthermore, deletion of an LEA protein can lead to sensitivity to osmotic stress (Garay-Arroyo, 2000). The TAS14 protein is part of the group 2 LEA proteins, named dehydrins (Dure, 1993). The motif by which the dehydrins are grouped is the K-segment, a 15 residue lysine rich motif. Structural analysis of this motif predicts the formation of amphipathic α-helices, with both hydrophilic and lipophilic characteristics (Dure, 1993). Because of this, the protein is thought to protect membrane structures. A few dehydrins are constitutively expressed, but most are induced upon drought, salt or cold stress (Battaglia et al., 2008). Some dehydrins are transcriptionally regulated by ABA through the ABA Responsive Element (ABRE). The proteins are present throughout the whole plant, although some dehydrins are found to specifically protect buds against cold damage (Muthalif, 1994). Besides a possible role as protectant of the membrane, ion binding properties of some dehydrins suggests a role in radical scavenging under oxidative stress (Battaglia et al., 2008).

1.3.2 TAS14 contributes to stress tolerance in potato and tomato

With a yeast functional screen, *Solanum tuberosum* TAS14 was found to significantly increase the survival of yeast under severe drought and salt stress and moderately to high temperature stress (Kappachery et al., 2013). Furthermore, overexpression of TAS14 in *Solanum lycopersicon* led to improved long term drought and salinity tolerance without yield loss under non-stressed conditions (Munoz-Mayor et al., 2012). The transgenic plants showed earlier and higher accumulation of ABA on the short term, whereas on the long term no difference was observed between transgenic and control plants. Furthermore the leaf turgor of the transgenic plants remained higher, both due to a more reduced osmotic potential and a less reduced water potential. Transgenic plants also showed accumulation of sugars, which are able to protect the stability of membranes and macromolecules upon severe dehydration (Hoekstra, 2001) and act in reducing the osmotic potential. Since the gene was found to contribute to salt stress tolerance as well as drought stress tolerance without yield loss in tomato, it is a very interesting target for research and possibly for stress tolerance breeding.

1.4 Gene regulatory network inference indicated ER24 as the central gene in the heat shock network

The eQTL experiment performed by Anithakumari gave a lot of food for thought (Anithakumari, 2011). The dataset provided by Anithakumari could be used as input for Gene Regulatory Networks analysis. The inferred network was visualised and clusters were annotated (figure 1.4) (van Muijen, 2013). Subsets of the network were selected for in depth analysis. The node with heat shock proteins was selected, since these have been found to protect the cell in several ways in the abiotic stress response (Wang et al., 2004). Network analysis of this subset of genes indicated that the ER24 gene may be a central regulator of the heat shock protein node (figure 1.5).

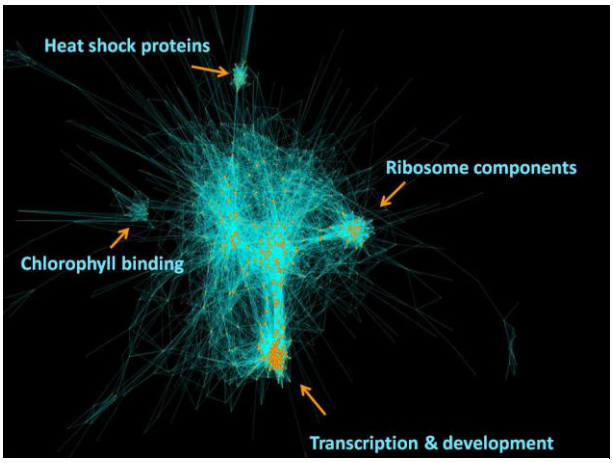


Figure 1.4 Visualisation of the Gene Regulatory Networks analysis. Clusters of co-regulated genes are present as nodes, such as the heat shock proteins node at the top (van Muijen, 2013).

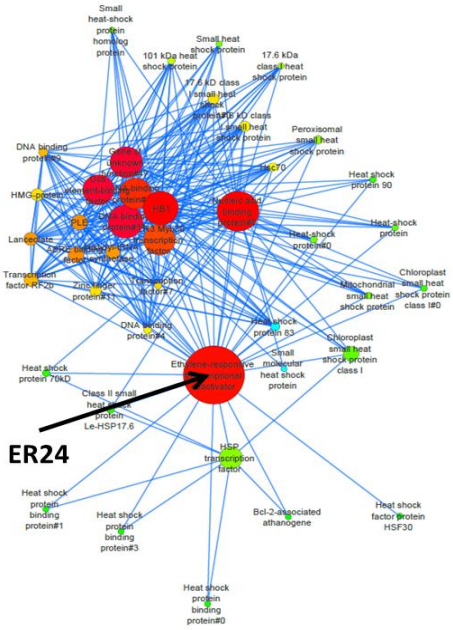


Figure 1.5 Network analysis of the subset of genes in the heat shock protein node. The size indicates the centrality of the gene in the network, the color indicates the amount of interactions. Red means many interactions, green means few (van Muijen, 2013)

1.4.1 Heat shock proteins protect the plant under various abiotic stress conditions

Heat shock proteins are a group of proteins that were found to be upregulated upon heat treatment, therefore the name Heat Shock Proteins was given (Lindquist, 1988). The response of upregulation of these kind of proteins is widespread, since it is found from Archaea to animals. The mode of action of these proteins is mainly protecting the proteins in the cell from aggregation and degradation, as well as refolding damaged proteins or breaking down proteins that are beyond repair (Wang et al., 2004). These actions are mainly necessary when the organism is confronted with abiotic stresses such as heat stress, which causes proteins to denature and aggregate. Heat shock proteins are not only deployed in heat stress. In fact, they are active in response to multiple other abiotic stresses as well. This is not hard to imagine, since proteins need protection and repair under osmotic and oxidative stress as well. Support for the deployment of heat shock proteins under drought is present in the network analysis performed by Dennis van Muijen, as well as in several other studies (Vierling, 1991; Sung, 2001). The heat shock proteins can be classified in five major families. The general function of each of these families are summarized in figure 1.6, which is adapted from the review of (Wang et al., 2004). Abiotic stress conditions can

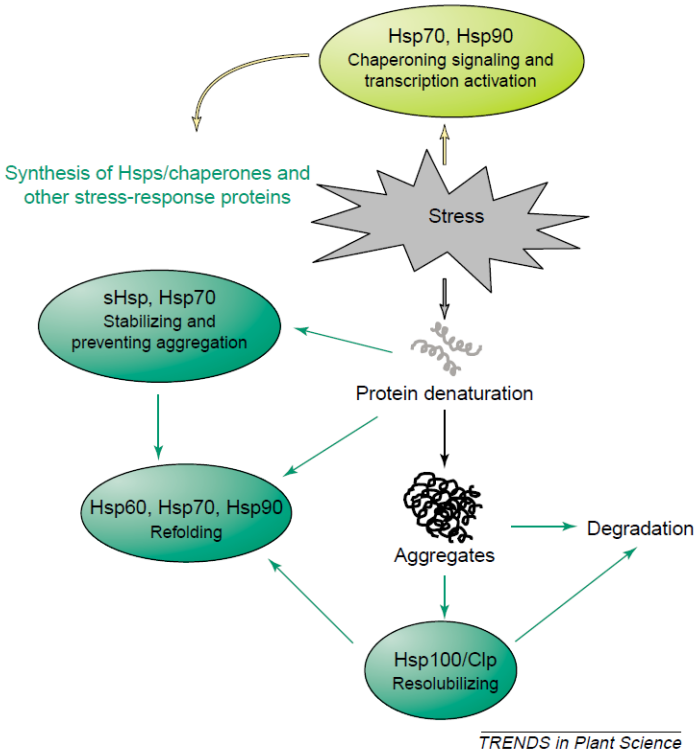


Figure 1.6 (Wang et al., 2004). The different types of heat shock proteins sometimes perform multiple roles or have an overlapping function. The Hsp70 and Hsp90 families have a signalling role, which regulates the synthesis of other Hsps or stress proteins. They are active in the direct protection of proteins as well. Proteins that are denatured as a result of stress conditions are either stabilized and refolded, or aggregated. Aggregated proteins are undesirable since they can form clumps of protein which are harmful for the cell. Aggregates are either resolubilized and refolded or degraded.

change the internal environment of the cell, which can have a big impact on the integrity of the proteins. Heat shock proteins prevent the proteins from damage by either stabilizing and refolding them, or by breaking down aggregates of proteins and subsequent degradation. Proteins which are still repairable are refolded. The exact mechanism by which the heat shock proteins decide which proteins to refold, which to break down and which to stabilize is still unknown (Wang et al., 2004).

1.4.2 Heat shock factors are transcription factors with widespread functions

The heat shock proteins are regulated by heat shock factors (HSFs). The HSF transcription factors regulate expression of the heat shock proteins via the Heat Shock Element (HSE) in their promoter region (Wu, 1995). It is assumed that disruptions in the protein homeostasis drive the Heat Shock system (Baniwal, 2004). More denatured proteins due to environmental stress deplete the pool of free heat shock proteins. The master regulator HSFA1 senses the shift in free heat shock proteins and becomes active (Mishra, 2002). It binds to the HSE of both Heat Shock Protein and Heat Shock Factor genes and activates the heat shock response. As said, there are multiple heat shock factors in plants, exemplified by 17 identified heat shock factors in tomato (Koskull-Doring, 2007). However, the functions of the heat shock factors are far from redundant. Heat shock factors have been found to be not only involved in the heat stress response, but in drought and biotic stresses as well (Bechtold et al., 2013).

1.4.3 The ER24 homolog MBF1c has been put into context of heat stress factors and the heat stress response before

The ER24 gene that was identified by Dennis van Muijen as the central gene in the HSP node of the gene regulatory networks analysis is a homolog of the *Arabidopsis thaliana* MBF1c (Multiprotein Bridging Factor) gene. Extensive research by Suziki et al. (Suzuki et al., 2005; Suzuki et al., 2008; Suzuki et al., 2011) has identified the MBF1c transcriptional (co)activator as a regulator of the heat response in *Arabidopsis thaliana*. The gene was found to be upregulated in cells subjected to heat and drought. The protein was localized to the nucleus. Constitutive expression of the gene conferred increased tolerance to several stresses, such as bacterial infection, heat and osmotic stress (Suzuki et al., 2005). It was found that the MBF1c protein could bind DNA, and thereby regulate several genes including two heat shock factors (HSFB2A and HSFB2B) (Suzuki et al., 2011). However, it was also found that the Heat Shock Transcription factor A1b (HSFA1B) binds a newly identified type of HSE in the promoter of MBF1c (Bechtold et al., 2013). This indicates that the MBF1c protein is intertwined in the heat shock response, which is also active in the drought response.

1.5 NF-YC4 is the gene that putatively underlies the eQTL hotspot on chromosome 5

In the eQTL experiment performed by Anithakumari (Anithakumari, 2011) a large peak of trans-acting eQTLs was found on chromosome 5 (figure 1.7). A likely explanation is that a transcription factor is underlying the eQTL hotspot, which may be a master regulator responsible for reprogramming the plant towards a drought response. Analysis by Dennis van Muijen pointed out that the eQTL hotspot is well separated from another major locus on chromosome 5, the maturity locus (Kloosterman et al., 2013). Nevertheless, all the genes located on chromosome 5 were included in Gene Regulatory Networks analysis to try and point out a candidate gene. The gene that scored the highest in all 4 networks constructed was a homolog of the tomato nuclear transcription factor Y subunit C4 (NF-YC4, (van Muijen, 2013)). Further inspection showed that this gene is located at 40 cM, which is at the position of the

highest peak visible in figure 1.7. Under drought, the NF-YC4 gene was found to be down regulated compared to the expression under control conditions.

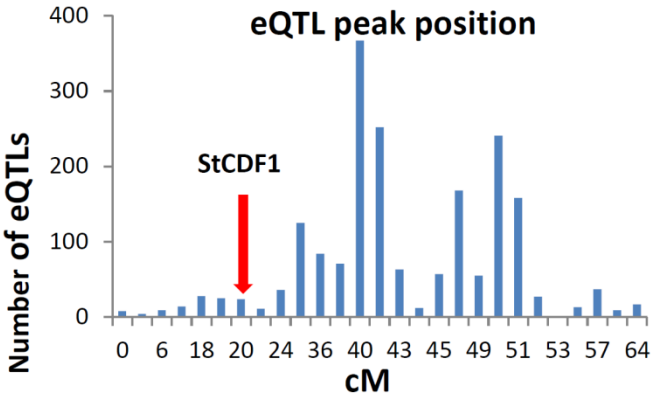


Figure 1.7 (van Muijen, 2013). The number of eQTLs that map to a certain location on chromosome 5 of potato. The StCDF1 locus is indicated with an arrow. The peak of eQTLs seems to be distinct from the StCDF1 locus.

1.5.1 NF-YC4 is a subunit of the heterotrimeric NF-Y transcription factor complex

The NF-YC4 protein is a subunit of a heterotrimeric transcription factor complex (Petroni et al., 2012). The transcription factor binds the CCAAT box in the promoter region of its target genes. Three different subunits constitute the transcription factor: NF-YA, NF-YB and NF-YC. Whereas in animals only 1 or 2 variants of each subunit are found, in plants this can be as much as ten different variants per subunit. This means that theoretically ~1000 different transcription factors can be constituted, which would allow for specific fine-tuning of the regulation. Different subunits have been linked to abiotic stress tolerance. The overexpression of the maize gene *ZmNF-YB2* conferred improved drought resistance. Transgenic plants expressing the *ZmNF-YB2* gene showed less wilting, better recovery and increased turgor pressure when compared to control plants (Nelson et al., 2007). The same goes for the homolog in Arabidopsis *AtNF-YB1*. Similar results have been found for Arabidopsis *NF-YA5* (Li et al., 2008). The NF-Y complex is not always composed of only NF-Y subunits, since CONSTANS (CO) has been found to be co-regulated by NF-Y B and C subunits (Cai et al., 2007). However, this statement has been doubted recently with the finding that complete NF-Y complexes probably recruit the CONSTANS protein to its transcriptional activation site (Cao et al., 2014). Figure 1.8 summarized functional involvement of some of the individual subunits of the NF-Y complex in different biological processes.

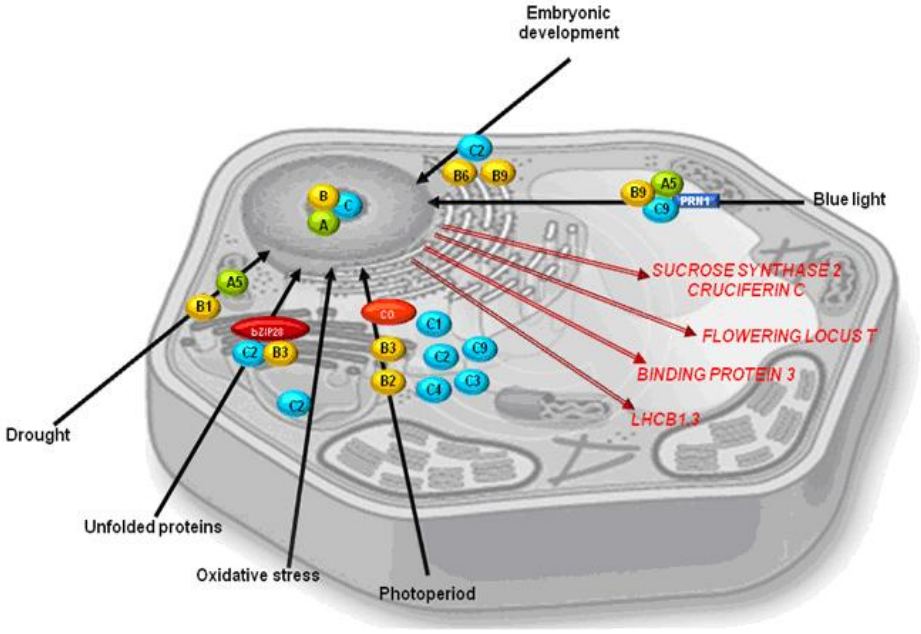


Figure 1.8 (Hackenberg, 2013). The figure presents proposed roles of individual subunits of the NF-Y complex in different situations. Individual subunits have been proposed to be involved in drought, oxidative stress, photoperiod and embryonic development. The subunits mentioned are the subunits that were found in *Arabidopsis thaliana*.

1.5.2 NF-YC4 as a possible master regulator for the drought response

Analysis of the eQTLs that map to the hotspot on chromosome 5 provides information of the possible mechanisms in which NF-YC4 may exert its influence. Among the genes that were found are *HMG, MCMs* and *NF-YA* subunits (van Muijen, 2013). The High Mobility Group protein *HMG* is after histones the largest group of chromatin proteins (Bianchi, 2005). Their involvement in the drought response is not

that surprising, since *HMG* proteins have been associated with the fine tuning of the transcription when there is a sudden change in the environment of the plant. The *MCM* Mini-Chromosome-Maintainance proteins were found among the eQTLs in the hotspot as well. These proteins are responsible for proliferation of the chromosomes prior to cell division (Tye, 1999). Down regulation of the NF-YC4 gene was accompanied by down regulation of these proteins. Furthermore, a link might be present between the photoperiod regulatory mechanism and the NF-YC4 subunit, since it was found that major components of the photoperiod regulatory mechanism were affected by drought as well (van Muijen, 2013). As the CONSTANS protein, which is involved in the photoperiod mechanism of the plant, has been associated with NF-YB and NF-YC subunits this is well possible. Based on these findings, a model was proposed by Dennis van Muijen (figure 1.9). The model describes active inhibition of the drought response by the NF-Y complex under well watered conditions. The model will be further discussed in the general discussion (chapter 6).

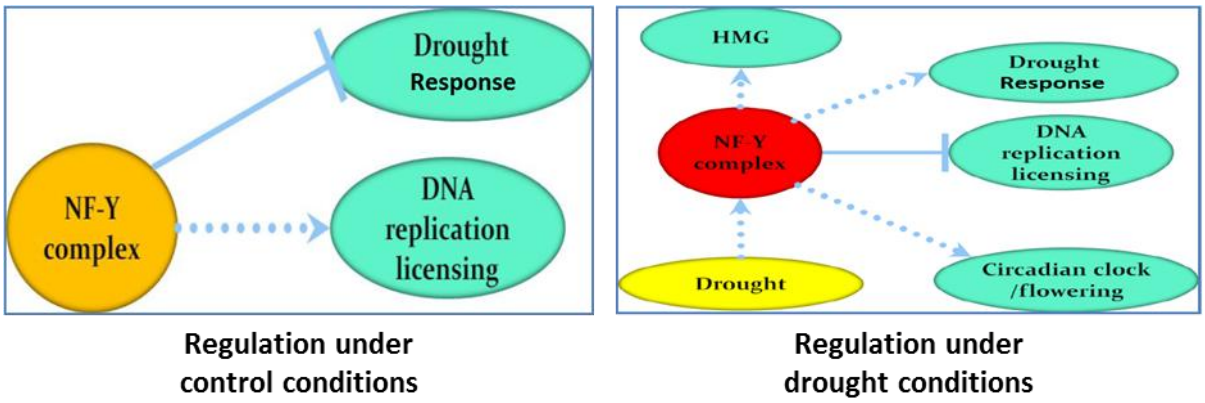


Figure 1.9 (van Muijen, 2013). A model proposed by Dennis van Muijen for the regulation exerted by the NF-Y complex. Under control conditions, the drought response is inhibited (indicated by the blocked line). The conformational change of the NF-Y complex as a result of drought by the absence of NF-YC4 and replacement by other NF-YC subunits results in a shift in the regulation. The DNA replication is inhibited, whereas the *HMG* and photoperiod systems are induced.

1.5.3 An interesting observation in the annotation of NF-YC4 on the potato genome

According to the annotation of the potato genome (Potato Genome Sequencing Consortium; *Solanum tuberosum* group Phureja DM1-3, version 4.03), the 3'UTR of the *StNF-YC4* gene appears to overlap with the 3'UTR of the PLE gene on the opposite strand (figure 1.10). The 3'UTR of the NF-YC4 gene appears to have overlap with the 3'UTR of the PLE gene. In fact, the 3'UTR of PLE may comprise the whole NF-YC4 gene. Simultaneous expression of both genes may therefore lead to complementary mRNA, which can hybridize and form double stranded RNA. Since dsRNA is a target for the plants endogenous RNAi system, silencing might occur (Napoli, 1990). It is imaginable that the overlapping UTRs act as a regulatory mechanism, where the overlapping genes regulate each other. Such mechanisms are known as Natural Antisense Transcripts (Wang, 2005) and have been found in many organisms. Besides regulation through RNAi silencing, NATs are involved in alternative splicing and RNA editing (Wang, 2005). On the other hand, it is well possible that the UTRs were falsely annotated. If the UTRs indeed overlap, this has interesting mechanistic implications.

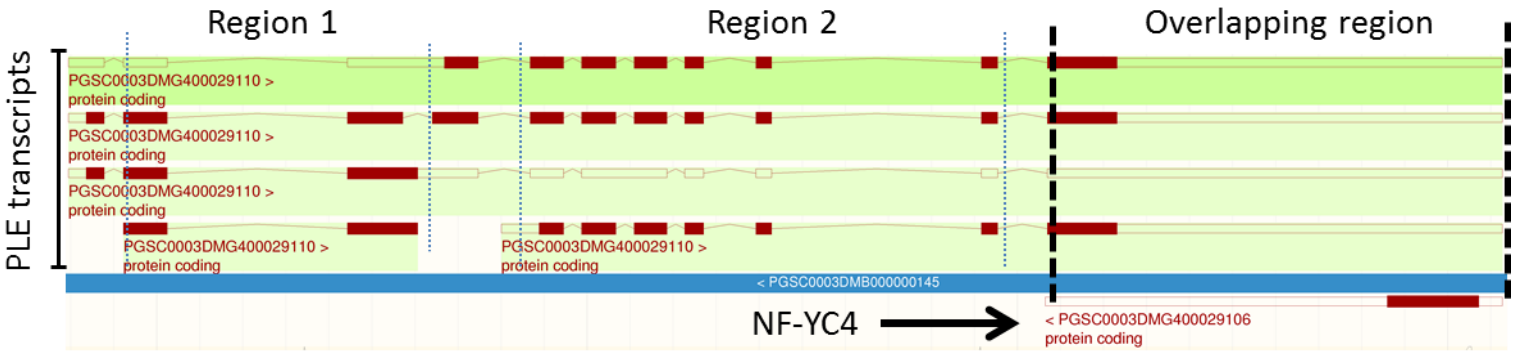


Figure 1.10 The PLE gene is present on the + strand of chromosome 5. In close vicinity of this gene the NF-YC4 gene is encoded, on the – strand. The 3' UTR of PLE is annotated in such a way that the 3'UTR comprises the whole NF-YC4 gene. The overlap is indicated by two bold dashed lines. The PLE gene consists out of 5 splice variants. To cover all five transcripts in a PCR, two probes have to be used. One developed on region 1, the other on region 2. The red squares indicate the exon sequences, whereas the unfilled squares indicate UTRs. The lines linking the exons together indicate the introns.

