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Role of the gene *At1g64110* for the development of syncytia induced by *Heterodera schachtii* in Arabidopsis roots

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für Sieglinde

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List of abbreviations

AAA+ ATPase = ATPases Associated with diverse cellular Activities

amiRNA = artificial micro ribonucleic acid

bp = base pairs

DNA = Deoxyribonucleic acid

dpi = days past infection

efP Browser = ”Electronic Fluorescent Pictograph” Browser

GUS = beta-glucuronidase

PCR = polymerase chain reaction

qRT-PCR = quantitative real time polymerase chain reaction

RT-PCR = reverse transcription polymerase chain reaction

TAIR= The Arabidopsis Information Resource

T1 Arabidopsis plants = first generation of transformed Arabidopsis plants

U = unit

WMD = Web microRNA Desinger

Abstract

The plant parasitic nematode *Heterodera schachtii* infects several crop species and also the model plant *Arabidopsis thaliana*. The parasites induce the formation of specific feeding structures (syncytia) in the plant roots that serve as their sole nutrient source.

Previous transcriptome analysis has shown that many genes are up regulated and down regulated in syncytia. One of the strongly up regulated genes, *At1g64110*, codes for an ATPase. Infection tests of *At1g64110* T-DNA mutants with *Heterodera schachtii* revealed that this gene is important for nematode development.

The aim of this thesis was to prepare transgenic Arabidopsis lines for a functional analysis and for a expression analysis of *At1g64110*. For the functional analysis, artificial microRNA constructs with tissue specific promoters were created to silence the gene in the whole plant (promoter *CaMV*) and in syncytia (Promoter *MIOX5*, Promoter *PDF2.1*). The constructs were successfully transformed into Arabidopsis. 15 T1 Arabidopsis lines for each construct were isolated for further studies.

For the expression analysis three promoter::GUS constructs with different length were created and also transformed into Arabidopsis. Preliminary analysis of randomly selected T1 Arabidopsis plants showed transgenic promoter activity in trichomes and siliques. 15 T1 Arabidopsis lines for each construct were isolated for further GUS expression analysis.

Moreover, the previous transcriptome analysis for the gene *At1g64110* was confirmed by quantitative real time PCR (qRT-PCR) during this project. The qRT-PCR showed a 97.2-fold up regulation of the gene in 15 dpi (days post infection) syncytia in comparison to uninfected roots which is in line with the previous GeneChip data.

The gene *At5g52882* was found to be highly similar to the gene *At1g64110*, but as no transcriptome expression data were available for this gene a qRT-PCR was done. The analysis showed no change in expression levels of this gene in 15 dpi syncytia in comparison to normal roots.

Keywords: *Heterodera schachtii* · Arabidopsis · syncytia · *At1g64110* · *At4g28000* · *At5g52882* · *MIOX5* · *PDF2.1*

Kurzfassung

Nematoden der Gattung *Heterodera schachtii* infizieren neben vielen anderen Pflanzen auch die Modellpflanze *Arabidopsis thaliana*. Der Schädling induziert in der Pflanzenwurzel den Aufbau eines Nährzellensystems (Synzytium), welches für die künftige Nährstoffversorgung des Nematoden dient. Vorhergehende Transkriptomanalysen von Synzytien haben offen gelegt, welche Gene im Nährzellensystem in ihrer Transkriptionsrate verändert werden. Ein stark hochreguliertes Gen, das Gen *At1g64110*, codiert für eine ATPase. Infektionsversuche von *Heterodera schachtii* an *At1g64110* T-DNA Mutanten haben gezeigt, dass dieses Gen für die Entwicklung von Nematoden eine wichtige Rolle spielt.