1.6 Aim and objectives of this thesis

The three genes, *StNF-YC4*, *StTAS14* and *StER24*, that were found by Dennis van Muijen in the dataset of Anithakumari (Anithakumari, 2011; van Muijen, 2013) are the subjects of my research. The aim of this thesis is to further characterize these genes regarding their role in the drought response and their contribution to drought tolerance. To reach this goal, two objectives have been set up. Firstly, expression analysis has been performed to confirm and further dissect the expression profile of the three genes. The analysis was done in plants that were exposed to different types of drought stress. Secondly, tools have been provided for functional analysis of these genes. For this, constructs were designed and made for constitutive, induced and transient expression and silencing of the genes *in planta*. For a short term indication of the function of the genes, the transient silencing constructs have been used for analysis in *Nicotiana benthamiana* plants. The other constructs that were made have been stored to be used for functional characterisation in potato plants in a follow-up study.

2. Investigation of the 3'UTR of PLE and NF-YC4

Characterisation of one of the three genes of interest, *StNF-YC4*, is possibly hampered by the phenomenon described in section 1.5.3. In short, the *StNF-YC4* gene is positioned on the antisense strand of chromosome 5 of potato (figure 1.10). Another gene is present in close proximity of the *StNF-YC4* gene, the microtubular protein encoding gene *StPLE*. The 3' UTR of the *StPLE* gene is annotated to encompass the complete *StNF-YC4* gene. This could be a biological relevant phenomenon, which is known as a Natural Antisense Transcript (Wang, 2005). NAT's can regulate the expression of genes through the formation of dsRNA after reciprocal expression of the genes, resulting in silencing through RNAi. Furthermore, alternative splicing can be regulated by NATs. Besides interesting implications of the phenomenon, it could present complications for the other experiments that are performed in this thesis. Expression of *StNF-YC4* cannot be distinguished from expression of *StPLE*, and targeting the *StNF-YC4* with RNAi would also target *StPLE*. To elucidate whether to take this into account, PCR experiments have been designed to amplify the transcripts of this region.

2.1 Material and Methods

2.1.1 Development of primers

Primers have been developed, both based on the NF-YC4 gene coding region as well as on the coding region of PLE. The cDNA sequences of the genes were derived from sequence information of *Solanum tuberosum* group Phureja DM1-3, genome browser version 4.03 (Potato Genome Sequencing Consortium). The primers have been developed with web-based primer design software (www.primer3plus.com). Primers have been designed both to indicate the expression of PLE, as well as to elucidate the 3'UTRs of PLE and NF-YC4 (table 2.1). Since there are 5 splice variants of PLE, a specific approach was undertaken to be able to indicate the expression of all of them. Two regions were identified, which together cover all transcripts (figure 2.1). Region I covers the splice variants PGSC0003DMT400074850 and PGSC0003DMT400074851, region II covers the other three splice variants PGSC0003DMT400074852, PGSC0003DMT400074853 and PGSC0003DMT400074854. The codes refer to the annotation by the Potato Genome Sequencing Consortium. Five primer pairs were developed for each region. A PCR on the genomic DNA of the C and E parent was performed to check whether they were able to generate the correct fragment (table 2.1). A forward primer was selected (PLE2 F2) to amplify the 3' UTR of PLE in combination with reverse primers designed on the 3'UTR (table 2.1, figure 2.1).

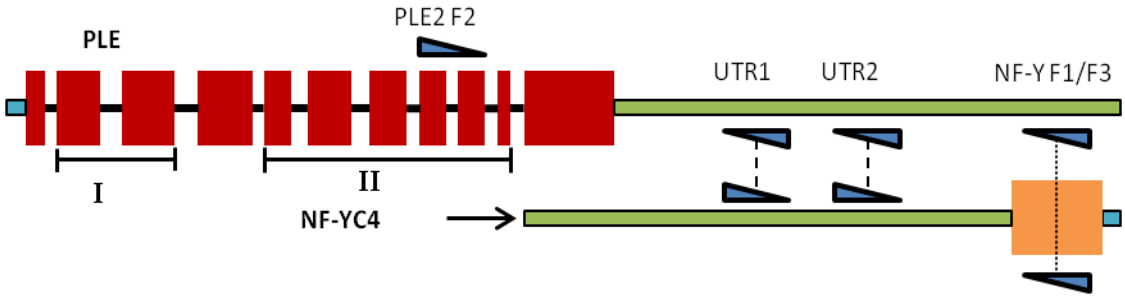


Figure 2.1 Visualisation of the position of the used primers on the cDNA of the PLE and NF-YC4 genes. The PLE gene, the top strand, is divided into two regions, indicated by I and II. These two regions cover all the splice variants of the PLE gene, as displayed in figure 1.9. Primer pairs that have been developed on these two regions to monitor the expression have not been visualised. These can be found in table 2.1. The PLE2F2 primer has been used in the experiments in combination with reverse primers on the putative UTRs, as indicated by the blue triangles. The NF-Y F1 and F3 primer have been used both as a forward for amplification of the 3' UTR of NF-YC4, and as a reverse for amplification of the PLE 3' UTR. The UTR1 and UTR2 primers have been used both sense and antisense, to amplify both the 3' UTR of NF-YC4 and PLE.

2.1.2 PCR

The combination of a forward primer in an exon of the genes and a reverse primer in the 3' UTR generates a relatively large fragment (table 2.1). To amplify these large fragments not only the commonly used DreamTaq DNA Polymerase was used, but also Phusion® High-Fidelity DNA Polymerase (see appendix). Several primer combinations were used, either to try to amplify the gene with its UTR, or to prove presence of the gene (with both primers in the coding region) (table 2.1). The PCRs that were done to check the presence of the gene were performed with normal DreamTaq polymerase, since only a small product is formed. Products were subjected to gel electrophoresis to visualise the products formed and to estimate their sizes. Negative controls were included with only water as template. There was no positive control, since a sample in which PLE was known to be expressed was not present.

Table 2.1 Primer combination that were used to either check the expression of the gene in the cDNA (expression) or to elucidate the UTR (UTR Check). The position of the primers on the genes is visualised in figure 2.1

Target	Forward primer	Reverse primer	Product length on cDNA (bp)	Product length on gDNA (bp)	Purpose
PLE I	Fw1	Rv1	78	916	Expression
	Fw2	Rv2	115	953	Expression
	Fw3	Rv3	92	92	Expression
	Fw4	Rv4	216	216	Expression
	Fw5	Rv5	202	202	Expression
PLE II	Fw1	Rv1	112	195	Expression
	Fw2	Rv2	75	75	Expression
	Fw3	Rv3	86	86	Expression
	Fw4	Rv4	218	300	Expression
	Fw5	Rv5	203	285	Expression
	Fw2	PLE UTR1	694	2140	UTR Check
	Fw2	PLE UTR2	1323	2769	UTR Check
	Fw2	NF-YC4 Fw3	2196	3642	UTR Check
	Fw2	NF-YC4 Fw1	2052	3497	UTR Check
NF-YC4	Fw3	NF-YC4 UTR1	1526	1526	UTR Check
	Fw3	NF-YC4 UTR2	893	893	UTR Check

2.2 Results

2.2.1 Selection of primer pairs

Primer pairs were developed for two regions of the PLE gene (table 2.1). The selected working primer for PLE region I was F3R3, for PLE region II F2R2. These were selected based on the results of a PCR on the genomic DNA of C and E, since these pairs showed clear bands at the expected height (data not shown). However, the amplified fragments of these primer pairs on cDNA were not distinguishable from the primer dimer (data not shown). Therefore, two additional primer pairs were designed for each region to result in larger amplicons (pairs 4 and 5, table 2.1). This makes it possible to clearly distinguish the actual product from primer dimer.

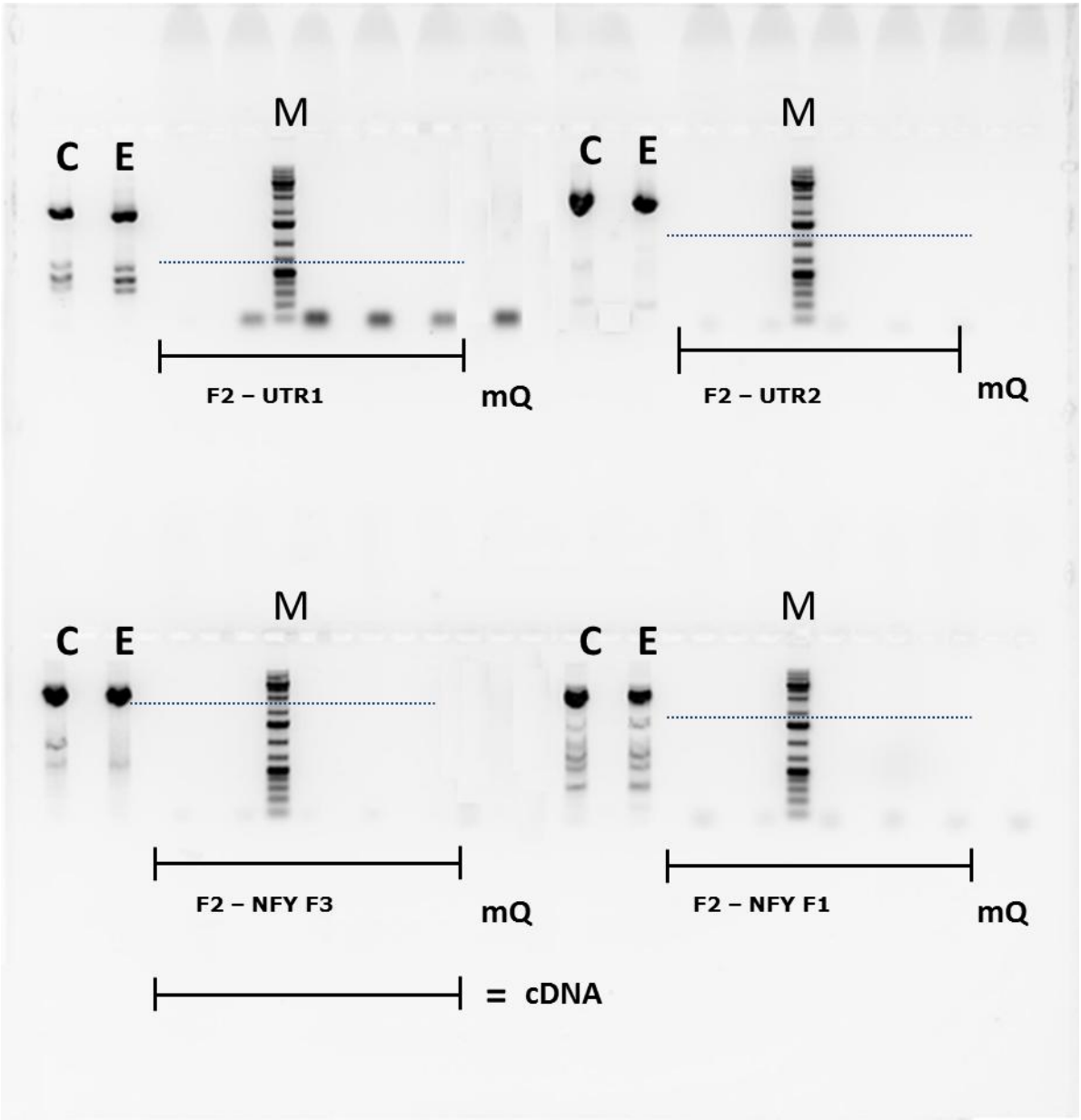


Figure 2.2 Four primer pairs were used on 8 different templates. Two used templates are genomic DNA from the C parent (C) and the E parent (E). 5 random cDNA samples and a water control (mQ) were added as well. The primer pair that was used is written below the dash indicating the cDNA. The expected products in the cDNA are visualised by a blue dotted line through the marker lane (M).

2.2.2 Verification of the UTR of PLE

A PCR was done with as forward primer the Fw2 primer of the PLE2 segment (figure 2.1) and the four different reverse primers UTR1, UTR2, NF-YC4 Fw3 and NF-YC4 Fw1 (figure 2.2). The PCRs that were performed on the genomic DNA of the C and E parent showed two clear bands, at the expected height. Some small side products were visible as well. For the cDNA samples however, nothing was present. Only for the F2-UTR1 combination there was some primer dimer, which was present in the water control as well.

2.2.3 Verification of the UTR of NF-YC4

A similar approach was used to verify the UTR of the NF-YC4 gene (data not shown). The used forward primer for this experiment was the NF-YC4 Fw3 primer. Two reverse primers were used: NF-YC4 UTR1 and NF-YC4 UTR2 (table 2.1). With both primer combinations a product did form on the genomic DNA. On the cDNA however, only primer dimer was formed.

2.2.4 Confirmation of the expression of PLE

A PCR was performed on the samples used for verification of the UTR with the primer pairs PLE1 F3R3 and PLE2 F2R2. Both showed a band in the lane with genomic DNA as template. In the cDNA however, no clear bands were visible. There was some primer dimer, which might be difficult to distinguish from real product since the size of the fragment is small (respectively 92 and 75 bp for PLE1 F3R3 and PLE2 F2R2). For this reason a second PCR was done on the same samples, with primers generating fragments of approximately 200 bp (figure 2.3). A product of the correct size was formed for the reactions with genomic DNA as template. The reactions with cDNA as template either had no signal, primer dimer or a band which is not of the correct size. The primer pair PLE2 F4R4 was selected to screen 96 of cDNA samples that were not screened yet for expression of PLE (data not shown). This PCR resulted in 96 primer dimer bands, indicating that the PLE gene is not expressed in any of these samples.

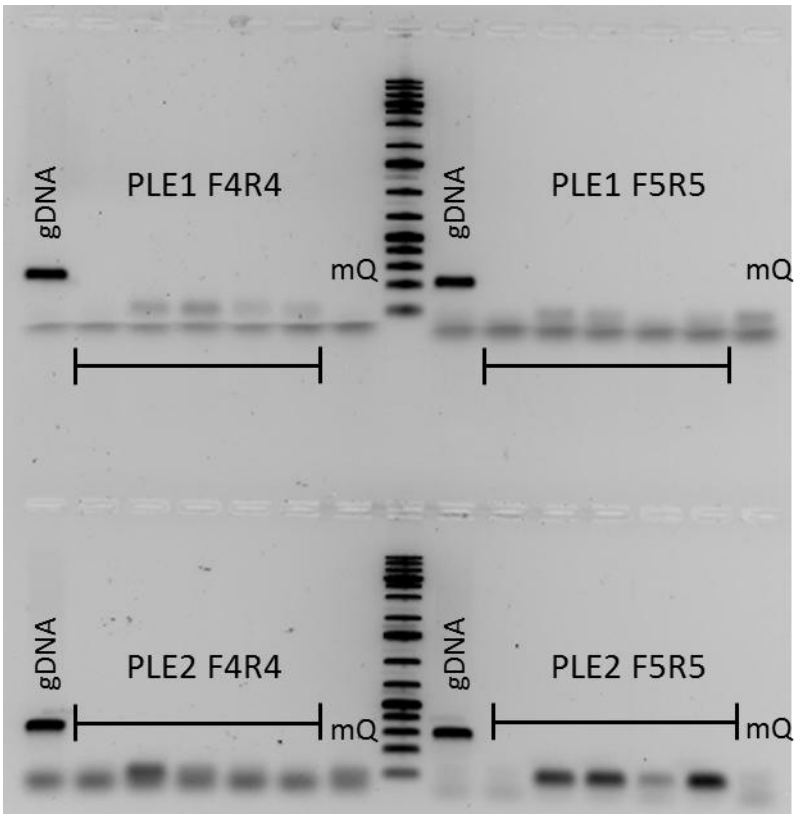


Figure 2.3 Product is present at the expected height in the lanes which contain genomic DNA as template. The cDNA lanes however do not have product. Some lanes contain primer dimer or another band, such as with PLE2 F5R5. Neither of these bands however are of the expected height.

2.3 Discussion

The 3'UTR of PLE has not been detected with the experiments. The PCRs that were done failed to amplify any product regarding the PLE gene. However, no expression was detected either. All the severely stressed samples were checked for expression, but neither of them had any. The C and E parent did not have any expression under control conditions either. The existence of an 3'UTR of PLE overlapping the *StNF-YC4* gene has not been rejected. However, with the rest of the experiments no attention has been paid to this issue, since there is no interference to be expected. An indication that the UTRs may have been annotated falsely can be found when the genes are studied in the genome browser in *Solanum lycopersicon*. Both genes are positioned next to each other there as well, but the UTRs do not overlap. One way or the other, the other experiments described in this thesis can be performed without any problems to be expected.

3. Expression analysis

To confirm the drought-induced changes in expression of the genes *StTAS14, StER24* and *StNF-YC4* as was found in the data of Anithakumari by Dennis van Muijen, expression analysis was performed. The expression of the genes might be different under various stress conditions. Therefore, three different datasets were used. The mild stress samples were originating from experiments performed by Daniela Bustos Korts in 2011. The severe stress samples were obtained from Ernest Aliche (2013), who sampled at two time points. A few samples came from Peter Bourke, who performed a heat stress experiment in 2013.

3.1 Material and Methods

3.1.1 Origin of mild stress material

83 genotypes of potato were investigated under mild drought stress conditions (Daniela Bustos, unpublished). Genotypes of Bustos' experiment that were used to analyse the expression of genes in this thesis are depicted in table 3.1. Seed tubers of the different genotypes were pre-germinated for one week, after which the homogeneously sprouted tubers were planted in pots. Three weeks after sowing the tubers, four out of eight replicates per genotype were subjected to mild drought conditions. The application of water was limited to 25% of pot capacity. The other four replicates received normal water application, being the control plants. Two weeks after initiation of the stress source and sink leaves were sampled, flash-frozen in liquid nitrogen and stored at -80°C. RNA was isolated from these samples by grinding the tissue with mortar and pestle in liquid nitrogen. The samples were further processed using the KingFisher™ Flex Magnetic Particle Processor using the protocol for RNA isolation. The resulting RNA was converted into cDNA using the iScript cDNA synthesis kit and stored in the freezer at -20°C. The measured phenotypic traits during the drought treatment and at harvest are given in table 3.2.

Table 3.1 Overview of the codes of the genotypes with their meaning and the experiments in which they are used.

Code of genotypes	Origin	Used in experiments
CE608	C x E population	Severe drought
CE628	C x E population	Severe drought
CE733	C x E population	Severe drought
CE765	C x E population	Severe drought
5	Albion	Severe drought; mild drought
24	Eos	Severe drought; mild drought
34	Great Scot	Severe drought; mild drought
79	Premiere	Severe drought; mild drought; heat stress
18	Cherie	Mild drought
49	Mondial	Mild drought
51	Nicola	Mild drought
9	Arran Chief	Mild drought
PrT2	Premiere at 11.30 am	Heat stress
PrT5	Premiere at 11.30 pm	Heat stress
SaT2	Andigena at 11.30 am	Heat stress
SaT5	Andigena at 11.30 pm	Heat stress

Table 3.2 Overview of the measured traits and the way they are measured.

Measured trait	Measured using
Chlorophyll content	SPAD meter
Leaf area	Camera at fixed position with blue background
Height	With a ruler, stretching the stem
Stolon appearance	Visual
Tuberisation	Visual
Yield	Fresh weight of tubers
Shoot biomass	Dry weight

3.1.2 Origin of heat stress material

The experiments described in this section were performed by Peter Bourke. Amongst others, the genotypes *Solanum tuberosum* group *Andigena* and *Solanum tuberosum tuberosum* Premiere were propagated from in vitro collections maintained within the Wageningen Plant Breeding Collection. Plantlets were put on Murashige and Skoog medium (MS) with vitamins and 2% sucrose. The plants received 16 hours of daylight at a temperature of 24°C. Another two weeks later apical cuttings were put on new medium for an additional two weeks, after which apical cuttings were taken for the second time.

After three weeks, these were planted in 0.5 liter pots with soil to grow for 26 days in climate chambers (LD 16/8; day temperature 20°C, night temperature 18°C, 75% humidity). The plants were transferred to bigger 3 liter pots and were separated into two similar batches. Both received short day conditions (LD 8/16) for 4 days, followed by the onset of heat treatment for one of the two batches. This heat treatment was gradually implemented, via one day of 26°C during daytime and 20°C at night-time, to 32°C at daytime and 28°C at night-time. The plants in the control batch received 20°C during day and 18°C during night-time. After 9 days of stress, leaves were sampled from both control and stressed plants. Four to six fully expanded young leaves were pooled and flash-frozen in liquid nitrogen. The leaves were sampled at different time-points over a period of 24 hours, two of which are analysed in this report. T2 leaves were sampled at 11.30 am, T5 leaves at 23.30 pm. The leaves were ground with mortar and pestle. RNA was extracted from the leaf powder with the Qiagen RNeasy kit, according to the manufacturers manual. The quality of the acquired RNA was checked by gel electrophoresis and by analysis with the Nanodrop spectrophotometer. Intact RNA was characterized by two bands representing the two largest subunits of the ribosome. RNA with a good quality typically had a value around 2.0 for both 260nm/280nm and 260nm/230nm ratios of absorbance. Once the quality of the RNA was confirmed, cDNA was synthesized using the iScript cDNA synthesis kit manufactured by BioRad. Prior to the actual synthesis, the RNA was cleaned up with DNAse to remove genomic DNA. As a negative control samples were included in the cDNA synthesis without reverse transcriptase. The resulting 20 µl of cDNA per sample was diluted with 380 µl mQ water and stored at 4°C.

3.1.3 Origin of severe stress material

Eight potato genotypes (table 3.1) were subjected to a severe drought treatment (Ernest Aliche, unpublished). This was done in order to further investigate and confirm previous results (Anithakumari 2011; Van Muijen, 2013). Four of these genotypes are common cultivars, selected for the diversity in their drought response. The other four genotypes are diploid potatoes from the CxE population, which resulted from a backcross between C (USW5337.3; Hanneman and Peloquin 1967) and E (77.2102.37 (CxVH34211); Jacobsen 1980). The CxE genotypes were selected based on their diverse drought response in the experiment conducted by Anithakumari. The plants were grown from seed-tubers, which before planting, were allowed to pre-germinate for one week at room temperature. Similarly germinated tubers were selected and planted in pots. The plants grew for four weeks when plant height was measured and the number of leaves was determined as well. Per genotype three replicates were subjected to the different treatments. One treatment, the control treatment, implies continuation of normal watering. Plants subjected to the other treatment did not receive water until all the pots contained 0 v/v% of water, measured with a Grodan water content meter. This was the case after 18 days of drought. After 4 and 9 days of drought, leaf discs were sampled from the upper leaves of all plants and flash-frozen in liquid nitrogen. The samples were stored for the long term at -80°C. After 18 days of drought, watering was restored to allow the plants to recover. The height of the plants, the senescence, root and shoot dry weight, amount of tubers and the weight of the tubers were measured as well.