Ziel dieser Masterarbeit war es, transgene Arabidopsis Linien bereitzustellen, um eine Funktionsanalyse sowie eine Expressionsanlyse an dem Gen *At1g64110* durchführen zu können. Für die Funktionsanalyse wurden künstliche Mikro-RNA Konstrukte mit gewebespezifischen Promotoren kombiniert, um das Gen zum einen in der gesamten Pflanze (Promotor *CaMV*) zum anderen nur im Synzytium (Promotor *MIOX5*, Promotor *PDF2.1*) runterzuregulieren. Die Konstrukte wurden erfolgreich in Arabidopsis transformiert und 15 verschiedene T1 Arabidopsis Linien für die Funktionsanalysen selektiert. Für die Expressionsanalyse wurden drei Promotor::GUS Konstrukte mit unterschiedlicher Promotor Länge hergestellt und erfolgreich in Arabidopsis transformiert. Eine vorläufige Analyse von zufällig ausgewählten T1 Arabidopsis Pflanzen hat eine transgene Promotoraktivität in Trichomen und Schoten gezeigt. 15 verschieden T1 Arabidopsis Linien wurden für weiterführende GUS Expressionsanalysen selektiert. Zusätzlich wurde die vorhergehende Transkriptomanalyse für das Gene *At1g64110* über quantitative Real Time PCR (qRT-PCR) bestätigt. Die qRT-PCR zeigte eine 97,2-fache Hochregulierung des Gens in 15 Tage alten Synzytien im Vergleich zu nicht infizierten Kontrollwurzeln.

Außerdem wurde gefunden, dass das nahe verwandte Gen *At5g52882* sehr große Ähnlichkeit zu *At1g64110* aufwies, jedoch dafür keine GeneChip Daten vorhanden waren. In der Vermutung, dass auch dieses Gen im Synzytium stärker exprimiert werden könnte, wurde eine qRT-PCR durchgeführt. Die Analyse zeigte weder eine Hochregulierung noch eine Runterregulierung von *At5g2882* in 15 Tage alten Synzytien im Vergleich zu nicht infizierten Kontrollwurzeln.

1 Introduction

Cyst forming nematodes of the genera *Heterodera* and *Globodera* have the potential to induce the formation of feeding cells by intervention in the developmental program of the host plant. Preparasitic second-stage juveniles enter the root preferentially at the elongation or differentiation zone. Juveniles migrate intracellularly toward the vascular cylinder. The nematodes invade the roots with the help of their stylet, assisted by secretions produced from two subventral pharyngeal gland cells that have been shown to contain cell wall degrading enzymes, such as cellulases and pectinases, as well as puntative expansin (Kudla *et al*., 2005; Smant *et al*., 1998; Vanholme *et al*., 2004). Depending on the nematode-host combination, a differentiated or non differentiated root cell is preferred as starting point for feeding cell induction. (Magnuson and Golinowski, 1991). In Arabidopsis, the initial syncytial cells are preferably procambium or pericycle cells within the central cylinder (Golinowski *et al*., 1996; Sobczak *et al*., 1997). Syncytia formation is presumably initiated in response to signal molecules released by the infective juvenile (Williamson and Hussey, 1996).

From the initial syncytial cell the syncytium is initiated through secretions of the nematode and by a coordinated expression of plant genes. Such plant genes include, for instance, expansins and cellulases, which are important for the degradation of cell walls to incorporate new cells into the growing syncytium (Goellner *et al*., 2001; Wieczorek *et al*., 2006, 2007). Very early in feeding cell development, cell wall openings between the initial syncytial cell and adjacent cells are formed. Initially, cell wall breakdown occurs by a gradual widening of plasmodesmata; later, large openings are created without the involvement of these natural cytoplasm bridges (Grundler *et al*., 1998). Progressive cell wall dissolution results in the expansion of the feeding cell toward and within the stele along the xylem vessels. Subsequent fusion of the protoplast results in a hypertrophied multinuclear cell complex, a syncytium, that can include up to 200 cells (Jones, 1981). The central vacuole of the cells is replaced by numerous small, secondary vacuoles and the dense cytoplasm contains numerous organelles and enlarged nuclei. Extensive cell wall protuberances are formed at those parts of the syncytium that are in close contact with xylem elements. These protuberances greatly enlarge the plasma membrane surface, thereby facilitating massive short-distance nutrient import that is essential for nematode development. The hypertrophic feeding

cell is lined by a thickened cell wall to resist the osmotic pressure that increases up to 9.000 to 10.000 hPa (Jones and Northcote, 1972; Böckenhoff and Grundler, 1994).
The syncytia are the only nutrient source for these nematodes, and are thus a severe nutrient sink for the host. The nematodes feed from the syncytium through a feeding tube that is produced at the tip of the stylet during each feeding cycle (Davis *et al*., 2004; Williamson and Kumar, 2006).

Many attempts were done to analyse the transcriptome of syncytia (Puthoff *et al*. 2003; Ithal *et al*., 2007; Klink *et al*, 2007). The latest and most accurate approach to identify the transcriptome of syncytia was done by Szakasits *et al*. and published in Plant Journal in 2009. Pure syncytium material was harvested by microaspiration without contaminating root tissue (Juergensen *et al*., 2003) which was then used to prepare RNA for hybridization to Affymetrix GeneChips. The data revealed that the transcriptome of syncytia is clearly different from roots and other plant organs. Out of a total of 21138 genes, 18.4% (3893) had a higher expression level and 15.8% (3338) had a lower expression level in syncytia, as compared to control roots. A gene ontology analysis of up and down regulated genes showed that categories related to high metabolic activity were preferentially up regulated (Szakasits *et al*., 2009).
Three highly up regulated genes that are relevant for this thesis are the following:

At5g56640.1 myo-inositol oxygenase (*MIOX5*)	M value 8.8
At2g02120.1 plant defensin (*PDF2.1*)	M value 7.7
At1g64110.1 AAA+ ATPase family protein	M value 7.7

The gene *At5g56640.1* (*MIOX5*) which codes for myo-inositol oxygenase is part of a small gene family containing four members (*MIOX1, MIOX2, MIOX4, MIOX5*). Myo-inositol oxygenase (MIOX) is responsible to channel carbohydrates into a pool of UDP sugars used for cell-wall biosynthesis. The genes have been cloned and promoter::GUS reporter gene lines revealed that two isoformes (*MIOX1*, *MIOX2*) are expressed in almost all tissue of the plant, whereas the expression of *MIOX4* and *MIOX5* is largely restricted to flowers, particularly maturing pollen (Kanter U. *et al.*, 2005; Zimmermann *et al*., 2004) and syncytia (Siddique *et al*., 2009).
The second highly up regulated gene in syncytia, *At2g02120.1* (*PDF2.1*), belongs to the gene family of plant defensins. They play an important role in the innate immune response of plants. Plant defensins are small (45-54 amino acids), highly basic, and

cysteine rich peptides that are apparently ubiquitous throughout the plant kingdom. Most plant defensins identified so far have eight cysteines that form four structure-stabilizing disulfide bridges. In *Arabidopsis thaliana* , 13 plant defensin genes (*PDF*) are present, encoding 11 different plant defensins which are known to inhibit the growth of a broad range of fungi (Penninckx *et al*., 1996; Epple *et al*., 1997; Thomma and Broekaert, 1998; Thomma *et al.*, 2002).
As the *PDF2.1* gene is highly up regulated in syncytia I used the promoter of this gene to trigger the syncytial expression of an artificial micro RNA to silence the highly up regulated gene *At1g64110* in syncytia.

At1g64110 codes for an AAA+ ATPase (TAIR, 2008). AAA+ ATPase proteins were first described by Erdmann *et al*., 1991, as a new family of "**A**TPases **A**ssociated with diverse cellular **A**ctivities". The family is characterized by a highly conserved P-loop NTPase domain of about 240 residues, which contains further regions of high sequence conservation. Most members of the P-loop NTPase fold hydrolyse the β-γ phosphate bond of a bound nucleoside triphosphate, most often, ATP or GTP. The free energy of this hydrolysis reaction is typically utilized to induce conformational changes in other molecules (Iyer *et al*., Snider *at al.*). The gene *At1g64110* shares common domains of AAA+ ATPases but it is unclear in which processes the protein is involved in the plant and especially in syncytia. Infection tests of *At1g64110* T-DNA Arabidopsis mutants with *Heterodera schachtii* revealed that this gene is important for nematode development as more males than female individuals developed during the infection process (El Ashry and Bohlmann, unpublished results).