3.1.4 RNA acquisition and cDNA synthesis

RNA was isolated from leaf samples collected by Ernest Aliche from the severe drought experiment performed in June 2013. The leaf samples were disrupted with the Qiagen TissueLyserII, which shook the samples with two metal beads at 25 movements per second for 46 seconds. The resulting lysed tissue was further processed using the Qiagen RNeasy kit, according to the manufacturers protocol. The quality of the RNA and the synthesis of the cDNA was performed as previously described. To confirm the synthesis of cDNA two housekeeping genes were used in a PCR, EF-1α and APRT. The PCR was performed with dreamTaq polymerase. The synthesized cDNA was stored at -20°C.

3.1.5 Design of qPCR primers

Primers were developed to monitor the expression of the NF-YC4, TAS14 and ER24 gene in the isolated cDNA samples. The primers were designed using the Primer3Plus tool (www.primer3plus.com). The cDNA sequences of the genes were used as template, derived from sequence information of *Solanum tuberosum* group Phureja DM1-3, genome browser version 4.03 (Potato Genome Sequencing Consortium). To ensure the specificity of the primers, a primer BLAST was performed against the superscaffolds and the EST database of DM1-3. Three primer pairs per gene were ordered at Biolegio BV. Upon arrival the primers were dissolved in mQ water to an end concentration of 100 µM. To test the functionality of the primers, PCRs were done on both C and E gDNA, as well as on two randomly selected cDNA samples and water controls. The products were loaded on a 1% agarose TAE gel and an electric current was used to separate the products. The bands were visualised using the Octopus F II UV camera. Based on the products formed the primer pair that performed best was selected for each of the three genes. These primer pairs first were tested on randomly chosen samples, three drought stressed samples and three control samples.

3.1.6 Performing the qPCR

After confirming the integrity and purity of the cDNA a qPCR was performed to measure the expression of the NF-YC4, TAS14 and ER24 genes. For each of the three genes of interest and for the housekeeping gene APRT a mastermix was composed (table 3.3). For the qPCRs 384 wells plates were used with 10 µl of reaction mix per well. For each of the loaded samples there were two technical replicates. In total three plates were done. The first plate contained the samples originating from the plants that had received 4 days of severe drought stress with their controls. On the second plate samples were present that were processed from plants that received 9 days of drought stress and the corresponding controls. The third plate contained both the mildly drought stressed samples and the heat stressed samples with their controls. The plates were loaded into the CFX384 Touch™ Real-Time PCR Detection System and the program was initiated (table 3.4).

Table 3.3 Composition of the mastermix per gene, per two technical replicates and the final concentration.

	MM per gene	MM per two technical replicates	Amount per single 10µl reaction
iQ™ SYBR® Green Supermix	621.5	11.3	5
Fw primer	37.125	0.675	0.3
Rv primer	37.125	0.675	0.3
cDNA		4.5	2
mQ	297	5.4	2.4

Table 3.4 Overview of the program and cycling conditions of the performed qPCRs.

	Conditions	Duration of steps
1	95.0°C	3:00 min
2	95.0°C	0:10 min
3	55.0°C	0:30 min
	Plate read; back to 2, 39 times	
4	95.0°C	0:10 min
5	Melt curve analysis: 65.0°C to 95.0°C; steps of 0.5°C; plate read	0:05 min per step

3.1.7 Analysis of the expression data

The BioRad software package CFX Manager 3.0 was used to analyze the melt curves and discard samples that had a deviating melt pattern. Samples that had a large difference in Ct value between two technical replicates were further analysed to see whether one of the two strongly deviated from the average. If one value clearly was wrong, this was discarded and the correct one was used in further analysis. Raw Ct expression values of approved samples were exported to Microsoft Excel from the BioRad CFX Manager 3.0 software. Normalized expression values were calculated for the samples according to the ΔCt method using a reference gene (Bio-Rad Laboratories, 2006):

$$\Delta Ct = Ct_{ref} - Ct_{target}$$

$$Normalized\ Expression = 2^{\Delta Ct}$$

Ct_{ref} is the Ct value of the reference gene APRT, Ct_{target} is the Ct value of one of the target genes NF-YC4, TAS14 and ER24. The variation was expressed using the standard error of the mean, which was calculated according to the following formulas:

$$SD_{\Delta Ct} = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \bar{X})^2}{n-1}} \quad SEM_{\Delta Ct} = \frac{SD_{\Delta Ct}}{\sqrt{n}}$$

$$SEM_{Normalized\ Expression} = 2^{(\Delta Ct + SEM_{\Delta Ct})} - 2^{\Delta Ct}$$

An intermediate step in the calculation of the standard error of the mean (SEM) is the sample standard deviation (SD). Individual values are denoted by the X, whereas $\bar{X}$ represents the mean of the values. N is the number of samples of which the variation is determined. Fold changes were calculated by dividing the normalized expression of the stressed samples over the control samples. The corresponding standard error of the mean was calculated in a similar approach as the standard error of the mean of normalized expression:

$$Fold\ change = \frac{Normalized\ Expression_{test}}{Normalized\ Expression_{control}}$$

$$SEM_{Fold\ change} = 2^{((\overline{\Delta Ct}_{test} - \overline{\Delta Ct}_{control}) + SEM_{\Delta Ct_{test}})} - 2^{(\overline{\Delta Ct}_{test} - \overline{\Delta Ct}_{control})}$$

In the calculation of the standard error of the mean of the fold change, the means of the ΔCt values are used. For the mean of the samples from stressed plants this is denoted by $\overline{\Delta Ct}_{test}$, for the control samples by $\overline{\Delta Ct}_{control}$.

3.1.8 Statistical analysis of the expression data

To test whether observed differences in expression are significant, the student's t-test was performed on the ΔCt values. The null hypothesis states that the mean ΔCt of both the control group and the stressed group is the same. The alternative hypothesis states that the mean ΔCt is not equal, thereby proving an effect of the treatment. The variances were not the same for the different subsets of samples, neither were the sample sizes. Therefore Welch's t-test was performed. This modified student's t test is used because it can handle unequal sample sizes and unequal variances. The t value is calculated according to the following formula, both for equal and unequal sample sized:

$$t = \frac{\bar{X}_t - \bar{X}_c}{\sqrt{\frac{s_t^2}{n_t} + \frac{s_c^2}{n_c}}}$$

The suprascript $_t$ represents the mean of the samples that were processed from stressed plants, whereas $_c$ stands for the mean of the control samples. $\bar{X}$ refers to the mean of the samples, *s* to the sample standard deviation. The amount of samples that are included within one group is symbolised by *n*. The degrees of freedom was calculated with the Welch-Satterthwaite equation, which has to be used in case of unequal variances:

$$d.f. = \frac{(\frac{s_t^2}{n_t} + \frac{s_c^2}{n_c})^2}{\frac{(s_t^2/n_t)^2}{n_t - 1} + \frac{(s_c^2/n_c)^2}{n_c - 1}}$$

The difference between two groups of samples is considered significant when the p-value is lower than 0.05. To further discriminate between barely significant and very significant, three classes of significance are used. The first class contains p-values <0.05, the second class <0.01 and the third class <0.001.

3.2 Results

3.2.1 Yield of the mildly drought stressed plants

Several different traits were measured on the plants that were used in the mild drought stress experiments. Of these traits, the relative reduction in yield is used as an indication for the drought tolerance of the plant (figure 3.1). The genotype with yield reduction to almost nothing upon mild drought stress is Great Scot (98.4%), and it was therefore drought sensitive. Mondial (65%), Nicola (72.5%), Arran Chief (71.3%) and Eos (62.6%) lost more than half of the yield, but they still yielded at least 25% of the control yield. Therefore they are classified as moderately drought sensitive. Cherie (45.7%) retained more than half of the yield, which is why this genotype can be considered moderately drought tolerant. Albion (32.6%) and Premiere (25%) however, had a very small yield loss. These can be considered drought tolerant.

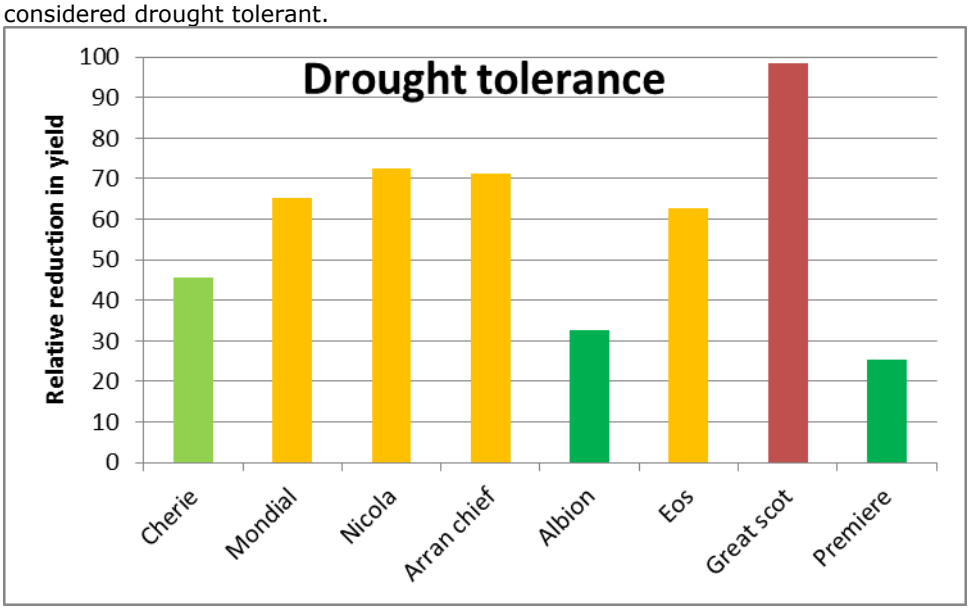


Figure 3.1 The drought tolerance of the different genotypes is determined with the relative yield reduction. The colour scales indicate the tolerance of the plants. Red means drought sensitiv, while dark green means that the plants are relatively drought tolerant.

3.2.2 Quality of the cDNA from the mild stress experiment

The cDNA originating from the mild stress experiment was checked for its integrity by running a few samples on gel. No product was visible on gel indicating degradation or absence of cDNA (figure 3.2a). Therefore, the original RNA was checked for its integrity. The RNA was intact (figure 3.2b), so it was used to generate new cDNA for analysis of the expression of the genes of interest.

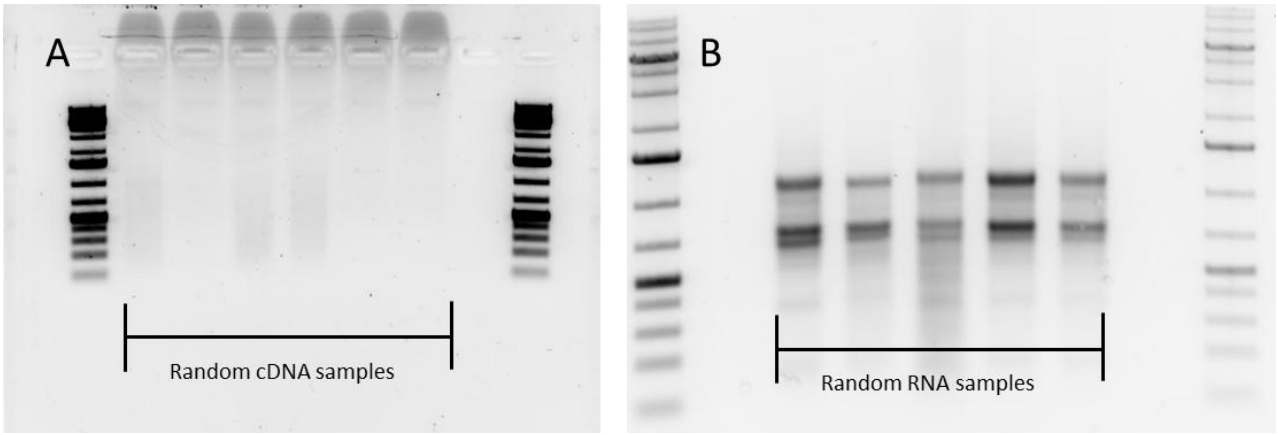


Figure 3.2 Visualisation of cDNA on gel. Section A of the figure shows the cDNA samples as they were present in the freezer. Section B shows the RNA stored in the freezer. Both intact cDNA and RNA are indicated by at least two clear bands, representing the two biggest ribosomal subunits, as in section B. Therefore, the cDNA that was present in the fridge was either degraded or absent, whereas the RNA was intact.

3.2.3 Tolerance of the heat stressed samples (Peter Bourke, unpublished)

Only two genotypes were included in the gene expression analysis. *Solanum tuberosum tuberosum* Premiere was included because this one appeared to be the most heat tolerant, whereas *Solanum tuberosum* group *Andigena* was the least heat tolerant. The selection was based on the relative yield loss under heat stress. For Premiere this was 12%, whereas for Andigena this was 68.7%.

3.2.4 Drought tolerance of the severely stressed plants (Ernest Aliche, unpublished)

Since almost none of the eight used genotypes had any yiel under severe drought stress, the yield is not an appropriate measure of the drought tolerance of the genotypes. Only Eos, Premiere and CE733 still yielded some potatoes. More interesting is that the only genotype that was able to recover from its original stem is Eos. Furthermore, Eos, Great Scot, Premiere and CE733 had re-growing stems emerging from the soil. Upon further inspection the stems appeared to emerge from newly formed tubers.

3.2.5 Synthesis of cDNA from the severe stress RNA

The integrity of the RNA that was obtained from the severely stressed leaf samples present in the -80°C freezer was confirmed by loading the RNA samples on gel (figure 3.3). Whereas only six samples are presented here, the other 286 samples were analysed as well. All of them were intact, except for the sample 765D1A (Genotype 765; drought stressed; 4 days of stress; biological replicate A). This sample was left out in further analysis, because it also allowed us to include water controls on the qPCR plates. The same sample for the second time point was excluded as well, again to allow the inclusion of a water control. The Nanodrop spectrophotometer indicated that the RNA was of good quality as well, with a concentration of around 120 ng/µl. The cDNA samples that were checked with the housekeeping genes EF-1α and APRT all showed a product (figure 3.4). The negative controls, cDNA that was synthesized without reverse transcriptase, did not show a band for APRT. For EF-1α they did however show primer dimer. For this reason the housekeeping gene APRT was used in further gene expression analysis.

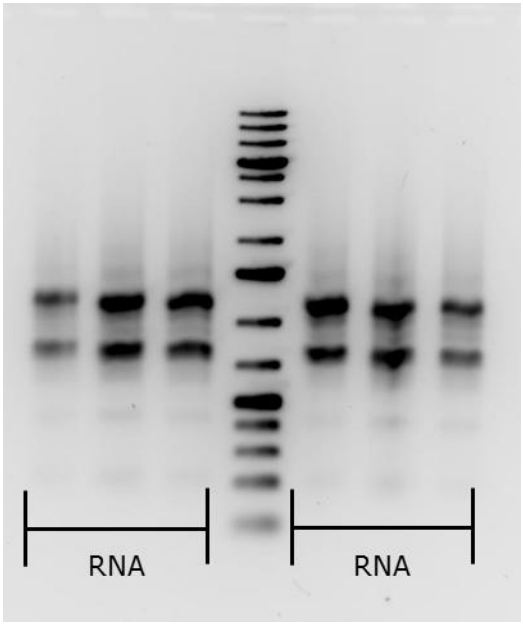


Figure 3.3 Visualisation of the RNA from the severe stress experiment on gel. The two bands that are visible in the RNA lanes represent the two biggest ribosomal subunits. As is visible, the RNA is of good quality.

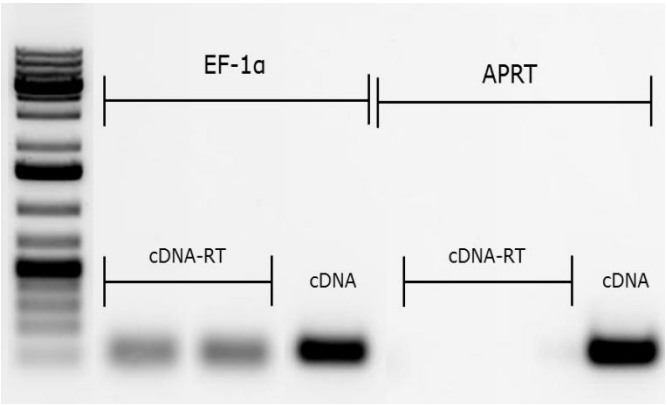


Figure 3.4 Quality check of the synthesized cDNA by using the housekeeping genes EF-1α and APRT. cDNA samples that were generated without reverse transcriptase (cDNA-RT) were included as a negative control. The cDNA samples show a product for both the housekeeping genes. For the EF-1α gene primer dimer is present as well. For the housekeeping gene APRT no primer dimer was formed.

3.2.6 Testing of the developed primers

The primers to be used for expression analysis were tested by performing a PCR both on genomic DNA and cDNA. First, the primer pairs were tested on genomic DNA of both the C and E parent and on cDNA (figure 3.5a). As a positive control the housekeeping genes APRT and EF-1α were included. As a negative control a reaction without template was loaded on the gel. APRT worked well as a control, since a product was formed in both the C and E parent genomic DNA, and in the cDNA. EF-1α did not show a band with the C parent, possibly due to a mistake with pipetting. Together with the fact that primer dimer formed, this was a reason not to include the EF-1α housekeeping gene in further analysis to not take any chances. The primer pairs NF-YC4_3; TAS14_2 and ER24_2 were selected because they formed

a product both in the gDNA of the C and E parents, as well as in the cDNA. Furthermore, minimal primer dimer was formed. For TAS14 the product that was formed on the genomic DNA was positioned higher in the gel than the product of the cDNA. The reason for this is that the primers span an intron region. With the selected primers a second test was performed, which could be used to get some preliminary expression results as well (figure 3.5b). APRT was included as a control. Indeed this gene appeared to be expressed constitutively in all the samples. The NF-YC4 gene showed a higher expression in control samples compared to samples from stressed plants. The opposite is true for TAS14 and ER24.

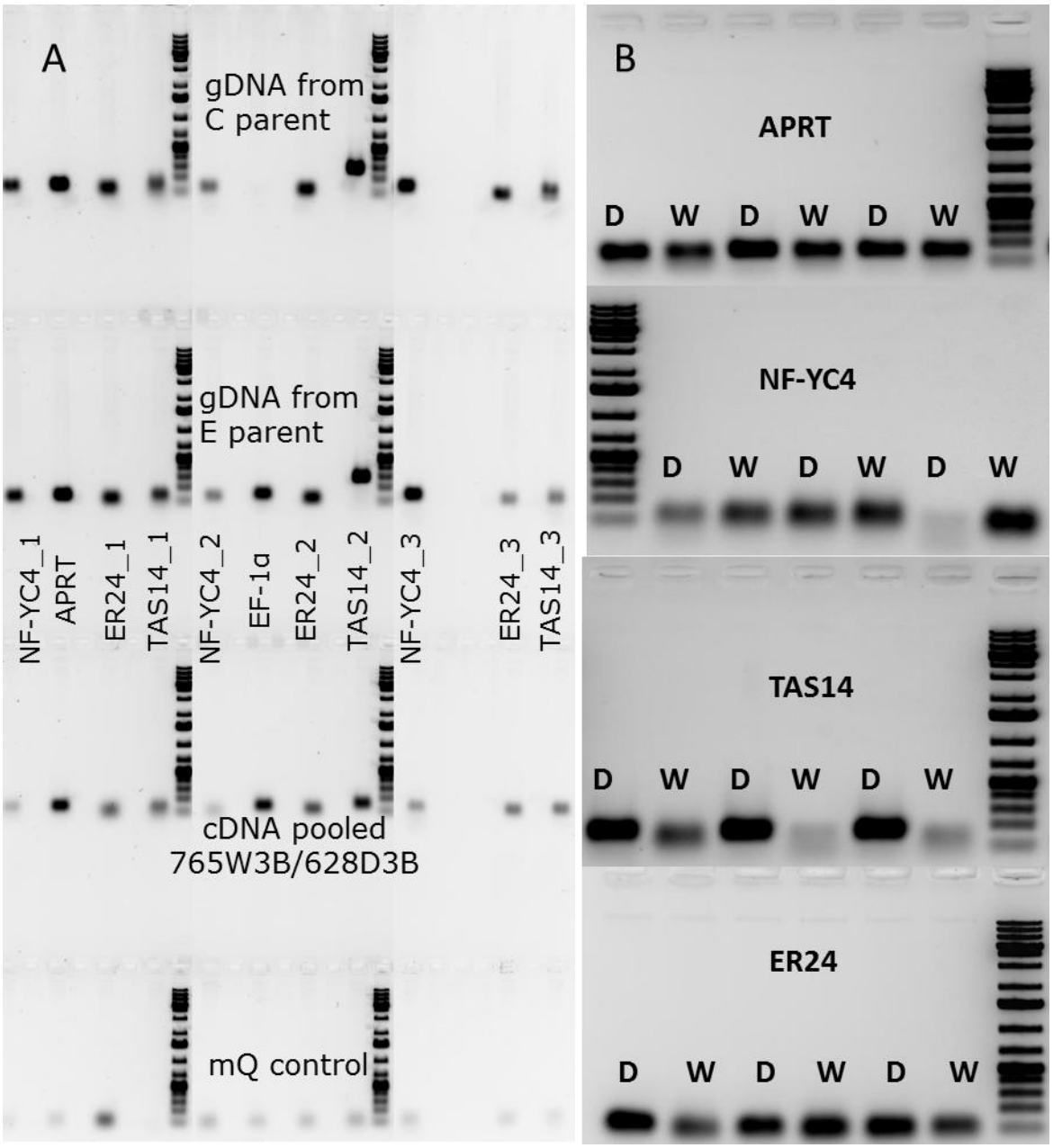


Figure 3.5 (A) Per gene of interest (GOI) three primers pairs have been developed, denoted as GOI_1, GOI_2 and GOI_3. APRT and EF-1α have been included as positive controls. Each row has two GeneRuler™ 1 kb plus marker lanes. Each row represents a template on which the primers have been tested. The primer pairs that were selected to be used in further analysis are NF-YC4_3; TAS14_2; ER24_2. These formed both a product on genomic DNA and on cDNA, while no product was present in the negative control wells. EF-1α did not show a band in the genomic DNA from the C parent. (**B**) The primer pairs NF-YC4_3; TAS14_2 and ER24_2 were tested again on three drought stressed samples and three control samples. APRT was included as a positive control. "D" means that the sample originates from drought stressed plants, "W" means that the sample originates from control plants.