The aim of this thesis was to prepare transgenic Arabidopsis lines for a functional analysis and for a expression analysis of the gene *At1g64110*. For the functional analysis, artificial microRNA (amiRNA) constructs were created to silence the gene. This should give additional evidence for the correlation between nematode development and the highly up regulated gene *At1g64110*, beside the T-DNA mutant infection essays. We used three different tissue specific promoters to express the amiRNA in the whole plant and in syncytia. A double *CaMV* promoter should express the amiRNA in the whole plant while the promoters of the gene *MIOX5* and *PDF2.1* are known to be highly active in syncytia.

For the expression analysis three promoter::GUS constructs with different length were created and also transformed into Arabidopsis.

A phylogenetic analysis of the gene *At1g64110* revealed that the gene is part of a small gene family (three genes in Arabidopsis) and related genes in other plant species. In Arabidopsis the three related genes are the following: *At1g64110* (AAA+ ATPase family protein), *At4g28000* (nucleoside-triphosphatase activity), *At5g52882* (nucleoside-triphosphatase activity), (TAIR, 2008).

As mentioned above, the transcriptome analysis for the gene *At1g64110* gave a 208 fold up regulation in syncytia in comparison to non infected roots (Szakasits *et al*., 2009). The related gene *At4g28000* was not expressed in syncytia. Unfortunately, the gene *At5g52882* is not included on the Affymetrix GeneChip and no GeneChip data are available. Therefore, qRT-PCRs were performed for the genes *At1g64110* and *At5g52882*.

2 Materials and methods

2.1 Beta-glucuronidase (GUS) reporter analysis

The three promoter fragments (promoter 1: 1464 bp; promoter 2: 1147 bp; promoter 3: 907 bp) were amplified by polymerase chain reaction (PCR) using 50 ng Arabidopsis Col-0 genomic DNA as template.

Primer pairs used for promoter 1 (annealing temp. 46°C):

forward primer: 5´-TCTCTGAACGAATTCATTAGGAAGTAAC-3´

reverse primer: 5´-TGGACAGCAAACCCATGGTGTTGTCGG-3´

Primer pairs used for promoter 2 (annealing temp. 52°C):

forward primer: 5´-GTATATGAATTCAAAATTTCGAATGG-3´

reverse primer: 5´-TGGACAGCAAACCCATGGTGTTGTCGG-3´

Primer pairs used for promoter 3 (annealing temp. 55°C):

forward primer: 5´-AGCCACGAATTCCAGATCTAACACAG-3´

reverse primer: 5´-TGGACAGCAAACCCATGGTGTTGTCGG-3´

Primers included restriction sites for NcoI (blue) and EcoRI (gray) for subsequent cloning into the binary vector pPZP3425. The vector is derived from the widely used pPZP100 series of binary *Agrobacterium* vectors (Szakasits *et al*., 2007). This plasmid harbors in its T-DNA the gene that confers kanamycin resistance, the double enhanced 35S promoter of the cauliflower mosaic virus, the translational enhancer (TMV omega element) and the reporter beta-glucuronidase (GUS) gene (figure 1). During the cloning procedure the double enhanced 35S promoter has been exchanged by the different promoter fragments. Promoter::GUS constructs were introduced into *Agrobacterium tumefaciens* GV3101 for transformation of *Arabidopsis* Col-0 plants by the floral dip method (Clough and Bent, 1998).

Leaves, flowers and siliques were taken from T1 Arabidopsis plants to perform a preliminary analysis of the GUS expression. The tissue was submerged in 100 mM $NaPO_4$ buffer (pH 7.0) containing 10 mM EDTA, 0.01% Triton X-100, 0.5 mM $K_3(Fe(CN)_6)$, 0.5 mM $K_4(Fe(CN)_6)$ and 1 mg ml^{-1} 5-bromo-4-cloro-3-indolyl glucuronide (X-gluc; Melford Laboratories Ltd, Ipswich, UK) and subsequently incubated in the dark for 16 h at 37°C. Tissues were destained in 70% ethanol and viewed using bright field optics on a Leica DMRB microscope. Images were captured with an Olympus C-5050 digital camera.