3.2.7 Quality check of the qPCR data

Before analysis of the expression data of the qPCR, the melt curves were investigated per target gene, per plate (figure 3.6). Overall, the melt curves showed a single curve (figure 3.6c). For two subsets however, the melt curves were not of perfect quality. To clean up the set of melt curves the samples that showed a deviating melt pattern were removed from further analysis. Samples from the genotype Premiere were excluded from the mild stress dataset, since there was not enough sample of stressed plants to do significant analysis.

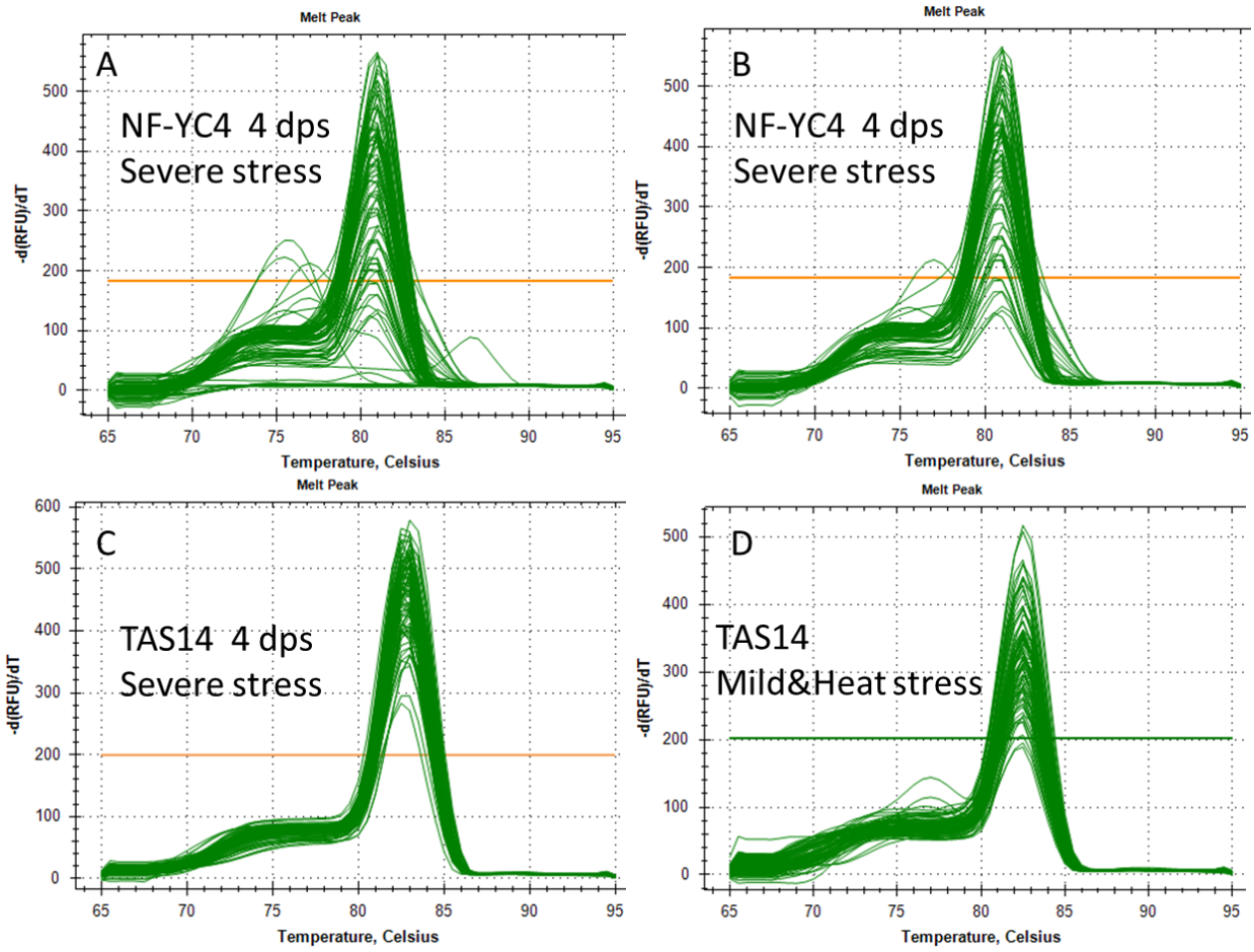
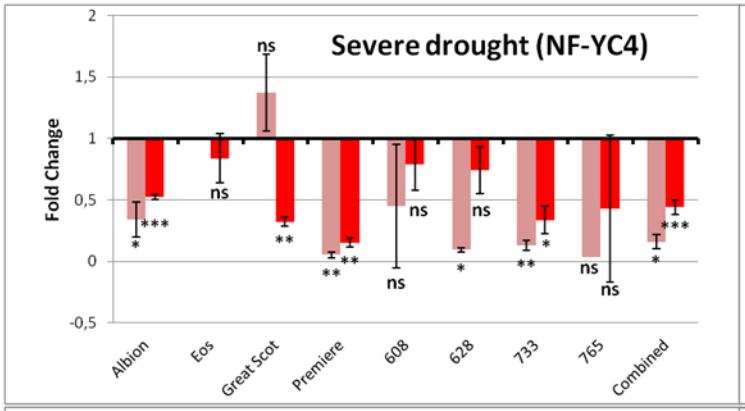
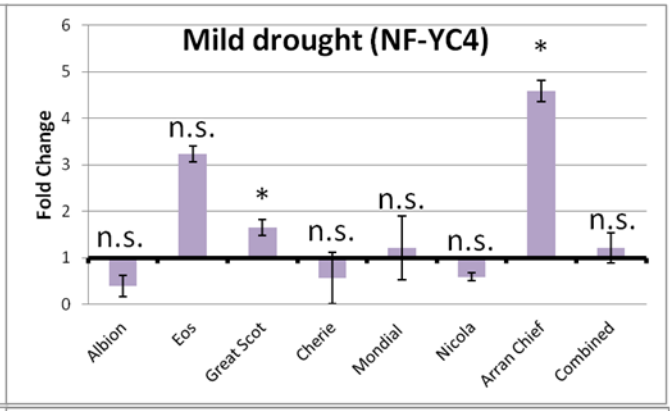


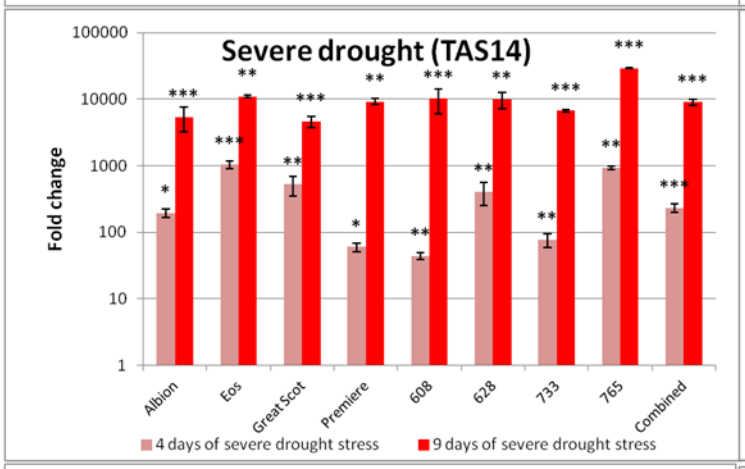
Figure 3.6 Melt curve output from the BioRad CFX Manager 3.0 software package. Part A shows all the melt curves of the samples with target gene NF-YC4 on the plate loaded with samples from plants that were severely stressed for four days (4 dps). The curves look messy, with flat lines and double peaks. After removal of the water controls and other samples that were of bad quality, the figure presented in part B was obtained. The figure in part C shows a typical figure representative for the other output as well, where all the samples of one target gene have the same melt curve. Only the melt curves for TAS14 on the third plate, with mildly drought stressed and heat stressed samples, were not of perfect quality.

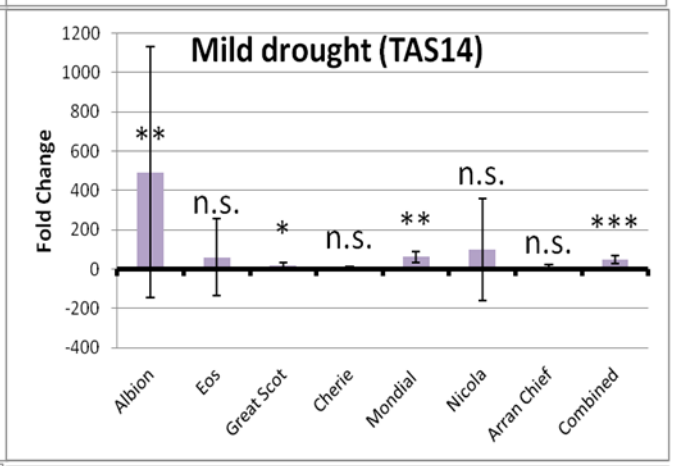
3.2.8 Expression of NF-YC4

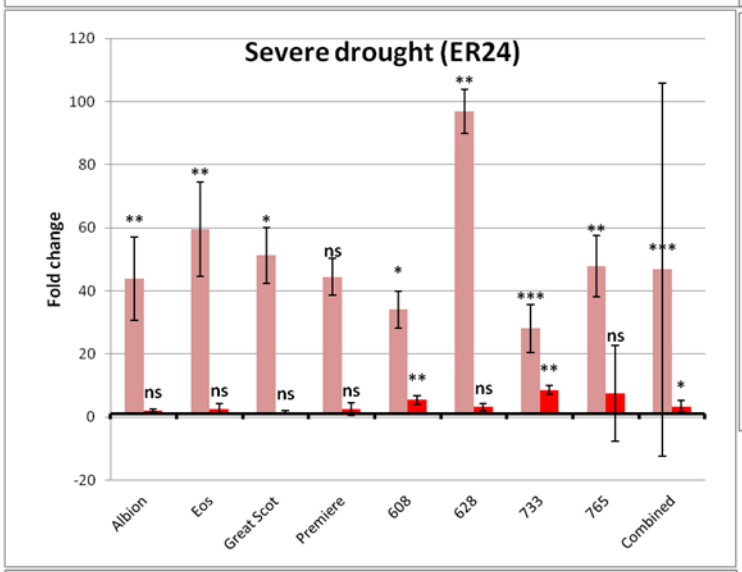
The gene NF-YC4 was down regulated for the different drought treatments (figure 3.7). Four out of eight genotypes were significantly down regulated at 4 days of severe drought stress. When the eight genotypes were taken together, a clear significant down regulation could be seen. The same was true for 9 days of severe stress. However, NF-YC4 appeared to return to the expression level under control conditions at 9 days of severe stress, although this was not statistically investigated. The expression after 4 days is 0.16 compared to the control, whereas the average after 9 days is 0.43. The expression of NF-YC4 under mild stress conditions is not significantly different from the expression under control conditions for most genotypes. Only the cultivars Great Scott and Arran Chief showed significant up regulation. When the genotypes were taken together this effect was nullified by the other genotypes, which mainly show insignificant down regulation.

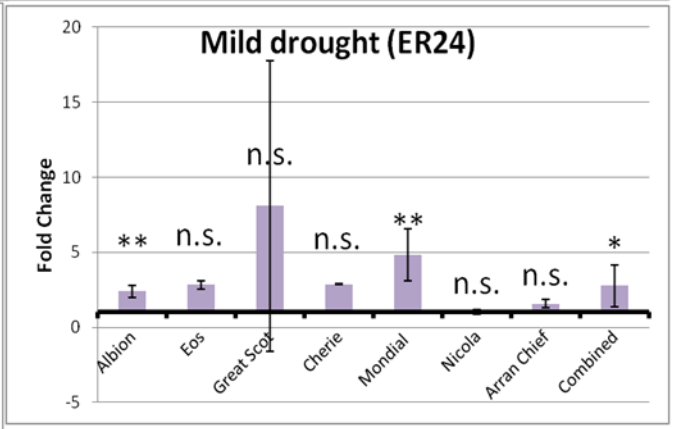


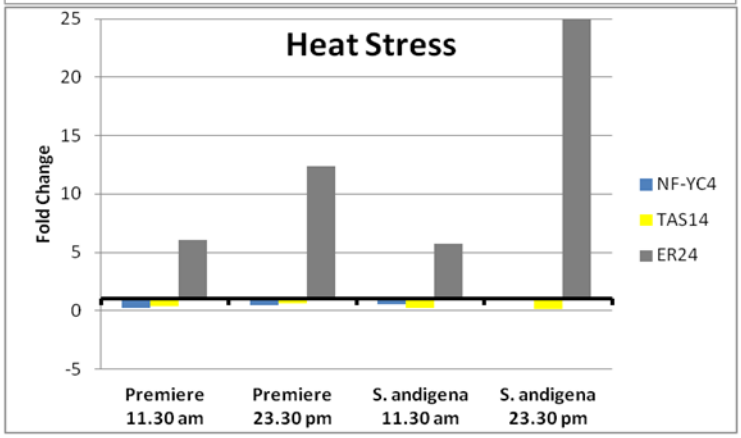












*	p≤0.05
**	p≤0.01
***	p≤0.001
ns	Not significant

Figure 3.7 The bar graphs represent the fold changes of the genes NF-YC4, TAS14 and ER24 under the different stress conditions. The error bars symbolize the standard error of the mean. The significance levels are based on a student's t-test performed on the underlying ΔCt values. The significance is indicated with a symbol above the bars in the chart. The individual genotypes are given below the bars. The genotypes that were subjected to the same treatment have been taken together as well, represented by "combined". The bar graphs visualising the change in expression under heat stress are based on one value only. Therefore no significance can be attributed to these values.

3.2.9 Expression of TAS14

Under control conditions *StTAS14* was expressed in a low amount (figure 3.7; figure 3.8). The increase in expression of the gene under drought conditions was very high. When the expression is compared in terms of fold change of the stressed samples over the control samples, an increase in expression is found as the drought persists. At 4 days of drought the fold change ranged between 44 (CE608) and 1040 (Eos). After 9 days of stress, the expression was even higher (4500 (Great Scott) to 30000 times increased (CE765)). The mildly stressed plants all showed upregulation of *StTAS14*, but for 4 out of 7 samples this was not significant. Combining the samples resulted in a fold change of 47, which was significant. However, the relative normalized expression gives rise to another perspective. The stressed samples at four days of drought show a slightly higher expression than the samples stressed for 9 days. The expression of the samples that received mild water stress did not exceed 1.3 (data not shown). On average the relative normalized expression under mild drought was 0.34, whereas under control conditions this was 0.007. Under severe drought, the genotype with the highest expression is 608 of the CxE population.

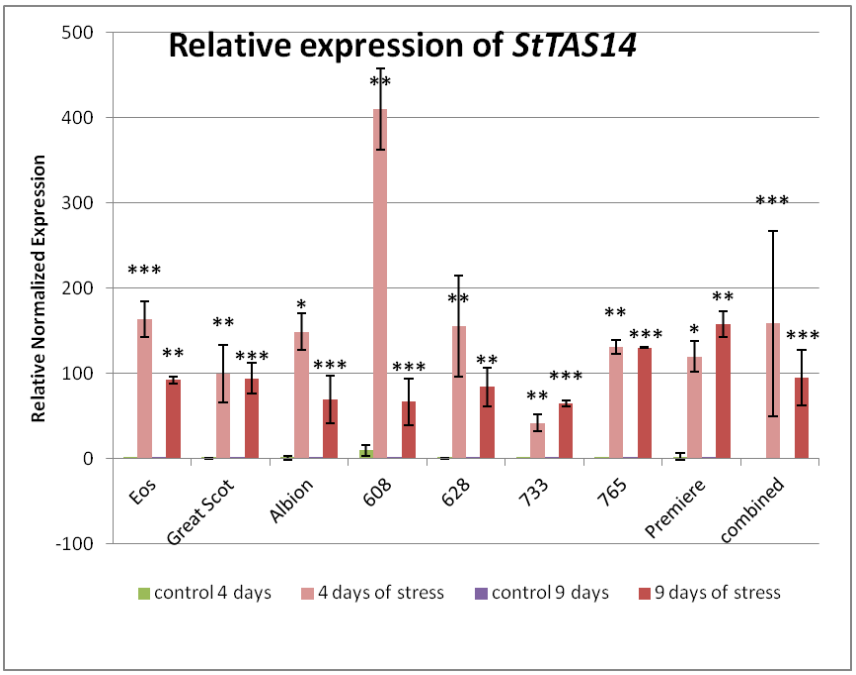


Figure 3.8 The expression of *StTAS14* is visualised, normalized to the expression of the reference gene *APRT*. The significance of the expression indicates whether the expression at the time-point is significantly different from the corresponding control.

* p<0.05; ** p<0.01; *** p<0.001

3.2.10 Expression of ER24

StER24 was significantly upregulated after 4 days of drought stress in all genotypes except Premiere. After 9 days of severe drought stress the expression of *StER24* had returned to the expression under control conditions. For the samples which still were upregulated, the value had dropped at 9 days of severe stress. Under mild drought stress conditions, most genotypes show upregulation of *StER24*. However, this is only significant for Albion, Mondial and the genotypes combined. The average expression was significantly increased for all three types of drought stress. For 4 days of severe drought stress the up regulation was highest. The up regulation at 9 days of severe stress and under mild drought stress were barely significant.

3.2.11 Expression of the genes under heat stress

No significance could be credited to the expression changes induced by the heat stress. However, a trend could be seen with one of the three genes. *StNF-YC4* and *StTAS14* were not differentially expressed from the expression under control conditions. *StER24* however was upregulated in both the evaluated genotypes. It's expression seemed to increase from 11.30 in the morning to 23.30 in the evening.

3.3 Technical discussion

The housekeeping gene Adenine Phosphoribosyl Transferase (APRT) was used as a reference gene. However, the gene Elongation Factor 1α (EF-1α) is recommended to be used as a reference gene in biotic and abiotic stresses (Nicot, 2005). Both had stable expression under the different biotic and abiotic stress conditions tested, but the expression of EF-1α was higher. In the experiments of this thesis EF-1α was not used as reference gene because primer dimer was found. This could pose a problem since the amplification efficiency is affected, which could result in misleading expression levels. Therefore not the recommended reference gene EF-1α was used, but the alternative reference gene APRT.

The first qPCR plate that was done, which contained the samples of plants that were severely drought stressed for 4 days, showed relatively many low quality data. Especially the first row of wells showed this pattern. A lot of air bubbles were present in these wells because the residue was blown out inside the well. Visual inspection indicated that it was not necessary to blow out, since no residue was left when the operating button reached the first stop. The remaining wells on the plate were loaded without blowing out. Therefore the first row of wells was removed from the dataset. Because of this, no expression data was available for Eos under 4 days of severe drought stress.

Most of the individual samples of the mildly drought stressed plants did not show significant differential expression. This can mainly be attributed to the amount of replicates. There were three control samples per genotype and two mildly drought stressed samples. For most of the genotypes there was a missing value, so this had great impact on the significance. Combining the genotypes allowed to at least get a general impression of the expression of the genes.

Based on the fold change of the expression of *StTAS14* in stressed samples over the expression in well-watered samples, an increase in expression was seen as the drought persisted. However, the normalized expression value of the *StTAS14* gene under control conditions is close to 0, whereas the expression under stressed samples ranges between 100 and 200. Small changes under control conditions have a big impact on the fold change, which is reflected by the apparent increase in expression as the drought persists. Looking at the normalized expression, the expression of *StTAS14* does not increase. On the contrary, it seems to decrease. However, the order of magnitude is the same.

4. Design of stable constructs

To characterize the function of the genes *StTAS14, StER24* and *StNF-YC4 in planta*, constructs have been designed enabling the generation of stable transgenic plants. The vectors that are generated exhibit stable overexpression, induced expression, stable silencing or induced silencing.

4.1 Material and methods

4.1.1 Design and amplification of the gene (fragment)

The annotated sequences of the *StNF-YC4*, *StTAS14* and *StER24* genes were downloaded from the SPUD DB Genome browser (http://solanaceae.plantbiology.msu.edu/). The sequences originate from Solanum tuberosum group Phureja DM1-3 pseudomolecules version 4.03 (Sharma, 2013). The online tool primer3plus (www.primer3plus.com) was used to design primers with the sequences of *StNF-YC4*, *StTAS14* and *StER24* as templates. Per gene, three different pairs were designed (table 1). Two of these include the coding sequence of the whole gene, since the designated vectors are aimed at measuring functional expression of the gene. For these the forward primers were fixed, starting at the first ATG of the coding sequence. The reverse primers were designed starting from the stop codon, directed upstream of the gene. Both forward and reverse primers were developed aiming at a length of 20 bp, ending with a G or C. For the whole gene amplicon destined for GATEWAY™ cloning, the short sequence CACC was added to the 5' end of the forward primer. To the forward primer of the ligation-targeted amplicon the sequence GTCGAC was added, for restricted digestion by SalI. To the reverse primer 5' end the sequence ACTAGT was added, allowing the product to be digested by SpeI.

Silencing of the genes can be achieved using a 250-300 bp fragment of the gene. The designed primers were blasted against the superscaffolds and the EST database of *Solanum tuberosum* group Phureja DM1-3 of the Potato Genome Sequencing Consortium. The program used is optimized for short sequences such as primers. The resulting gene fragment was checked for specific silencing as well. The fragments were cut into 20bp pieces in silico and blasted against the EST database. This was to make sure no off-target silencing would occur in the experiments. The 4 base pair sequence CACC was added to the 5'end of the forward primers for compatibility with the GATEWAY™ system.

A gradient PCR was performed to determine the optimal temperature for amplification (table 4.1), using pooled cDNA of the C and E parent as template. For the amplification of the whole *StNF-YC4* gene not pooled cDNA, but pooled genomicDNA of the C and E parent was used as template. The optimal temperature was used in a 2x50µl PCR reaction using Phusion® High-Fidelity DNA Polymerase. This DNA Polymerase was used because of its proofreading capability and its property of generating blunt ends. After amplification the products were loaded on gel. Bands with the correct fragment size were excised and purified using the MinElute® Gel Extraction kit by Qiagen. To make sure the right bands were excised without contamination, the purified products were loaded on gel again and checked for single bands.

Table 4.1 The table represent the different amplicons that were designed. The type indicates whether the whole gene is amplified for expression *in planta,* or whether only a fragment is amplified for silencing purposes.