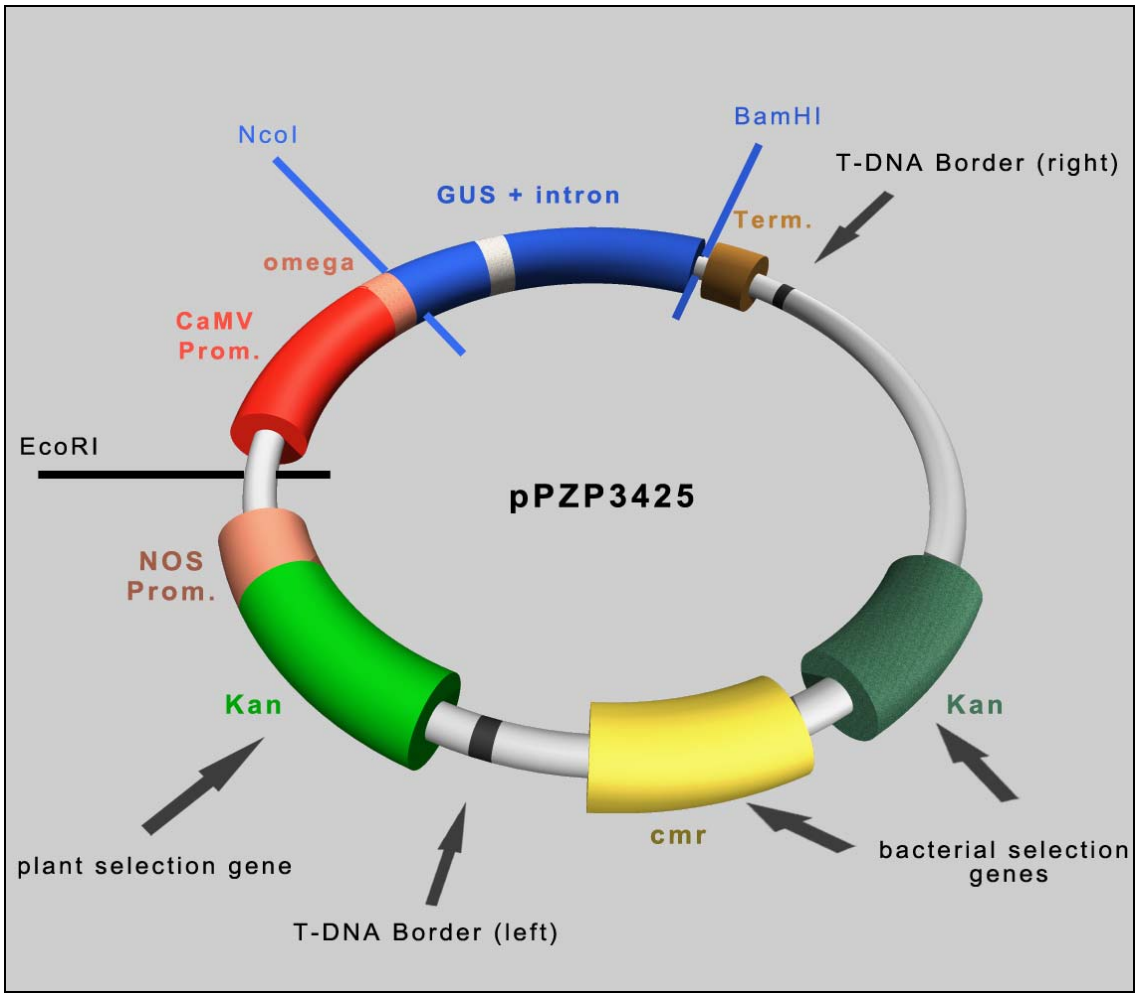


Figure 1. The plasmid pPZP3425. Restriction sites EcoRI and NcoI were used to change the promoter, NcoI and BamHI were used to exchange the reporter gene GUS by the amiRNA (Szakasits *et al*., 2007).