Type	Target gene	Length	Optimal temperature of PCR
Whole gene CACC overhang on 5' end of amplicon	**NF-YC4**	430	61°C
	TAS14	429	60°C
	ER24	447	56°C
Gene fragment CACC overhang on 5' end of amplicon	**NF-YC4**	261	61°C
	TAS14	291	59°C
	ER24	266	60°C
Whole gene SalI overhang on 5' end SpeI overhang on 3' end	**NF-YC4**	438	64°C
	TAS14	435	64°C
	ER24	453	61°C

4.1.2 Vectors used

Four different vectors were designed for stable transformation (table 4.2). Three of these contain a gateway cassette, which allows the insert to be recombined into the vector via an efficient LR reaction. The pK7WG2 vector, pHELLSGATE12 vector and the pER8 vector were present in the lab as glycerol stocks stored at -80°C in the freezer. The pOpOff2 vector was ordered (Wielopolska, 2005). All four vectors contain the resistance gene for spectinomycin resistance for selection of transgenic bacteria. The pK7WG2 vector contains a 35S promoter that constitutively drives the gene inserted into the Gateway cassette (figure 4.1a). The pHELLSGATE12 vector contains two Gateway cassettes, allowing the gene fragment to be inserted twice (figure 4.1b). The fragments are inserted sense and antisense, separated by an intron. This whole region is driven by a 35S promoter. Once transcribed, the resulting RNA forms a dimer, hybridising the sense and antisense gene fragment together. The intron is spliced

out and the double stranded RNA is processed by the plants RNAi mechanism, resulting in silencing of the target genes. The pOpOff2 vector is essentially the same as the pHELLSGATE12 vector, but the difference lies in the expression of the silencing domain (figure 4.1c). The domain is controlled by the pOp6 promoter system, which operates under control of the LhGR transcription factor. The LhGR transcription factor, which is expressed constitutively under control of the 35S promoter, activates the pOp6 promoter system only when dexamethasone is present. In this way, silencing only occurs in the presence of dexamethasone. The fourth vector that is used is a vector enabling inducible expression (figure 4.1d). The chimeric LexA/VP16/ER (XVE) domain confers inducibility by β-estradiol. The fusion protein that is transcribed from this domain contains the DNA binding region of LexA, the transactivating domain of VP16 and the estrogen binding domain of the human estrogen receptor (ER). Upon perception of estrogens, the fusion protein activates the LexA operon region downstream of the XVE domain. This then enables the 35S promoter fused to the LexA operon region to activate the gene inserted into the multiple cloning site.

Table 4.2. Overview of the used stable vectors. The antibiotic resistance is given between brackets, where spec means spectinomycin resistance. Gateway vectors are presented in bold.

Name vector	Mode of action	Type	Used insert	Vector specific primers
pK7WG2 (Spec)	Overexpression	Constitutive	Whole gene	pK7WG2 Fw
pHELLSGATE12 (Spec)	Silencing	Constitutive	Gene fragment	AL216+218
pOpOff2 (Spec)	Inducible silencing	Dexamethasone inducible	Gene fragment	AL216+218
pER8 (Spec)	Inducible expression	B-estradiol inducible	Whole gene with restriction site overhangs	-

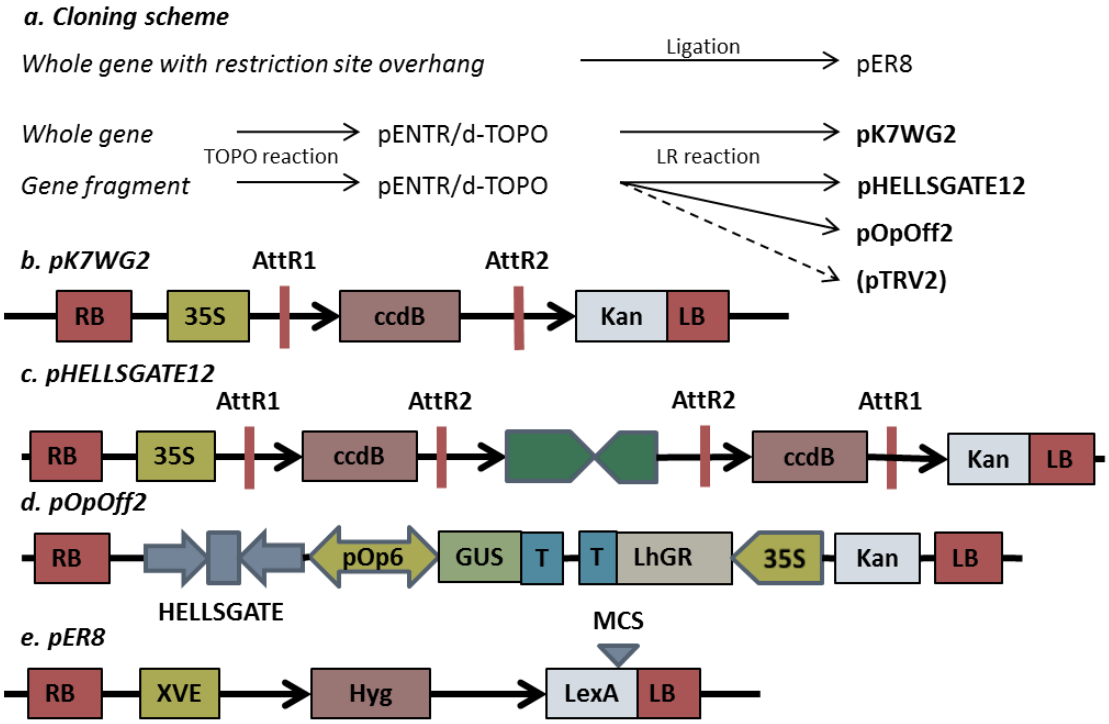


Figure 4.1 Cloning scheme and features of the used vectors. (**a**) A schematic overview of the cloning steps to perform to achieve the expression vectors. The gateway destination vectors are in bold. pTRV2 is visualised with a dashed arrow since this construct is treated in section 5.1.3. (**b**) The pK7WG2 vector contains the 35S promoter and a Gateway cassette (AttR1-ccdB-AttR2) in the tDNA, between the right border (RB) and the left border (LB). The whole gene is inserted downstream of the 35S promoter, thereby being constitutively overexpressed in the plant in which the construct is inserted. (**c**) The pHELLSGATE12 vector contains a 35S promoter followed by two gateway cassettes, one oriented opposite of the other. The green pentagons represent introns separating the two gateway cassettes. (**d**) The pOpOff2 vector is based on the pHELLSGATE12 vector. The grey part with HELLSGATE below it contains the two gateway cassettes and the intron that separates them. This silencing construct is not driven by the 35S promoter as is the pHELLSGATE12, but by the LhGR-pOp6 system. The pOp6 promoter system operates under control of LhGR, which activates the pOp6 promoter upon the perception of β-estradiol. The construct contains a kanamycin resistance gene for selection of transgenic plants. Functionality of the silencing can be monitored via the GUS gene that is active under the pOp6 promoter as well. (**e**) The pER8 construct does not contain a gateway cassette.

Instead, the desired fragment is inserted into the multiple cloning site. The XVE domain controls the LexA operon. The XVE system contains the DNA binding domain of the LexA repressor protein (X), the VP16 transcription activation domain (V) and a human estrogen receptor (E). This system is expressed constitutively under the G10-90 promoter, which exhibits 8 times the expression level of the CaMV 35S promoter (Zuo et al. 2000). The protein that is transcribed becomes active upon β-estradiol perception, which then binds to the LexA operon and activates the CaMV 35S promoter driving the gene inserted in the multiple cloning site (MCS).

4.1.3 TOPO reaction

Once the purity of the amplicons was confirmed, the TOPO reaction was performed. For this purpose Invitrogen™'s pENTR™ Directional TOPO® Cloning kit was used. Blunt-end gene fragments were inserted into the pENTR™/d-TOPO® vector according to a modified protocol (Untergasser, 2006) and according to the original protocol (Invitrogen, 2007). The plasmids were inserted into chemically competent *Escherichia coli* TOP10 cells with a 30 second heat shock of 42°C. After 1 hour of recovery in 250 µl LB (37°C/200rpm) they were plated out on 3% LB plates with 50 µg/ml kanamycin. Colonies formed overnight at 37°C were picked with sterile toothpicks and inoculated in 96 wells plates with LB and antibiotics. For one hour the plate was placed in the stove at 37°C, after which a colony PCR was done with the fragment synthesis primers. Colonies with the right insert were selected and inoculated in 3ml LB with antibiotics. The tubes with the cultures were put in the stove overnight at 37°C while shaken at 200 rpm. Plasmid DNA was isolated using the QIAprep® Spin Miniprep Kit by Qiagen. Plasmids were send for sequencing by GATC BioTech using the pENTR™/d-TOPO® primers M13F and M13R spanning the insert site. The inserted allele was identified by comparing the returned sequence to raw sequence alignment data of C and E obtained from Theo Borm. Comparison of the returned sequence with the raw sequence alignment data was done using the Integrative Genomics Viewer, version 2.3 (Robinson, 2011).

4.1.4 LR reaction

The gene (fragments) of *StNF-YC4*, *StTAS14* and *StER24* were inserted into the GATEWAY™ vectors (table 4.2) by means of the LR-reaction, according to a modified protocol (Untergasser, 2006) and according to the original protocol (Invitrogen, 2004). The pENTR™/d-TOPO® vectors were used as template, as represented in figure 1a. The LR-reaction mix was transformed into chemically competent *E. coli* dH-5α cells. Single colonies that were present on the plate the day after were inoculated in 3 ml LB with spectinomycin (200µg per ml). For the purpose of quality check the gene specific primers were used, both as pairs to confirm the presence of the insert, as well as in combination with vector specific primers (table 4.3). The primers were used both in colony PCR and in direct PCRs on the isolated plasmid. Glycerol stocks were made of bacteria containing the correct vectors to be used for subsequent transformation into *Agrobacterium tumefaciens*.

Table 4.3 The primer combinations that are used to check the insertion of the gene (fragment) into the specific destination vector are indicated in the table. More information on the primers is present in the appendix. "Gene" primers refer to the primers that have been used to amplify the inserted fragment.

Vector	Forward	Reverse
pK7WG2	pK7WG2 Fw	Gene reverse
	Gene forward	Gene reverse
pHELLSGATE12/pOpOff2	AL216	AL218
	AL216/AL218	Gene forward
	AL216/AL218	Gene reverse
	Gene forward	Gene reverse

4.1.5 Ligation cloning

The pER8 vector, which contains a multiple cloning site instead of a Gateway cassette, was cloned using restricted digestion and ligation. The amplicons containing the whole genes and overhangs for restricted digestion were double digested with XbuI (alias SpeI) and SalI as depicted in table 4.4. Simultaneously, the pER8 vector was digested in the same way. The reactions were stopped by incubating the samples for 15 minutes at 65°C. After digestion the vector and the amplicons were incubated in a 1:3 vector to amplicon ratio overnight at 16°C. Of the incubated samples 2 µl was added to chemically competent TOP10 cells. The transformation occurred as described before and the bacteria were spread out on plates containing spectinomycin (200µg per ml) and incubated overnight (37°C). The plates were stored in the cold room at 4°C.

Table 4.4 Reaction conditions of the double digestion of the amplicons and the pER8 vector. First only 0.5 µl SpeI is added, to a final volume of 20 µl with 1 µl sample and 1x tango buffer. After one hour 1 µl of SalI is added, together with 2.5 µl tango buffer. Both reactions occur at 37°C.

Enzyme	Buffer	Duration	Amount of enzyme
XbuI (SpeI)	1x tango	1 hour	Normal
SalI	2x tango	1 hour	2 fold excess

4.1.6 Transformation of the expression vectors into *Agrobacterium tumefaciens*

Correct expression vectors were inserted into chemically competent *Agrobacterium tumefaciens* cells of the strains AGL1 with virG or AGL0. AGL1 is resistant to spectinomycin, chloramphenicol and carbenicillin. AGL0 is resistant to spectinomycin and rifampicin. These cells were transformed with electroporation (resistance 200Ω, capacitance 25µF, voltage 1.4kV). A successful transformation should have a time constant between 4 and 5 ms. The recovery of the bacteria after the electro shock takes 3 hours at 28°C with 200 rpm. After recovery the bacteria were plated on LB plates with 3% agar and the associated antibiotics. The plates were inoculated at 28°C for two days, after which formed colonies were inoculated in 96 wells plates with LB and antibiotics. The plate was put at 28°C for at least 3 hours, after which it was possible to use them in colony PCR. The same primer combinations were used as mentioned before (table 4.3). Colonies that proved to be correct were inoculated in 3 ml LB with the proper antibiotics and a glycerol stock was made. The stocks were stored at -80°C.

4.2 Results

4.2.1 Amplification of the whole gene and insertion into pENTR™/d-TOPO®

To obtain the amplicons of the genes *StNF-YC4*, *StTAS14* and *StER24* multiple PCRs were done using cDNA as template. The first PCR that was done did not yield bands of the expected fragment size. A gradient PCR with 6 different temperatures did result in bands with the correct position on the gel (data not shown). The optimal temperature for each amplicon was determined and new 50µl reactions were set up. These yielded the desired fragment for each amplicon, but several a-specific products were formed as well (figure 4.2). The products were cut out of the gel and purified. The fragments were inserted into the pENTR™/d-TOPO® vector with half of the reaction volume as is described by the manufacturer

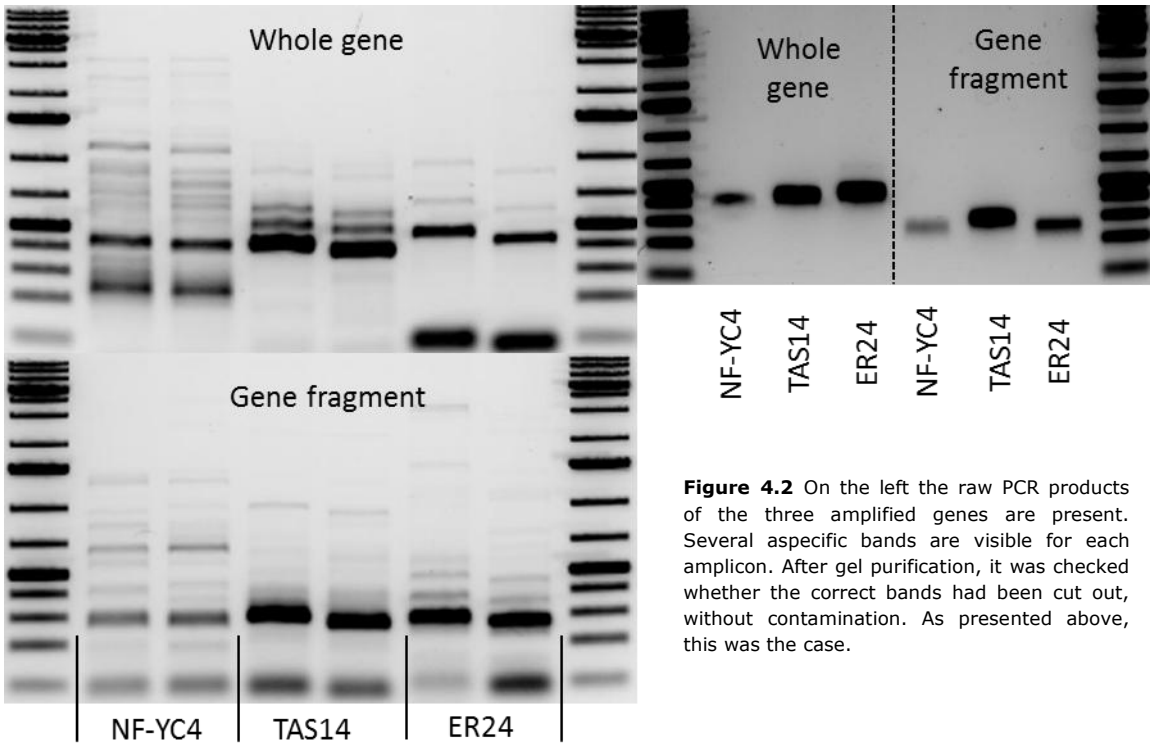


Figure 4.2 On the left the raw PCR products of the three amplified genes are present. Several aspecific bands are visible for each amplicon. After gel purification, it was checked whether the correct bands had been cut out, without contamination. As presented above, this was the case.

(Invitrogen, 2007). This did not yield any colonies. A new TOPO reaction was performed with the reaction volume as described by the manufacturer of the pENTR™/d-TOPO® kit (Invitrogen, 2007). Colonies were present on the plates. 10 colonies per construct were screened with a colony PCR using the pENTR™/d-TOPO® specific M13 forward primer and the gene specific reverse primer that is used in expression analysis (supplemental data).

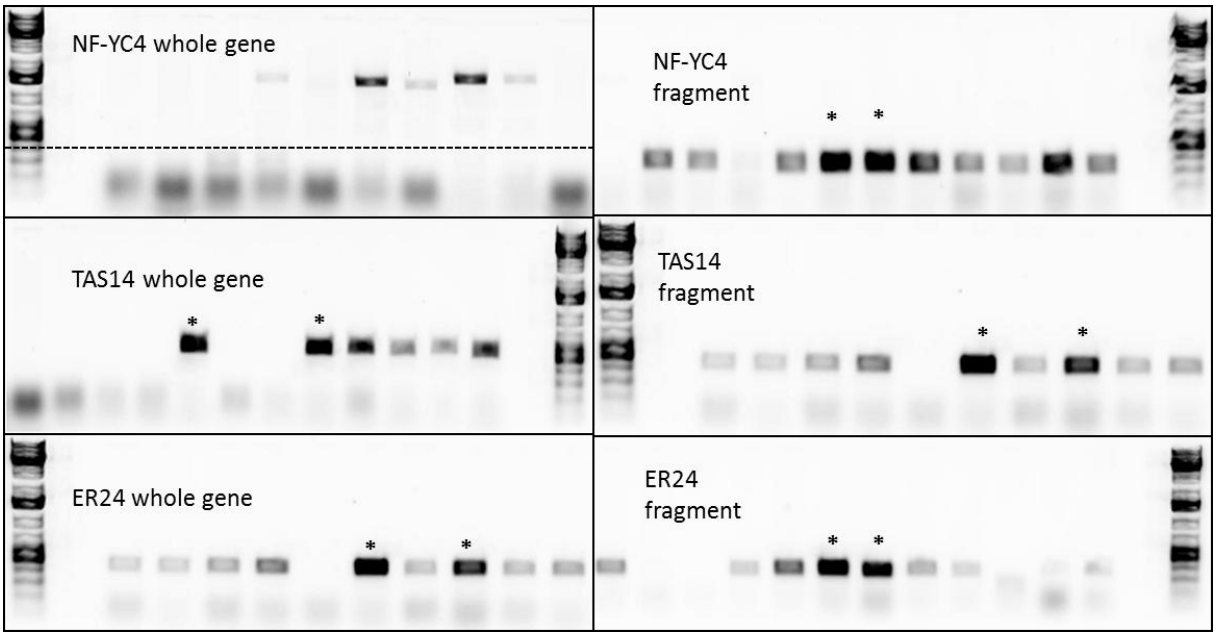


Figure 4.3 Colony PCR on the colonies transformed with pENTR™/d-TOPO®. Bands indicated with an asterisk represent the colonies that have been selected because their insert appears to be correct. Only for the whole gene amplicon of NF-YC4, such a colony has not been found.

Colonies with the expected product size were selected for all the amplicons, except for the colonies carrying the whole *StNF-YC4* gene (figure 4.3). For this amplicon, no colonies were screened that showed the right product. To achieve the *StNF-YC4* gene as well, a new PCR was performed. cDNA was not used as template but genomic DNA of the C and E genotype, which is possible because the *StNF-YC4* gene does not contain introns. Instead of multiple bands as in figure 1, a strong single band was present (data not shown). The PCR product was purified, and a TOPO reaction was performed using this gene product and *E. coli* cells were transformed. The resulting colonies were screened with the M13 forward primer and the NF-YC4 reverse primer used for expression analysis. Two colonies were selected that were positive for this primer combination. The plasmids of the selected colonies were isolated and submitted for sequencing using the pENTR™/d-TOPO® specific M13 forward and reverse primer (supplemental data). Details about the alleles that were found are given present in the supplemental data.

4.2.3 Cloning of the pER8 construct

The PCR with primers to amplify the genes NF-YC4, TAS14 and ER24 with restriction site overhangs (SpeI/SalI) resulted in bands of the correct size for all three genes (figure 4.4). Amplified gene products were present for NF-YC4 and TAS14. The PCR mix was purified using a PCR purification kit. There was no need for gel purification since contamination of the sample only consists out of bigger fragments. There are inserted more difficult than the fragments of the smaller, desired length. The amplified genes were digested and ligated into the pER8 vector. Only 2 colonies were present on the plate with TOP10 *E. coli* cells transformed with NF-YC, and 10 on the plate with TAS14 transformed cells. The plate with ER24 transformed TOP10 *E. coli* cells was empty. The plates with colonies were stored at 4°C. No further steps were taken with these constructs, to allow the cloning of the other constructs to be completed.

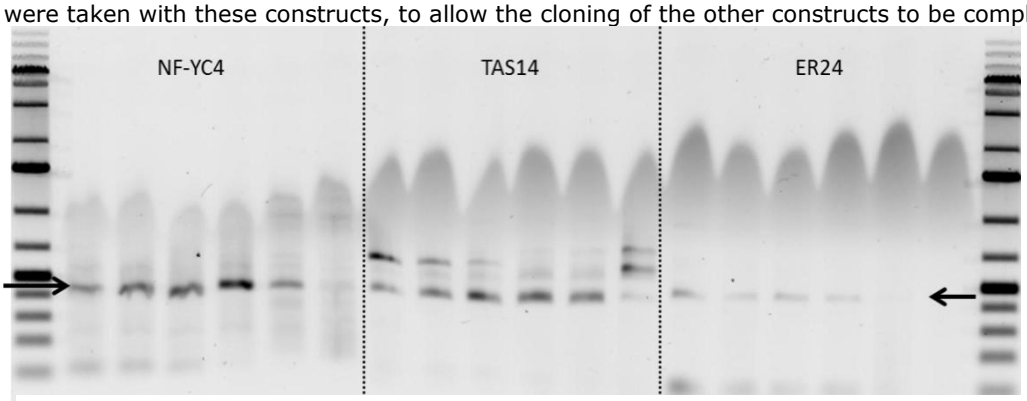


Figure 4.4 The products of a PCR with primers to amplify the genes NF-YC4, TAS14 and ER24 with restriction site overhangs (SpeI/SalI). The arrows indicate the height at which correct fragments are expected.