2.2 Artificial microRNA (amiRNA) to target *At1g64110*

Engineering of the precursor containing the artificial microRNA (amiRNA) was performed according to the procedure described by Schwab *et al.* in 2006. The required primer sequences to create the precursor of the artificial miRNA were generated by the web based tool "WMD, Web microRNA Designer", Schwab *et al.* in 2006, (http://wmd.weigelworld.org/cgi-bin/mirnatools.pl).

Primers used to engineer the precursor:

Sequence of the amiRNA:

5´-TAAACGTTTATGAAACTCGCC-3´

Oligo Primer I:

5´-gaTAAACGTTTATGAAACTCGCCtctctctttgtattcc-3´

Oligo Primer II:

5´-gaGGCGAGTTTCATAAACGTTTAtcaaagagaatcaatga-3´

Oligo Primer III:

5´-gaGGAGAGTTTCATATACGTTTTtcacaggtcgtgatatg-3´

Oligo Primer IV:

5´-gaAAAACGTATATGAAACTCTCCtctacatatatattcct-3´

Oligonucleotide A

5´-CTGCAAGGCGATTAAGTTGGGTAAC-3´

Oligonucleotide B

5´-GCGGATAACAATTTCACACAGGAAACAG-3´

The amiRNA containing precursor was generated by overlapping PCR (table 1). A first round amplified fragments (a) to (c). These were subsequently fused in PCR (d). Oligonucleotide primers I to IV were used to replace miRNA regions with artificial sequences. Primers A and B were based on template plasmid sequence. Regeneration of functional miRNA precursors was achieved by combining PCR products A-IV, II-III, and I-B in a single reaction with primers A and B.

	forward oligo	reverse oligo	template
(a)	A	IV	pRS300
(b)	III	II	pRS300
(c)	I	B	pRS300
(d)	A	B	(a)+(b)+(c)

Table 1. Primer combinations for the overlapping PCR to create the amiRNA precursor. I: microRNA forward, II: microRNA reverse, III: microRNA* forward, IV: microRNA* reverse

The PCR product was digested with NcoI and BamHI, purified, and inserted into the vector pPZP3425 (figure 1). Subsequently the promoter *CaMV* was exchanged by the *MIOX5* and *PDF2.1* promoters (Szakasits *et al*., 2007). The three constructs were sequenced (AGOWA, Berlin; sequences are listed in the attachment) to check the precursors for correct PCR amplification and introduced into *Agrobacterium tumefaciens* GV3101 for transformation of Arabidopsis (see below).

2.3 RNA isolation

Root segments containing syncytia were cut with a razor blade 5 days and 15 days after nematode infection and immediately frozen in liquid nitrogen. Two kinds of root tissues were taken from non-infected plants as controls. One control was collected from the root elongation zone and one control consisted of younger and older root tissue. In both controls no root tip was included. The harvested tissue was stored at -80°C for further usage. Total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen) according to the manufacture's instructions, including DNaseI (Qiagen) digestion. Quality and quantity of the RNA was assessed using an Agilent 2000 bioanalyser (Agilent Technologies, Palo Alto, CA, USA). Reverse transcription was performed with a Superscript III reverse transcriptase (Invitrogen) and random primers (oligo$(dN)_6$) according to the manufactures` instructions.

2.4 Polymerase chain reactions

Standart polymerase chain reactions (PCR) for DNA amplifications were performed on a gradient cycler (Peltier Thermal cycler, PLT-200). For one reaction 10 ng to 200 ng template DNA was used in 2.5 µl 10 x PCR buffer containing 15 mM $MgCl_2$ (Biotherm), 0.5 µl dNTP Mix (10 µM), 0.5 µl forward primer, 0.5 µl reverse primer (10 µM), 0.5 U *TAQ* (Biotherm) and water to make a total of 25 µl total reaction volume.

2.5 Quantitative real time PCR

Quantitative real time PCR of *At1g64110* and *At5g52882* expression in syncytia was performed on a ABI PRISM 7300 Sequence Detector (Applied BioSystems). Each qRT-PCR sample contained 12.5 µl Platinum SYBR Green qPCR SuperMix with UDG and ROX (Invitrogen), 2 mM $MgCl_2$, 0.5 µl forward and reverse primer (10 µM), 2 µl cDNA and water to make a 25 µl total reaction volume.