4.2.4 Recombination of the pENTR™/d-TOPO® vectors with the destination vectors

The LR reaction was performed to insert the genes or gene fragments of *StNF-YC4*, *StTAS14* and *StER24* in the destination vector. The reactions resulted in >50 colonies for each of the constructs. Different primer combinations were used to confirm insertion of the correct fragment into the vectors (table 4.3). Initially none of the plasmids that were cloned with the modified protocol using less LR clonase (Untergasser, 2006) showed a band that corresponded to the expected size (data not shown). After several tries and failures the original protocol (Invitrogen, 2004) was followed, which resulted in a few confirmed plasmids (figure 4.5). These were adopted by Marian Oortwijn, who continued with the rest of the constructs and transform them to *Agrobacterium tumefaciens.*

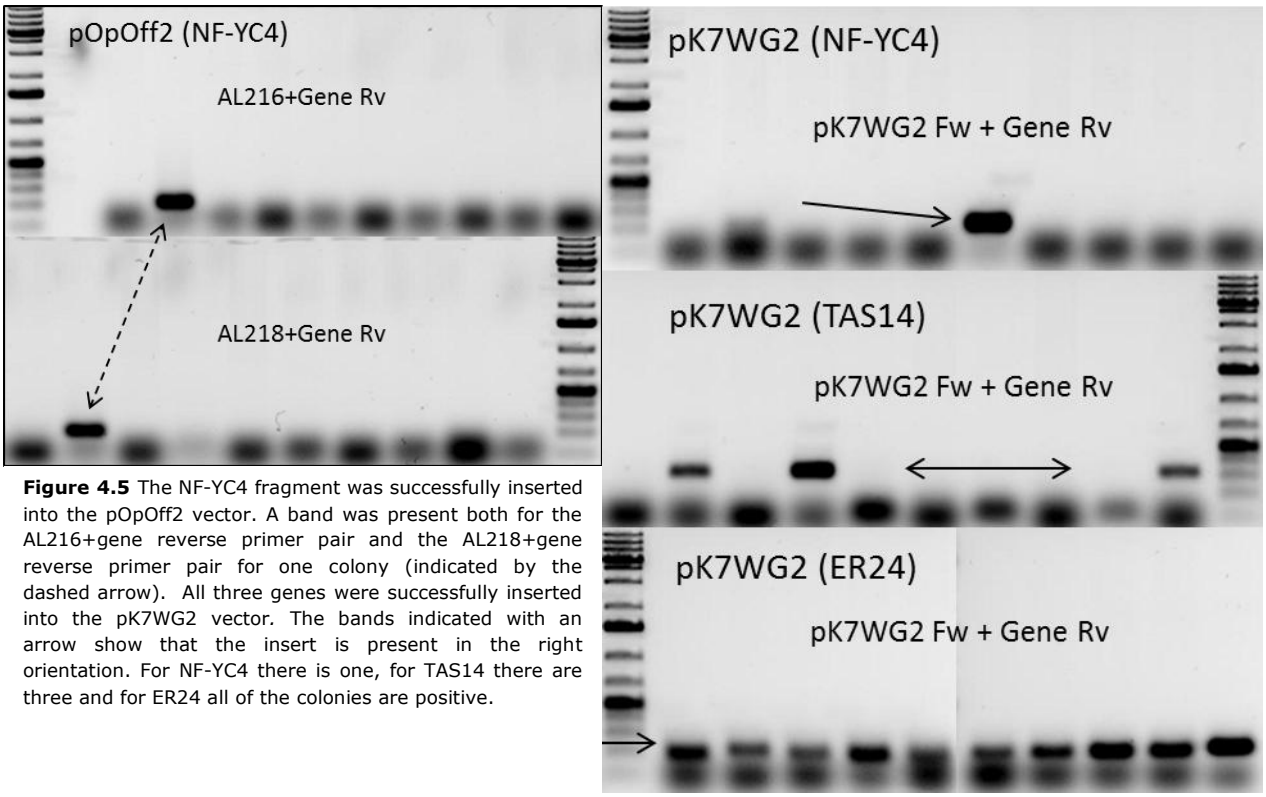


Figure 4.5 The NF-YC4 fragment was successfully inserted into the pOpOff2 vector. A band was present both for the AL216+gene reverse primer pair and the AL218+gene reverse primer pair for one colony (indicated by the dashed arrow). All three genes were successfully inserted into the pK7WG2 vector. The bands indicated with an arrow show that the insert is present in the right orientation. For NF-YC4 there is one, for TAS14 there are three and for ER24 all of the colonies are positive.

4.2.5 Further attempts to acquire the expression vector (Marian Oortwijn)

The LR reaction was repeated for the constructs that were not correct yet. Some constructs had a wrong orientation of the inserts, others only had one insert instead of two (figure 4.6). These were discarded and a new LR reaction was performed. Other constructs show a correct plasmid in *Escherichia coli* cells, but when they are transferred to *Agrobacterium tumefaciens* the insert was lost (data not shown). The transformation to *A. tumefaciens* had to be repeated. The vectors that were confirmed and stored as a glycerol stock at -80°C in the freezer are given in table 4.5.

Table 4.5 The vectors that are intact and present as glycerol stock in the freezer at -80°C are given in the table. The gene (fragment) that is present in the vector, as well as the type of cells as which it is present in the freezer, are given as well.

Vector	Gene (fragment)	Bacteria
pK7WG2	NF-YC4	*E. coli + A. tum*
pK7WG2	TAS14	*E. coli + A. tum*
pK7WG2	ER24	*E. coli + A. tum*
pHELLSGATE12	NF-YC4 (frag.)	*E. coli*
pHELLSGATE12	TAS14 (frag.)	*E. coli + A. tum*
pHELLSGATE12	ER24 (frag.)	*E. coli + A. tum*
pOpOff2(hyg)	NF-YC4 (frag.)	*E. coli*
pOpOff2(kan)	TAS14 (frag.)	*E. coli + A. tum*

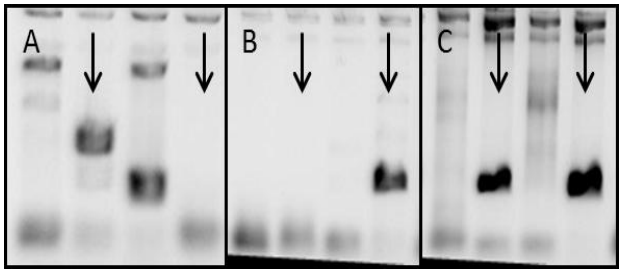


Figure 4.6 The figure presents examples of what results were found when the integrity of the silencing constructs was checked. Lanes in which the correct gene fragment should be present are indicated with an arrow. (**A**) One gene fragment is inserted in the right orientation. The second one is inserted the other way around, which causes the gene product to be smaller since the reverse primer is closer. (**B**) One insert is absent, whereas the other one is present. (**C**) Both fragments are inserted in the correct orientation.

4.3 Technical discussion

The amplification of the whole gene sequence of *StNF-YC4* did not work on cDNA. One could speculate that this is because of the low expression levels of *StNF-YC4* in the cDNA, but that is refuted by the success of the amplification of the silencing fragments. It is however well possible that the primers were not optimal. The forward primer had a melting temperature of 61.9°C, whereas the reverse primer had a melting temperature of 56.5°C. This is a big difference, which may affect the efficiency of the primers. The resulting low amount of produced NF-YC4 gene DNA might severely affect the effectiveness of the TOPO reaction. Amplification from the genomic DNA resulted in sufficient gene product for an effective TOPO reaction.

The insertion of the gene fragments into the pENTR™/d-TOPO® vector did not work at once. The protocol with half the reaction volume might work for other samples, but the fragments used here might not be suitable for this protocol. Using the original protocol solved this problem. However, because the modified protocol is less costly (half of the original protocol) I would not recommend to dispose of it and use the normal amounts further on. The modified protocol has proven to be successful in most of the cases (Untergasser, 2006). For the LR reaction however, the modified protocol was not successful at all. It uses half of the resources needed for the original protocol as well, but it also prescribed a shorter incubation time of one hour at room temperature (Untergasser, 2006). I would recommend to stick to the resources of this modified protocol, but to increase the incubation time to overnight at 25°C. This should work in most of the cases. The reason why the LR reactions did not yield many correct vectors, is unknown. The efficiency does not come near the efficiency as advocated by Invitrogen of >5000 colonies with the right insert (Invitrogen, 2004). However, because the reaction with pENTR™/d-TOPO® was problematic as well, the nature of the inserted fragments might be causing efficiency loss.

The transformation of vectors into *A. tumefaciens* led to complications as well. The loss of insert in *A. tumefaciens* may be caused by the fact that having a big plasmid is disadvantageous for bacteria. There might have been a strong selection in favour of bacteria carrying the vector without the insert. Eventually almost all constructs were made. The only constructs that still need to be made are the pER8 constructs.

5. Virus Induced Gene Silencing

The generation of transgenic potato plants exhibiting stable expression or silencing takes at least 6 months. Because this time is not available a faster alternative was used. The genes *StTAS14, StER24* and *StNF-YC4* have been silenced in *Nicotiana bethamiana* using the Virus Induced Gene Silencing (VIGS) technique. This allows me to get an impression of the effects of the absence of the genes within the timeframe of this thesis project.

5.1 Material and Methods

5.1.1 Plant material

Nicotiana benthamiana seeds were placed in a petridish with demi water containing gibberellic acid (0.5 g per liter). After absorption of the water the seeds were allowed to dry. The seeds were sown by placing them on the top of the soil. The conditions of the climate chamber were 23°C during day time and 19°C during night time, with 16 hours of light. After 10 days the plantlets were transferred to small pots with soil and placed in the greenhouse. In the greenhouse the temperature during daytime was 20°C, during night time 18°C. In the greenhouse there was 16 hours of light per day as well, with a humidity of 60%. The plants stayed under these conditions for the rest of the experiment.

5.1.2 pTRV is used for Virus Induced Gene Silencing

The destination vector that is used to perform the actual VIGS is pTRV (figure 5.1). The system is derived from the Tobacco Rattle Virus (TRV) that contains two positive sense single stranded RNA molecules (MacFarlane 1999). cDNA copies of these molecules have been inserted between the tDNA borders of the *Agrobacterium tumefaciens* Ti plasmid. pTRV1 is essential to encode the RNA dependent RNA Polymerase that generates double stranded viral RNA. This is targeted by the plant's endogenous RNAi system. pTRV2 carries a GATEWAY™ cassette for insertion of the gene fragment. Both of the vectors contain other proteins essential for successful infection of the plant, such as the coat protein and the movement protein of the TRV virus.

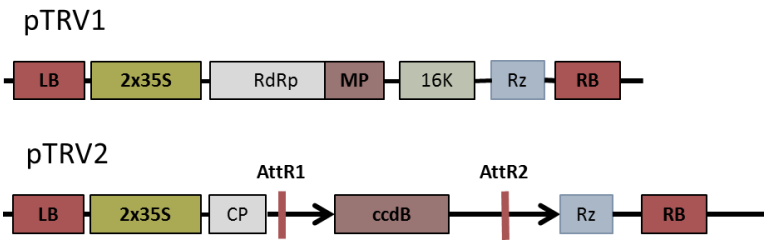


Figure 5.1. The pTRV system is used to perform VIGS. The system consists out of two vectors, pTRV1 and pTRV2. The pTRV1 vector contains the RdRp sequence which encodes both a helicase and a RNA dependent RNA polymerase. Furthermore a movement protein (MP) and a 16 kDa protein are encoded. The pTRV2 vector carries the insert. Next to that a coat protein is present. Together the two vectors can successfully infect the plant and spread the silencing.

5.1.3 LR reaction

The gene fragments of *StNF-YC4*, *StTAS14* and *StER24* were inserted into the pTRV2-Gateway vector by means of the LR-reaction, according to a modified protocol (A. Untergasser, 2006) as well as according to the original protocol (Invitrogen, 2007). As donor of the gene fragments the pENTR™/d-TOPO® vector was used (see section 4.1.3). An alternative approach avoided the problems of antibiotics selection by using a fragment of the vector: PCR was performed using the pENTR™/d-TOPO® specific M13 primers, which span the recombination sites AttL1 and AttL2 and the insert. The product was used as donor for the LR reaction. The LR-reaction mix was transformed to *E.* Coli DH-5α. The bacteria were plated out on LB plates with 50 µg/ml kanamycin or ampicillin and placed o/n in the stove at 37°C. Single colonies that were present on the plate were analysed in a colony PCR as described before and inoculated in 3 ml LB with 50 µg/ml kanamycin or ampicillin o/n at 37°C and 200 rpm. The primers used for colony PCR are the pTRV2 specific primers AL275 and AL276, the primers used for synthesis of the gene fragment and the pENTR™/d-TOPO® vector specific primers M13F and M13R. Combinations were made with the reverse primer of the fragment as well to confirm the insertion and it's orientation. When the intact pENTR™/d-TOPO® vector was used to do the LR reaction, both the gene specific primers were used, as well as the TOPO specific primers. The first one is to indicate the insert of the gene fragment, the second one to make sure that the colonies did not still contain the TOPO vector. The plasmid was isolated the day after and submitted for sequencing using the pTRV2 specific primer AL276 or a primer used for synthesis of the gene fragment.

5.1.4 Transformation into Agrobacterium tumefaciens

pTRV2 plasmids were transformed to *Agrobacterium tumefaciens* strain GV3101 with resistance to rifampicin. The heat shock procedure is the same as described before, but the recovery of the bacteria after the heat shock takes 3 hours at 28°C with 200 rpm. After recovery the bacteria were plated on LB plates with 3% agar and combinations of the antibiotics rifampicin (25-100 µg/ml), kanamycin (50 µg/ml) and ampicillin (50 µg/ml). The plates were inoculated at 28°C over the weekend, after which formed colonies were inoculated in 96 wells plates with LB and antibiotics. The plate was put at 28°C for at least 3 hours, and subjected to colony PCR. The same primer combinations were used as mentioned before for the pTRV2 vector. Colonies that were confirmed were inoculated in 3 ml LB with antibiotics.

5.1.5 VIGS

To prepare the cultures for VIGS, the protocol based on the protocol by Reinier van der Hoorn was followed (van der Hoorn, unknown). The constructs of the three genes of interest, as well as a PDS construct, a GUS construct and the pTRV1 construct were prepared. The PDS construct serves as a confirmation that the plant indeed is able to perform virus induced gene silencing by silencing the phytoene desaturase gene. Result of that is that the plant is no longer able to synthesize chlorophyll, thereby showing a bleaching phenotype. This result also confirms that the pTRV1 construct is intact, since this is responsible for the spread of the silencing. The GUS construct is included to provide control plants to compare with the plants that are targeted by the constructs carrying a gene fragment of interest. The constructs were inoculated in LB with kanamycin (50 µg/ml) for one day at 28°C with 200 rpm. The cultures were inoculated a day later in YEB medium after measuring the OD of the cultures at 600nm. The amount of culture that had to be added to the YEB medium was calculated with the following formulas:

$$z = \frac{8000}{2^{(\Delta t/\alpha)}} \qquad x = \frac{z/6.6}{OD}$$

In which Δt is the time in hours between inoculation and harvest of the cultures, α is 2 for GV3101 and 3 for AGL1, x is the amount of culture to add to 15 ml of YEB medium. 1.5 microliter of 200 mM acetosyringone, 150 microliter of 1 M MES and kanamycin (50 µg/ml) had to 15 ml YEB as well. The cultures were put at 28°C with 200 rpm o/n and centrifuged for 10 minutes at 4000 rpm the following day. The pellet was resuspended in 15 µl Minimal Medium A (MMA) with acetosyringone, which was prepared fresh on the same day. The OD was determined and the total amount of MMA to add to the pellet was calculated as follows:

$$Endvolume = {}^{OD}/_{0.3} \times 15$$

Each of the cultures was mixed 1:1 with pTRV1 and acclimatised for at least one hour at room temperature. The lower side of the *Nicotiana benthamiana* leaves was infiltrated with culture with a syringe without needle. For NF-YC4, TAS14, ER24 and GUS six plants were inoculated per construct. Only one plant was infiltrated with bacteria carrying the PDS construct. In total three sets of 25 plants were inoculated, with one week intervals.

5.1.6 Analysis of the plants

Only the first set of 25 plants was analysed because of time constraints. The *StNF-YC4* silenced plants, along with three controls, did not receive a drought treatment, but were watered throughout the experiment. For the *StTAS14* and *StER24* silenced plants a severe drought treatment was initiated 14 days after transformation of the plants. To synchronise the water in the pots at the beginning of the drought treatment, the pots were placed in trays with water for two days, and then the watering was stopped. The height of the plants was measured regularly throughout the experiment. The stomatal conductance was measured mainly for the *NF-YC4* silenced plants and their controls using the Decagon SC-1 Porometer. 12 days after initiation of the drought treatment, the chlorophyll content was measured for all the plants using a SPAD 502 PLUS Chlorophyll meter. The generated data was analysed using a one-way ANOVA to test whether the means of the different silenced genes was different. With a post hoc Fisher's LSD test, the silenced genes were compared pairwise. The *StNF-YC4* silenced plants were only compared between drought and control with an independent student's t-test. The *StTAS14* and *StER24* samples were compared with their controls using a one-way ANOVA.

5.2 Results

5.2.1 Attempt to insert the gene fragment into pTRV2 using the intact pENTR™/d-TOPO® vector

Different approaches were attempted to insert the gene fragment in the pTRV2 vector. The first approach was using the intact pENTR™/d-TOPO® vector in an LR reaction with the pTRV2 vector. The transformation to bacteria did not yield any colonies on selective media, most likely because the antibiotics used (Kanamycin, rifampicin and ampicillin together) were not correct. A new LR reaction was performed, where half of the transformed bacteria was plated out on plates containing ampicillin, the other half on plates containing kanamycin. The plates with ampicillin contained approximately 20 colonies per gene, where the kanamycin plates contained more than 200. A colony PCR was performed on 10 colonies for each gene (figure 5.2). For the ampicillin colonies the used primer pair (used for the synthesis of the gene fragment) did not give a product. For this reason the use of ampicillin in further cloning steps has been stopped. The colonies of the kanamycin plates were screened with two primer pairs and selected colonies sequenced. These contained the native pENTR™/d-TOPO® vector. Therefore, new LR reactions were performed and a new stock of pTRV2 was used to try to get the correct pTRV2 constructs. For the colony PCR of the LR recombined vectors the primer pair AL275-AL276 was used in combination with the gene specific primers, since these can indicate the presence of the pTRV2 vector. Per gene 32 colonies were screened with these primers. Three colonies per gene that showed a band with the AL275-AL276 primers were selected (figure 5.3). Of these colonies the plasmid was isolated and checked with a PCR using the same primers as for the colony PCR (figure 5.4). All the colonies had a band corresponding to the band of the pTRV2 vector that was included as a positive control. Almost all the colonies had a band representing the gene fragment too. However, a second band was present as well. The plasmids were transformed into *A. tumefaciens* GV3101 cells and plated on LB with kanamycin and with a reduced amount of rifampicin. The formed colonies were screened with the colony PCR as before. This time the pTRV2 specific primers were not positive (data not shown). The gene however did show a single band of the right size, indicating presence of the native pENTR™/d-TOPO® vector. Therefore, we decided to use another approach to insert the gene fragment into pTRV2.

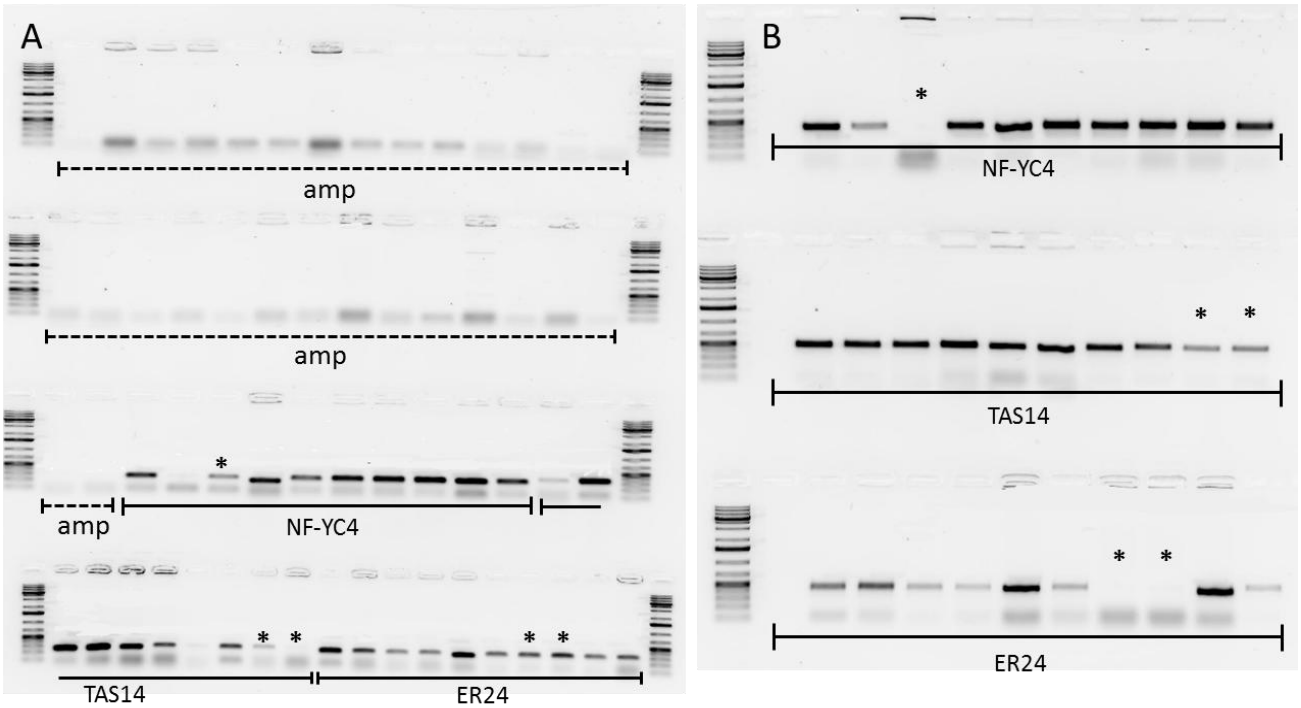


Figure 5.2. On the gel are the products of a colony PCR on the *E. coli* cells transformed with pTRV2. (**A**) Gene specific primers have been used to prove the presence of an insert. The dashed line represents the colonies that have been plated out on plates with ampicillin. Only primer dimer is present there. The uninterrupted lines represent the colonies that were plated out on plates with kanamycin. (**B**) To make sure that the colony found on the LB plates with kanamycin is not the unrecombined pENTR™/d-TOPO® vector the primer pair M13Fw and an internal gene specific reverse primer was used. This amplifies part of the TOPO vector with the insert. Colonies that were positive for the first primer pair and negative for the second primer pair were selected, indicated with an asterisk (*).

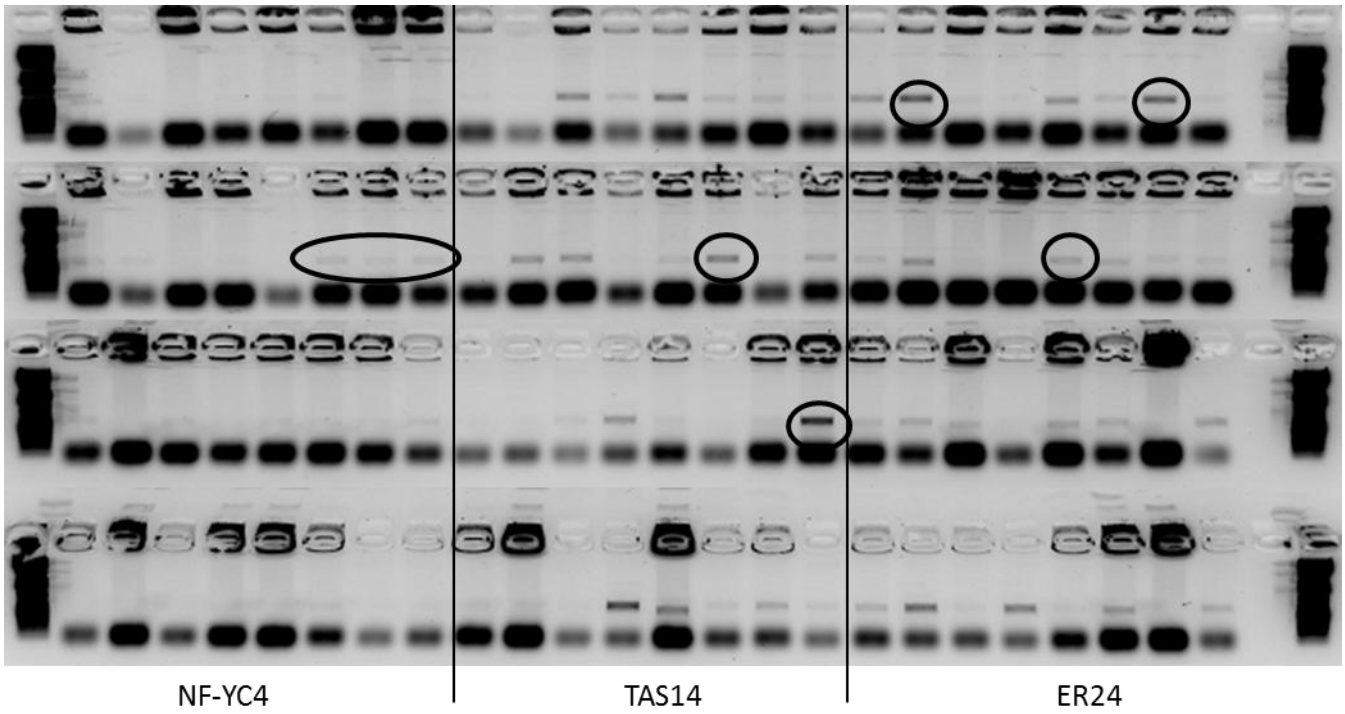


Figure 5.3 A colony PCR was performed on transformed *E. coli* with the pTRV2 vector specific primers AL275 and AL276. Per gene three colonies were selected that showed a band, which are indicated by a circle.

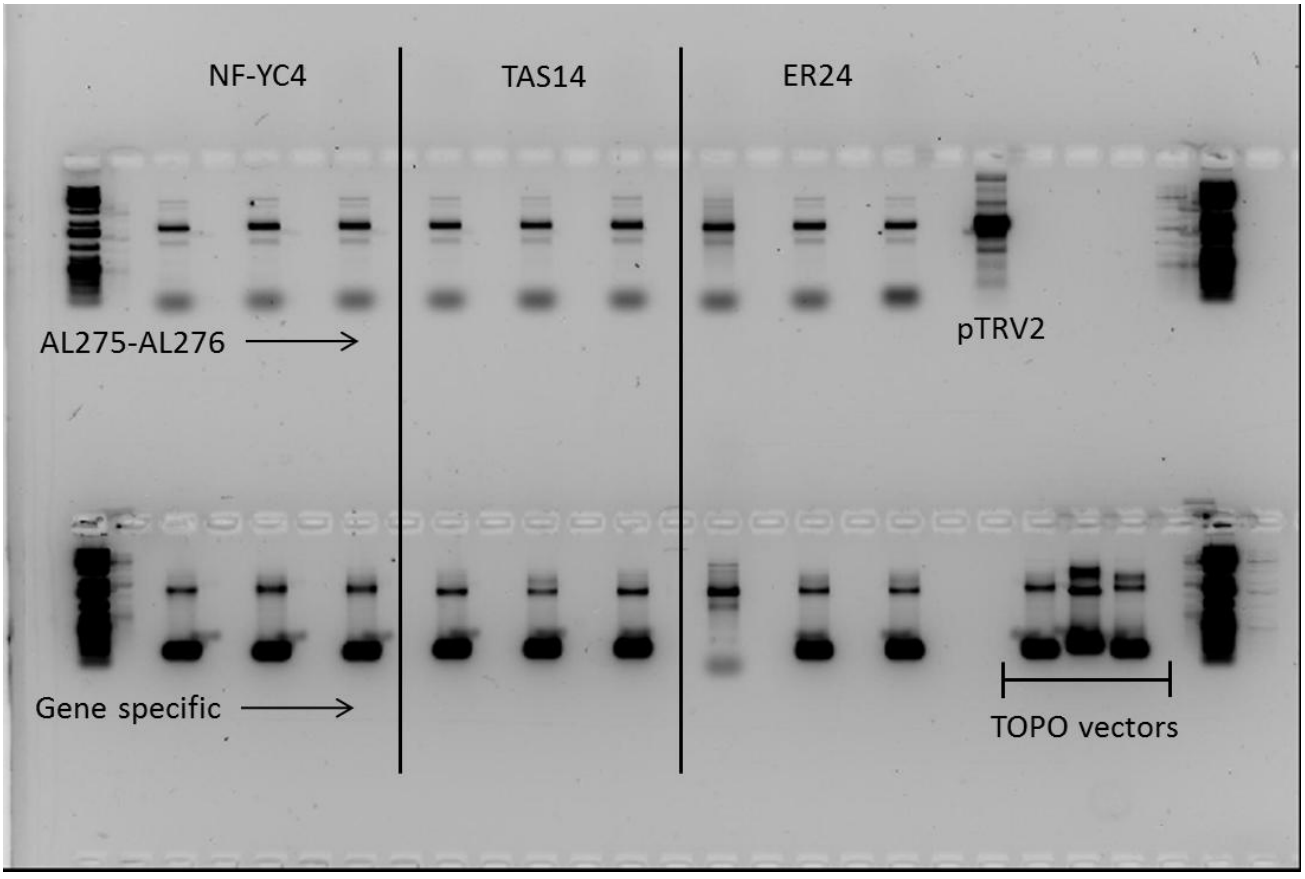


Figure 5.4 A colony PCR was done on transformed *A. tumefaciens* colonies with the pTRV2 specific primers AL275/AL276 and the gene specific primers designed for expression analysis. As a positive control the pTRV2 vector was included for the first primer pair and the TOPO vectors for the second primer pair. A band of the correct size was present in all colonies for the pTRV2 vector. For the other primer pair two bands were present for each colony. The lower one indicates the insert, where the upper one is unknown. This band is present for the positive controls as well.

5.2.2 Insertion of the gene fragment using a fragment of the pENTR™/d-TOPO® vector

The M13F and M13R primers were used to amplify a fragment containing the recombination sites and the insert (figure 5.5). This fragment contains all the essential elements for successful recombination, and eliminates the presence of colonies with pENTR™/d-TOPO® on the plates. The LR reaction mix was transformed into chemically competent *E. coli* and plated out on plates with kanamycin. Eight colonies per gene were screened with a colony PCR using the primer pairs AL275-AL276; Gene Fw-Gene Rv; AL275-Gene Rv and AL276-Gene Rv (figure 5.6). Bands are expected for all the primer combinations, except for AL275-Gene Rv. Of the colonies that met these conditions two were used to transform *Agrobacterium tumefaciens*. Approximately 100 colonies per gene were present on the plates inoculated with these bacteria. A colony PCR was done with the AL276 primer and the reverse of the gene specific primers. All colonies were positive (data not shown). The constructs were prepared for VIGS and *Nicotiana benthamiana* plants were infected.

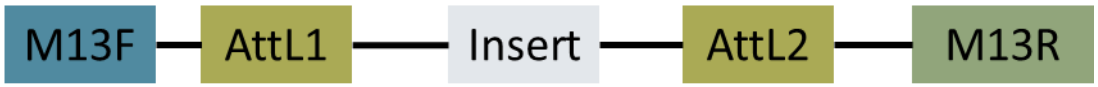


Figure 5.5 A fragment of the pENTR™/d-TOPO® vector was amplified using the M13F and the M13R primer. The fragment contains the recombination sites AttL1 and AttL2, as well as the insert.

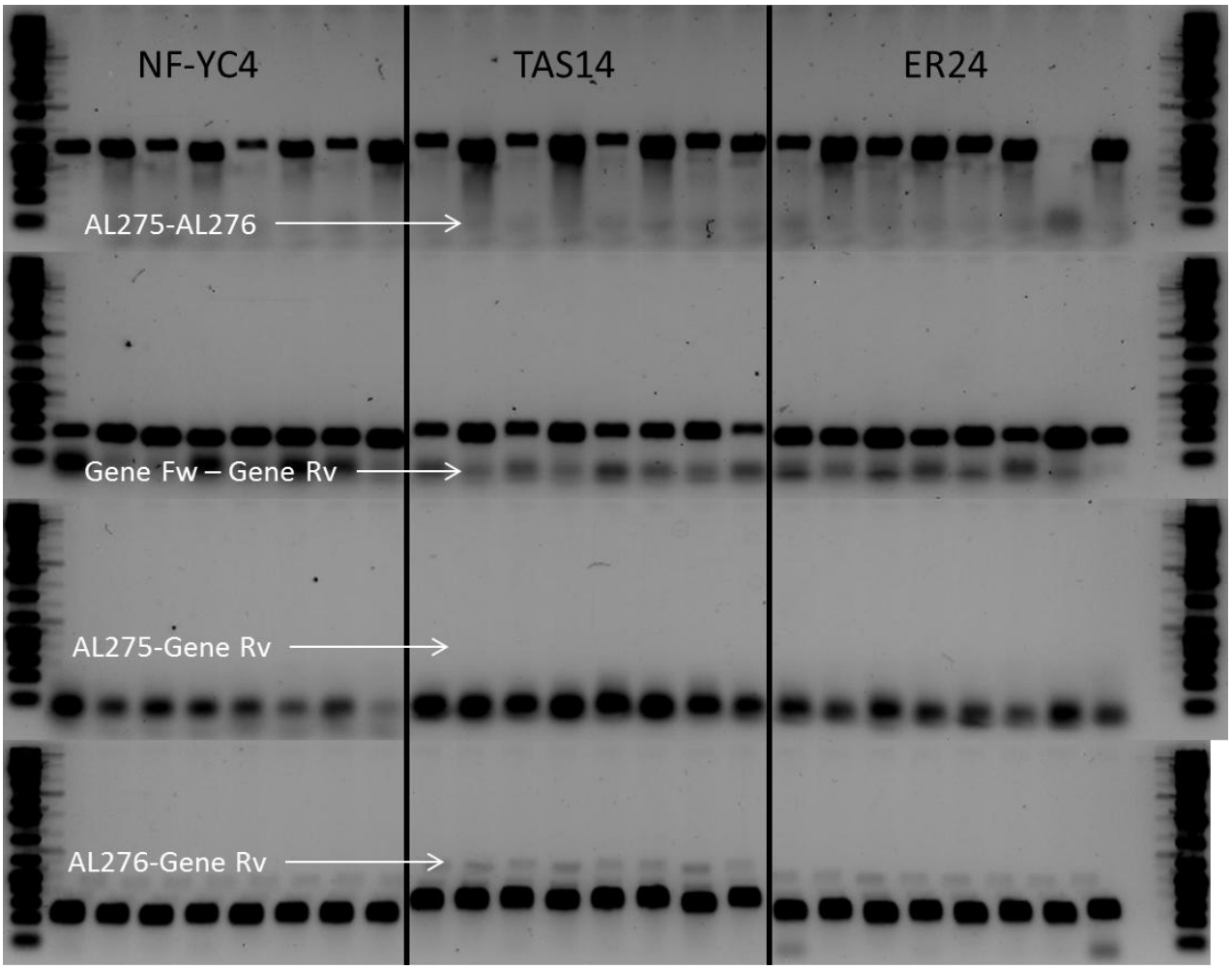


Figure 5.6 Four primer pairs were used to perform a colony PCR on the *E. coli* transformed with pTRV2 generated with the TOPO fragment. The primer pair AL275-AL276 is pTRV2 vector specific. A band is visible for almost all of the constructs. The second primer pair consists out of the gene specific primers used for synthesis of the gene fragment. A band is present for all of the colonies. The third pair, AL275 with the reverse of the genes, is not supposed to give product when the fragment is inserted in the correct orientation. Indeed, no product is present, only primer dimer. For the fourth primer pair, AL276 with a reverse of the gene, is showing product for all of the colonies.

5.2.3 Observations on the transiently silenced plants

During the experiment, the stomatal conductance increased as the plants grew bigger (figure 5.7). There was no difference in the conductance between control plants in which GUS was silenced and plants in which *NF-YC4* was silenced, except on the 28th of February. For the plants in which the other two genes were silenced, two measurements were taken at 12 and 14 days after initiation of the drought treatment. Stomatal conductance is decreased in the drought stressed plants and further decreases as the drought continues to stress the plants. No significant difference was found between the *StTAS14* and *StER24*, or between these plants and their control.

All the plants that were subjected to the drought stress were impacted by the drought in the same way. 14 days after initiation of the drought treatment wilting started to show. Both the plants in which the TAS14 or ER24 genes were silenced, as the control plants, had the same degree of wilting. The leaves had lost their turgor, but were still green.

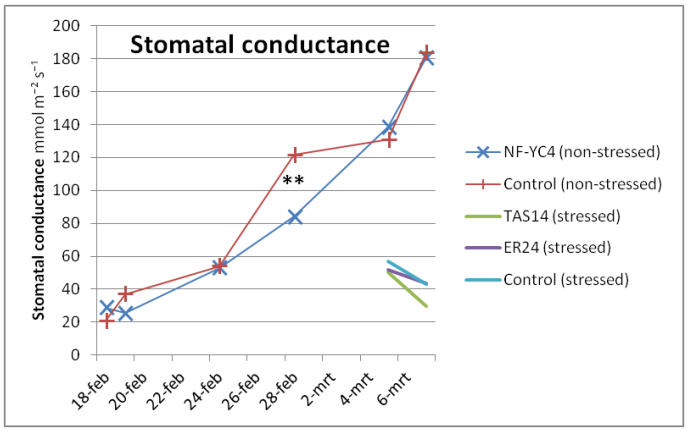


Figure 5.7 The stomatal conductance of the plants has been given for the NF-YC4 silenced plants and the corresponding controls. A pattern is visible where the conductance increases over time, as the plants grow. Although there are fluctuations in the difference between the plants, the overall pattern is the same. Only for the measurement on the 28th of February there is a significant difference ($p<0.01$). For the TAS14 and ER24 plants, along with the controls that did not receive water, the stomatal conductance has been measured twice, 12 and 14 days after initiation of the drought treatment. No significance has been found between these genotypes.

The chlorophyll content was measured 12 days after initiation of the drought treatment. No significant differences were found between well-watered and drought stressed plants.

The length of the stems of the plants was mainly measured for the *StNF-YC4* silenced plants (figure 5.8). The difference between *StNF-YC4* and the control plant was found to be significant only on the 28th of February and the 4th of March. Besides that, the overall appearance, i.e. the leaf size, of these plants was smaller. The height was measured for the drought treated plants as well, but no significant differences have been found.

To see whether the silencing has indeed spread in the plant, a plant was included in which the PDS gene was silenced. This plant showed bleaching throughout the experiment. On the 14th of March however, it was seen that the silencing began to break (figure 5.9).

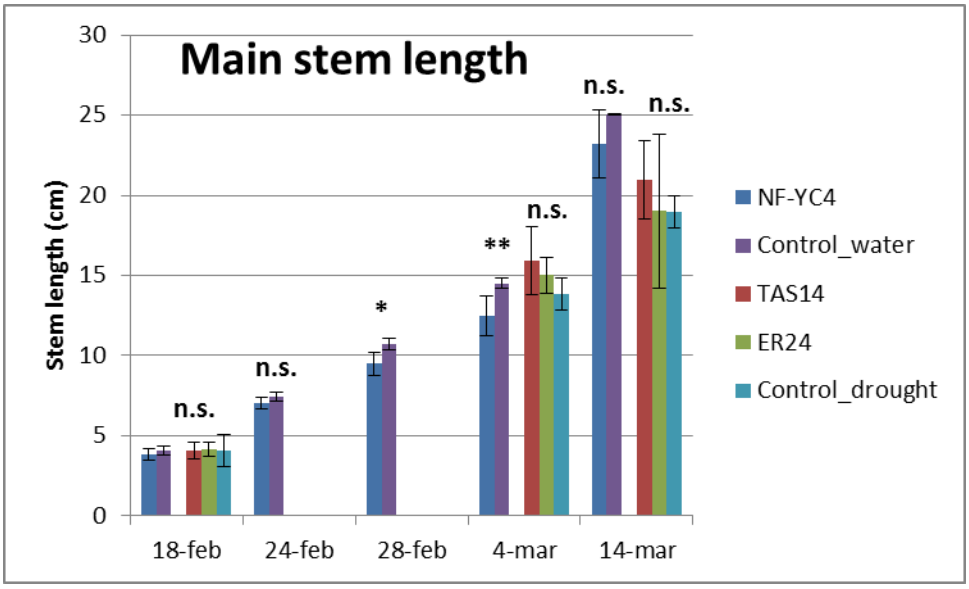


Figure 5.8 The height of the plants is plotted against the date. Initiation of the drought treatment occurred at the 21th of February. The heights are not significantly different, except for the difference between *StNF-YC4* silenced plants and their controls on 24 February ($p<0.05$) and 28 February ($p<0.01$).

Figure 5.9 The plant in which the PDS gene was silenced showed a bleaching phenotype throughout most of the experiment. On the 14th of March however, green spots began to show that indicate the silencing began to break.

5.3 Technical discussion

The generation of the constructs aimed at transient silencing in the *Nicotiana benthamiana* plants did pose some challenges. When the intact pENTR™/d-TOPO® vector was used to insert the gene fragment into the pTRV2 vector, the absence of a good selection marker caused overrepresentation of untransformed vectors on the plates. As a result correctly transformed pTRV2 was difficult to find. Some colonies were found that showed a band when they were screened with gene-specific primers (figure 5.2A), while they showed no or a faint band with the TOPO specific primers (figure 5.2B). Sequencing pointed out that the vectors still were native TOPO vectors. This can only mean that the PCR with the TOPO primers failed. Unfortunately no positive control was included, because the primers for the pTRV2 vector were not available at that time.

The second attempt with the intact pENTR™/d-TOPO® failed as well, although both the insert and the pTRV2 vector appeared to be present in E. coli. Possibly the vector was pENTR™/d-TOPO®. The false positive probably was caused by contamination in the samples, indicated by the presence of an unexpected PCR product at the same height in all samples. The height corresponds to the positive control of the pTRV2 vector. Therefore, the contamination probably was the pTRV2 stock solution.

The solution was found by using an amplified fragment of the pENTR™/d-TOPO® vector. So hereby I suggest to use this approach in the future for recombination of a gene fragment into the pTRV2 vector. The extra time it takes to do this is mostly less than 4 hours, since the extra step to take is only a single PCR with purification. It certainly saves time because there is no need to screen dozens of colonies in the hope of finding a colony that does not contain the pENTR™/d-TOPO® anymore.

Performing the VIGS on *Nicotiana benthamiana* was not an ideal approach to characterize the function of *StNF-YC4*, *StTAS14* and *StER24.* Although there was enough homology between the genes in potato and *N. benthamiana* to enable successful silencing, these plants are quite distinct from each other. Furthermore, a second homolog of the *NF-YC4* gene is targeted in *N. Benthamiana* (Bombarely, 2012, *N. benthamiana* Nb-1 genome v.0.42). This increases the chance of encountering effects in the plants that cannot be contributed to the silencing of the actual target gene. The observation that the *NF-YC4* plants appeared smaller therefore cannot be attributed to *NF-YC4* alone.

No differences were observed between the plants with the silenced genes *StTAS14* and *StER24* and their controls. It is well possible that there are some effects that are not reflected by the measurements during the treatment. It is therefore interesting to assess the recovery of the plants after a drought treatment. Furthermore, an electrolyte leakage assay can be applied during the drought treatment to assess the Cell Membrane Stability (CMS; Blum, 1981). Instead of a drought treatment on the whole plant level, 1 cm leaf discs can be used to characterize the drought tolerance properties of the silenced plants. These leaf discs sustain silencing for more than 6 weeks on Callus Induction Medium (Ramegowda, 2013) and are therefore suitable for such assays. These leaf discs can be stressed in several ways, to elucidate the mode of action of the target genes. The effect of the silencing of the genes on osmotic stress can be tested by treating the leaf discs with PEG (Verslues, 2006). A menadione treatment can be used to study the response to oxidative stress (Ramegowda, 2013). These alternative ways to measure the drought tolerance of the silenced plants might show effects unnoticed by the measurements that were done.

6. General discussion and conclusion

The expression patterns as were found by Dennis van Muijen (van Muijen, 2013) in the dataset provided by Anithakumari (Anithakumari, 2011) were confirmed in this thesis. The plants that received severe drought stress, as was the case in the experiments of Anithakumari, showed down-regulation of *StNF-YC4*, whereas both the *StTAS14* and *StER24* genes were up-regulated.

NF-YC4

The *StNF-YC4* gene encodes a subunit of the trimeric NF-Y complex (Petroni, 2012). Each of the subunits (A, B and C) is encoded by ~10 genes, which means the transcription factor complex has ~1000 different potential compositions. This allows fine-tuning of the transcription of genes for specific environments, stresses and developmental phases (Petroni, 2012). The *StNF-YC4* gene showed a 6.25 fold down-regulation in most of the genotypes after 4 days of severe drought stress compared to well-watered plants. For the samples harvested after 9 days of stress, this down-regulation was only 2.3 fold. *StNF-YC4* was therefore down-regulated upon drought perception, but the extent to which it was down regulated after 4 days is not necessary to sustain the drought response. *StNF-YC4* could act as a trigger to initiate the reprogramming of the cell towards the drought response. The mode of action then would be different than that of the subunit found in Arabidopsis, *AtNF*-YA5; the upregulation of this subunit was induced and increased as the drought persisted (Li et al., 2008). The *StNF-YC4* factor was found as a putative regulator of the genes found in the eQTL hotspot on chromosome 5. Among the genes of which the expression mapped to this eQTL locus were the *StNF-YA3* and *StNF-YA5* subunits. The down-regulation of *StNF-YC4* may up-regulate these genes via compositional change of the NF-Y complex (figure 6.1). These subunits then are responsible for yet another compositional change of the complex. The two A subunits of the NF-Y complex were found as central regulators of the drought response as well (van Muijen, 2013). Therefore it is well possible that the down-regulation of *StNF-YC4* mainly triggers the pathway, whereas the other subunits (such as *StNF-YA5*) or even other transcription factors sustain the drought response.

Of the NF-YC subunits that were present on the micro-array as used by Anithakumari, no candidates were found to replace the down-regulated *StNF-YC4* subunit. Two reasons can be imagined to explain this, each with different implications. The first reason might be that the NF-YC subunits replacing the C4 subunit are already present in the cell. It could be that these are outcompeted by the C4 subunit and can only become part of the NF-Y complex in the absence of NF-YC4. The second possible reason that no other NF-YC subunit was found up-regulated is that not all NF-YC subunits were present on the microarray. Therefore, another C subunit might be up-regulated that has not been detected so far. The second reason is well possible, since only two other NF-YC subunits were present on the microarray, where most plants have as much as ~10 variants of each subunit (Petroni, 2012).

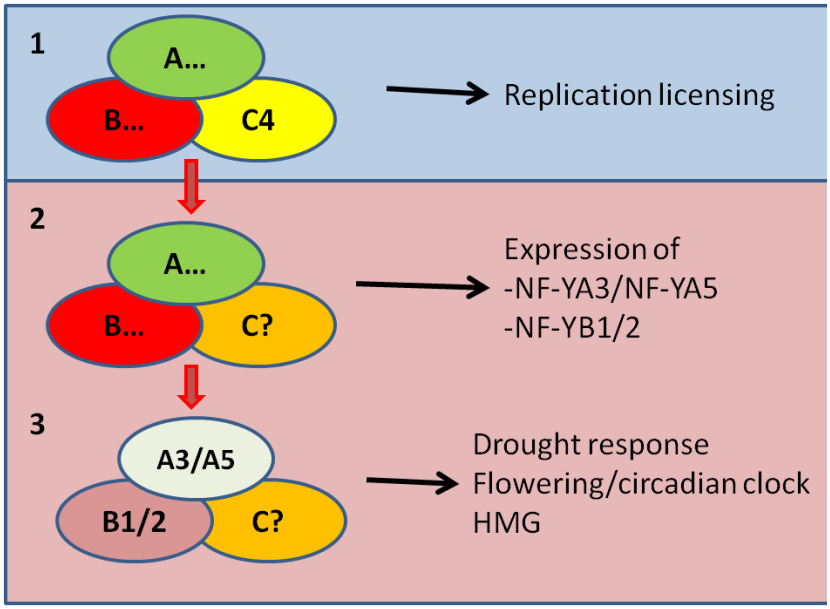


Figure 6.1 A revision of the model proposed by Dennis van Muijen (figure 1.9). **(1)** Under well-watered conditions the NF-Y complex with the NF-YC4 subunit regulates the genes for proliferation of the chromosomes prior to cell division. Upon drought perception, this function lapses because the NF-YC4 subunit is replaced by an unknown other NF-YC subunit. Either this other unit is up-regulated by drought, or the down-regulation of *StNF-YC4* allows the replacing constitutively present subunit to become part of the NF-Y complex. **(2)** The compositionally changed NF-Y complex without the NF-YC4 subunit drives expression of *StNF-YA3, StNF-YA5* and *StNF-YB1* or *StNF-YB2*. This leads to yet another compositional change. **(3)** The NF-Y complex with either NF-YA3 or NF-YA5, NF-YB1 or NF-YB2 and an unknown NF-YC subunit reprograms the plant towards the drought response.

The plants in which the gene was silenced were significantly smaller than the control plants, 21 and 24 days after infiltration of the plants. However, no wilting, no difference in chlorophyll content and no difference in stomatal conductance were observed. No expression analysis was done to confirm the silencing of the NF-YC4 gene in *Nicotiana benthamiana.* The fact that the plants appear to be smaller than the control plants, indicates that silencing of the target gene has an effect in the plant. Assuming that the *NF-YC4* gene was successfully

silenced, this did not lead to a drought response.

Under mild stress no differential expression was found of *StNF-YC4*. This could mean that for tolerance to mild stress down-regulation of *StNF-YC4* is not the strategy that is employed by the plant. However, the time of sampling for this dataset was 14 days after initiation of the stress. This is 10 days later than the sampling time at which the down regulation of *StNF-YC4* was most distinct under severe drought stress. Since the expression for the severely stressed samples appears to return to control values as well, this does not exclude the possibility of *StNF-YC4* being involved in mild stress too. However, the response of a plant to drought stress mainly acts to minimize the water loss by closing stomata and accumulation of solutes to lower the osmotic potential (Hsiao, 1973; Bray, 1993). Severe drought demands a more rigorous approach (Xiong, 2002), for which complete reprogramming of the plants' expression profile is necessary. For a relatively subtle adjustment to cope with mild drought, this complete reprogramming is not necessary. This might be the reason that down-regulation is not observed as is with the severely drought stressed plants.

Based on the confirmed expression profile of *StNF-YC4* and literature, the model proposed by Dennis van Muijen (figure 1.9) is revised. In the previous model, the NF-Y complex with the NF-YC4 subunit actively represses the drought response. Down regulation of *StNF-YC4* causes a compositional change of the NF-Y complex that is the signal for the drought response and the inhibitor of replication licensing. In the revised model (figure 6.1), the drought response is initiated by compositional change of the NF-Y complex, driven by initial substitution of the NF-YC4 subunit with an unknown up-regulated NF-YC subunit. The substitution of the C subunit drives expression of the NF-YA3 and NF-YA5 subunits, of which the expression linked with an eQTL to the *StNF-YC4* gene. Possibly a NF-YB subunit is up-regulated as well, since only three of these were present on the microarray. A good candidate for this is either the *StNF-YB1* or *StNF-YB2* gene, since these were found in respectively *Arabidopsis thaliana* and *Zea mais* (Nelson, 2007). Together, the NF-Y A, B and C subunits reprogram the plant towards the drought response. Furthermore, the ability of the NF-Y complex to assist CONSTANS in binding the promoter of flowering locus T is utilized to promote flowering (Cao, 2014). The reprogramming of the genes involved in the drought response is aided by the up-regulation of HMG, which fine-tunes transcription in response to sudden changes in the environment (Bianchi, 2005).

TAS14

The involvement of *StTAS14* in the drought response in potato has been indicated by several studies (Kappachery, 2013; Van Muijen, 2013; Munoz-Major, 2012). The gene encodes a dehydrin, which lowers the osmotic potential and probably protects cellular membranes through sugar accumulation. The *StTAS14* gene was up-regulated in all drought conditions. Severe drought stress increase the normalized expression of *StTAS14* under control conditions from close to 0 to 100-200. One genotype had normalized expression exceeding 400, which was CE608. This genotype however was not part of the recovering or re-germinating genotypes. The ones that did (Eos; Great Scot; Premiere; CE733) did not show expression deviating from the other genotypes. So based on the normalized expression of *StTAS14*, it is not possible to predict the drought tolerance of the potato plant. For each of the genotypes however, the *StTAS14* gene is employed in the drought response.

Although the *StTAS14* gene seems to involved in the response of potato to drought stress, no phenotype was observed with the drought-stressed plants that received a VIGS treatment to silence the gene. The main difference for the plants that were grouped based on their expression profile in the analysis of Dennis van Muijen was that they had the ability to recover very well, along with other traits such as early wilting. No early wilting was observed for the plants in the VIGS treatment. Furthermore, no recovery experiment was done since the time was limited. At the end of the experiments the plants that did not receive water looked severely stressed. The leaves had lost their turgor, but were still green. In the experiments of Anithakumari, the plants were stressed until the leaves showed senescence. Considering the putative mode of action, the stress that the plants received in this experiment was not enough to show a clear phenotype. To do so, the plants need to be stressed to the extent that the recovery of the plants is compromised. This means that there should be at least some visible senescence.

ER24

The expression of the *StER24* gene showed a characteristic pattern. After 4 days of stress, the expression was increased 46.8 fold on average, whereas after 9 days there was no significant differential expression compared to the watered controls for most of the samples. On average, the *StER24* gene was still expressed 3 fold more in the drought-stressed plants than in the well-watered plants. The expression of *StER24* in the investigated samples confirms the involvement of the gene in the drought response. Since the *StER24* is important for the heat shock response (Suzuki et al., 2011; Bechtold et al., 2013; van Muijen, 2013) its function might be to establish the heat shock response aimed at protection of proteins against damage induced by the effects of drought. Either the heat shock proteins sustain

themselves independent of *StER24*, or they are only needed as an early response mechanism. However, since proteins in the plant will be damaged more and more as the drought persists, the need for heat shock proteins remains (Wang, 2004). Therefore a role as initiator of the heat shock response seems most likely.

Considering a role as initiator of the heat shock response, it is interesting to see that at the moment the *StER24* protein is returning to levels comparable to control conditions under severe drought, the *StER24* gene still seems to be up-regulated. However, the normalized expression value of these samples is much lower than the ones found under severe drought. After 4 days of severe drought this ranged between 4 and 13, after 9 days between 0.2 and 3, whereas in the investigated heat stress samples this does not exceed 0.18. Certainly heat shock proteins are still needed after 10 days of heat stress, since the conditions in the cell will be detrimental for proteins. Possibly the *StER24* gene has initiated the heat shock response under heat stress conditions as well, but this cannot be proven based on the expression results of this thesis. Sampling after 4 days, as was done under severe drought, might show the involvement of *StER24* under heat as well.

As was the case with *StTAS14,* one genotype (CE608) showed higher expression than the rest of the genotypes. No specific phenotype can be linked to this, since CE608 did not recover particularly well or bad. Furthermore, no phenotype was observed with the VIGS experiment. The plants showed drought stress, but no differences were observed. Since the gene should possibly initiates the heat shock mechanism, its role is mainly to protect proteins in the cell against damage. The drought stress might have not been enough to induce the damage needed to visualise differences between plants where *StER24* is silenced and plants where it is still being expressed.

7. Recommendations

This thesis was aimed at gaining insight in the role of the three candidate genes *StNF-YC4, StTAS14* and *StER24*. The expression profile as was found in the experiments of Anithakumari by Dennis van Muijen (Anithakumari, 2011; van Muijen, 2013) has been confirmed. The transient silencing experiment that was performed using Virus Induced Gene Silencing did not yield any interesting phenotypes. However, constructs have been designed and made aimed at the formation of transgenic potato plants in which the genes can be expressed or silenced stably or inducible. Recommendations based on the proceeding of this thesis, as well as based on literature, are listed below.

- Both for the *StNF-YC4* and the *StER24* genes there appears to be a pattern in which the genes are respectively up-regulated or down-regulated shortly following the initiation of severe drought stress. As the stress persists, the expression appears to return to the expression under control conditions. To prove this, more expression analysis can be recommended using more samples and multiple time points later than 9 days after initiation of stress as well.

- Whereas the *StNF-YC4* gene seems to be down-regulated only temporarily, literature suggests that at least the *NF-YA5* gene is overexpressed throughout the whole duration of drought stress in *Arabidopsis thaliana* (Li et al., 2008). Since the *StNF-YA5* and *StNF-YA3* transcripts map to the eQTL on chromosome 5, and because they both appeared as a central regulator of the drought response (van Muijen, 2013), it would be interesting to see whether this is the case for these subunits in potato as well.

- As a follow-up for the previous recommendation, I recommend the generation of overexpression constructs for the *NF-YA* subunits mentioned, if they indeed show the patterns of increasing upregulation. These might form a target for the formation of transgenic plants, or as a breeding target.

- With the down-regulation of *StNF-YC4*, a gap needs to be filled in the trimeric NF-Y transcription factor. It could be that either subunits that were already present fill this gap, or that the transcription is initiated of another NF-YC subunit. Therefore I would recommend to list the different NF-YC genes and do expression analysis with these, since most of the NF-YC subunits were not present on the microarray.

- A putative mechanism of the drought response following the down-regulation of *StNF-YC4* is that the NF-YC4 subunit makes place for another one. This then would initiate the chain of events leading to the drought response. Artificially maintaining the expression of *StNF-YC4* using the provided stable constructs might postpone the drought response. Furthermore, induced silencing of the *StNF-YC4* gene could induce the drought response. A time series could be done, using the provided inducible silencing vector and the overexpression vector, in which the gene is silenced constitutively, two weeks before initiation of the drought, at the moment of drought initiation and maintaining the expression of the gene. This time series could provide information on the mechanism by which the down-regulation of *StNF-YC4* regulates the drought response.

-With the mild stress experiments the differential expression of *StNF-YC4* and *StER24* was not, or hardly, detected 14 days after initiation of the stress. Possibly this is because mild drought stress demands another approach than severe drought stress, as discussed before. A pattern was detected in which the expression of the genes returned to the expression value of control samples as the severe drought stress persisted. Therefore I recommend expression analysis of the genes shortly following the stress initiation, for instance after 4 days of stress. This might show that with mild drought stress the genes do indeed not play a role, or that the expression is much higher.

-Both for the *StTAS14* and the *StER24* genes overexpression I recommend to use the overexpression vectors that were made during this thesis. For the TAS14 gene it was found that overexpression led to improved drought and salt tolerance in tomato (Munoz-Mayor et al., 2012), whereas for the ER24 homolog MBF1c it was found that overexpression increased the tolerance of *Arabidopsis thaliana* to osmotic stress, abiotic stress and heat stress (Suzuki et al., 2005). Both genes appear to improve the tolerance to multiple stresses, so they are interesting targets indeed. To circumvent any pleiotropic disadvantageous effects, an inducible expression vector could be used. In this thesis I have attempted to produce such constructs, but these still need to be finished.

-As overexpression of *StTAS14* and *StER24* could increase the tolerance of *Solanum tuberosum* plants, the silencing should then decrease the tolerance. Therefore I recommend to use the constitutive silencing vector that has been made.

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9. Supplementary material

9.1.1 Used primers

Table 9.1 The primers that were used in the experiments as described in chapter 2 are listed below. In bold is the forward primer that was mainly used in combination with the different reverse primers.

Primers for confirmation of the UTR			
Target	**Pair**	**Sequence 5' -> 3'**	**Product length**
PLE1	Fw 1	TGAAAGATCACTCCCCGGAAG	78
	Rv 1	AGAGCAGGTGCAATCGACTC	78
	Fw 2	AGCTGAATTCACCCATCTCC	115
	Rv 2	TCTCAGAGCAGGTGCAATCG	115
	Fw 3	CCAGAGCTCAACTGCATCAAG	92
	Rv 3	CCTTCCGGGGAGTGATCTTTC	92
	Fw 4	AGAGTCGATTGCACCTGCTC	216
	Rv 4	TTTTGAAGCCGCTGAAGCTC	216
	Fw 5	TCGATTGCACCTGCTCTGAG	202
	Rv 5	GCTGAAGCTCGTTCTGGTAC	202
PLE2	Fw 1	AAGACCGTGCAAGGTTCTCC	112
	Rv 1	ACTTCTGTCTCAGCCTGCTG	112
	Fw 2	**AGGGACGACAACCGGTATTC**	**75**
	Rv 2	CATGACTCTGGCCCGTTCTG	75
	Fw 3	TGGGGAGATTGACCATGCTG	86
	Rv 3	TAGCCTTCCTGCTAGATGCC	86
	Fw 4	ATGCTGATCTCCTCACCAGC	218
	Rv 4	TTAACCATGACTCTGGCCCG	218
	Fw 5	AAGACCGTGCAAGGTTCTCC	203
	Rv 5	TGGGACCGTTTGCATATCTCC	203
PLE UTR	PLE 3' UTR 1	AGGTGACACAATTGTAGAAAGTCT	Variable
	PLE 3' UTR 2	ATACTCGACCTTCCGGGACA	Variable
NF-YC4 UTR	NF-YC4 3' UTR 1	AGACTTTCTACAATTGTGTCACCT	Variable
	NF-YC4 3' UTR 2	TGTCCCGGAAGGTCGAGTAT	Variable

Table 9.2 Primers that were used to analyse the expression of the three genes of interest. The primer combinations that were selected to use in the actual experiments, are represented in bold.

Primers for expression analysis			
Target	**Pair**	**Sequence 5' -> 3'**	**Product length**
NF-YC4	Fw 1	ATCTGCACAAGTCGCTCTGG	113
	Rv 1	TGGGAAGCGAATCGAGAAGG	113
	Fw 2	ACGCAGAAGCCCTACATCTC	88
	Rv 2	TTCTCCAGAGCGACTTGTGC	88
	Fw 3	**CGGAGATACCCACCAACTCC**	**122**
	Rv 3	**AAAGCTCGGTGGAACTAGCG**	**122**
TAS14	Fw 1	GATGCCAGGAGGAGGACATG	107
	Rv 1	GCATCCCAGGGATCTTGTCC	107
	Fw 2	**CAACAGCAGCTTCGTCGATC**	**118**
	Rv 2	**CATGTCCTCCTCCTGGCATC**	**118**
	Fw 3	CATGGGTACTGGCGGTATGG	87
	Rv 3	TGTTGCCCCTCATGGTGATG	87
ER24	Fw 1	AAGAAGCTAGATGAGGCGGC	78
	Rv 1	CGCTTTCTGTATCGCTTGCC	78
	Fw 2	**GAATCAGGCATTGCGAGCTG**	**75**
	Rv 2	**GCCGCCTTCTTGTTCAATCC**	**75**
	Fw 3	GGATTGAACAAGAAGGCGGC	101
	Rv 3	TGCCTCACATCCACTGTCAC	101

Table 9.3 Primers that were used to generate the gene (fragment) amplicons to be cloned. The overhangs that are included for cloning purposes are given in bold. These overhangs are included in the product length.

Primers for gene (fragment) amplification			
Target	**Primer**	**Sequence 5' -> 3'**	**Product length**
NF-YC4	Whole gene Fw	**cacc**ATGGCCGGAGAGGATGAAG	426
	Whole gene Rv	TTAAGTGCACTTCTGAAAGAATGC	426
	Gene fragment Fw	**cacc**CGGAGATACCCACCAACTCC	257
	Gene fragment Rv	TGGGAAGCGAATCGAGAAGG	257
	Gene restriction Fw	**gtcgac**ATGGCCGGAGAGGATGAAG	438
	Gene restriction Rv	**actagt**TTAAGTGCACTTCTGAAAG	438
TAS14	Whole gene Fw	**cacc**ATGGCACAATACGGCAACC	423
	Whole gene Rv	TCAATGCATCCCAGGGATCTTG	423
	Gene fragment Fw	**cacc**GTACCGGTATGGGAGGCATG	287
	Gene fragment Rv	TCCAGCTCCTTCACCTGTTG	287
	Gene restriction Fw	**gtcgac**ATGGCACAATACGGCAACC	435
	Gene restriction Rv	**actagt**TCAATGCATCCCAGGGATCTTG	435
ER24	Whole gene Fw	**cacc**ATGCCGATGCGACCAACAGG	441
	Whole gene Rv	TCATGACTTATGAATTTTACC	441
	Gene fragment Fw	**cacc**ACCAACAGGCGGATTGAAAC	262
	Gene fragment Rv	CGCTTTCTGTATCGCTTGCC	262
	Gene restriction Fw	**gtcgac**ATGCCGATGCGACCAACAGG	453
	Gene restriction Rv	**actagt**TCATGACTTATGAATTTTACC	453

9.1.2 Supplemental pictures

Pictures of the plants in which the genes StNF-YC4, StTAS14 and StER24 were silenced
The top picture shows the control plants that were infiltrated with the pTRV2::GUS construct. The left plant did receive water, whereas the right one did not. The second picture shows one plant for each of the three drought treated groups of plants. All three, pTRV2::TAS14, pTRV2::ER24 and pTRV2::GUS had the same phenotype. The last picture shows that there was no observed difference between the pTRV2::NF-YC4 and the pTRV2::GUS plants.

9.1.3 Overview of the alleles of the different genes

Table 9.3.1 The SNPs of the gene *StNF-YC4* are represented in the table below. The positions of the SNPs are indicated compared to the A of the ATG at the begin of the gene. The green row represents the SNP that does not result in different amino acids. Light green symbolises a synonymous mutation, whereas the red color in the last column indicates a non-synonymous mutation. C1, indicated in blue, is the allele that is present in the constructs.

NF-YC4	Allele					Codon	Amino Acid
SNP position	C1	C2	E1	E2	DM		
6	C	C	C	C	T	GC **(C/T)**	Ala
22	A	G	G	G	G	**(A/G)** GA	Arg/Gly
34	G	G	A	G	G	**(G/A)** TC	Val/Ile
41	A	T	T	T	T	G **(A/T)** C	Asp/Val
57	G	T	G	T	T	GA **(G/T)**	Glu/Asp
62	G	A	A	A	A	C **(G/A)** C	Arg/His
72	G	A	G	A	A	CA **(G/A)**	Gln
133	T	G	T	G	G	**(T/G)** CA	Ser/Ala
327	T	C	T	T	T	GA **(T/C)**	Asp
384	C	T	C	C	C	TC **(C/T)**	Ser
414	G	G	G	A	G	CA **(G/A)**	Gln

Table 9.3.2 The SNPs of the gene *StTAS14* are represented in the table below. The positions of the SNPs are indicated compared to the A of the ATG at the begin of the gene. The green row represents the SNP that does not result in different amino acids. Light green symbolises a synonymous mutation, whereas the red color in the last column indicates a non-synonymous mutation. C1, indicated in blue, is the allele that is present in the constructs.

TAS14	Allele				Codon	Amino Acid
SNP position	C1	C2	E	DM		
167	G	C	C	G	A **(G/C)** C	Ser/Thr
173/174	ACC	ATG	ATG	ACC	A **(CC/TG)**	Thr/Met
177	T	A	A	T	GG **(T/A)**	Gly/Gly
305	T	C	C	T	A **(T/C)** G	Met/Thr
307	C	G	G	C	**(C/G)** AG	Gln/Glu

Table 9.3.3 The SNPs of the gene *StER24* are represented in the table below. The positions of the SNPs are indicated compared to the A of the ATG at the begin of the gene. The column of C2 is coloured red since the deletion at position 116 causes a frame shift, thus in a defective gene. Rows that are green represent the SNPs that do not lead to another amino acid. Light green symbolises a synonymous mutation, whereas the red color in the last column indicates a non-synonymous mutation. Blue indicates the allele that is present in the expression constructs, orange is the allele that is present in the silencing constructs.

ER24	**Allele**					**Codon**	**Amino Acid**
SNP position	**C1**	**C2**	**E1**	**E2**	**DM**		
56	A	A	G	A	A	C **(A/G)** C	His/Arg
63	G	G	A	G	G	CC **(G/A)**	Pro
84	G	A	G	G	G	CT **(G/A)**	Leu
105	T	T	C	T	T	AA **(T/C)**	Asn
116	G..A	X	G..A	G..A	GAGCTGGA		
118	G	G	A	G	G	**(G/A)** CT	Ala/Thr
179	C	C	T	C	C	A **(C/T)** G	Thr/Met
186	T	T	A	T	T	GC **(T/A)**	Ala
197	A	A	G	A	A	A **(A/G)** G	Lys/Arg
240	G	A	A	G	G	GT **(G/A)**	Val
330	G	T	G	G	G	AC **(G/T)**	Thr
430/432	CAT	TAT	CAC	CAT	CAT	CAT/CAC	Gln