Primers for *At1g64110* were:
forward primer: 5´-GTGGGTTTAGGCTTGGCTTCT-3´
reverse primer: 5´-TGTTGGTAAAGCTCGGCAGG-3´

Primers for *At5g52882* were
forward primer: 5´-GAGCTTGGGCAGATAACAGA-3´
reverse primer: 5´-TTTTCCTTGAGCCTCCTTCT-3´

Control reactions with no cDNA template ruled out false positives. Dissociation runs were performed to asses the possible formation of primer dimers. The *UBP22* gene was used as a internal reference as described previously by Hofmann and Grundler in 2007. Results were calculated using the Sequence Detection Software SDS v2.0 (Applied BioSystems). Relative expression was calculated by the $(1+E)^{-\Delta\Delta Ct}$ method.

2.6 Transformation of Arabidopsis

The plants used for the transformation were *Arabidopsis thaliana* ecotype Col. Arabidopsis plants were grown in soil in 7.5 x 7.5 cm pots (density 5 – 8 seeds / cm^2) in a growth chamber under long-day conditions with 16 h light and 8 h dark at 21-24°C. Plants with inflorescences of about 10 cm were transformed.
Arabidopsis plants were transformed by the floral dip method (Clough and Bent, 1998). *Agrobacterium tumefaciens* strain GV3101, harbouring the vector pPZP3425, was grown over night at 28°C in 4 ml YEB medium (1 g Bacto yeast extract, 5 g Bacto tryptone, 5 g Bacto peptone, 5 g sucrose, 0.4 g magnesiumchloride, 1 l deionised water) with antibiotics rifampicin, gentamycin and kanamycin at concentrations of 35 µg ml^{-1},

25 µg ml^{-1}, and 50 µg ml^{-1}, respectively. The overnight culture was added to 1000 ml of fresh YEB medium containing the same antibiotics and was grown for 24 h at 28°C. The cells were harvested by centrifuging at 4300 rmp and the pellet was resuspended in 2 l infiltration medium (10 g sucrose, 2.15 g MS salts, 200 µl Silwett L-77, 1 l deionised water). Arabidopsis plants were inoculated in an exiccator by submersing the inflorescence in the Agrobacterium suspension and by applying vacuum for 4 minutes (figure 2). Plants were then taken into a growth camber under long-day conditions with 16 h light and 8 h dark at 21–24°C to produce seeds.

Figure 2. Inoculation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens*. Plants were submersed in infiltration medium and vacuum was applied for 4 minutes.

Seeds were harvested when shoots became dry (four to five weeks after transformation) and kept in paper bags at 4°C for more than two weeks to overcome dormancy. Dry, vernalized Arabidopsis seeds were transferred into 50 ml Falcon tubes and sterilized by washing them in a solution of 50% ethanol and 10% sodium hypochlorite. After 10 minutes the bleach solution was removed and the seeds were rinsed 4–6 times with ethanol 96%. The seeds were left open to dry in the falcon tube in a laminar flow bench for two hours.

2.7 Selection of transgenic plants

The plant selection growth medium was composed of 4.3 g MS salt (Sigma) (Murashige and Skoog, 1962), 0.5 g MES [2-(4-morphonlino)-ethane sulfonic acid] buffer, 30 g sucrose and 8 g Daichin Agar. The medium was brought to pH 5.8 with KOH. After autoclaving, 45 µg ml^{-1} kanamycin (selection antibiotic) and timetin 250 µg ml^{-1} was added to the medium at a temperature of 35-45°C. The medium was poured into sterile 14.5 cm Petri dishes. Dry seeds were sprinkled onto the selection plates at about 25–35 seeds per cm^2. The plates were incubated at 25°C with a photoperiod of 16 h light and 8 h dark until the selection for kanamycin-resistant plants was evident (Figure 3). Arabidopsis plants resistant to kanamycin were transferred to soil in a growth chamber to produce seeds.

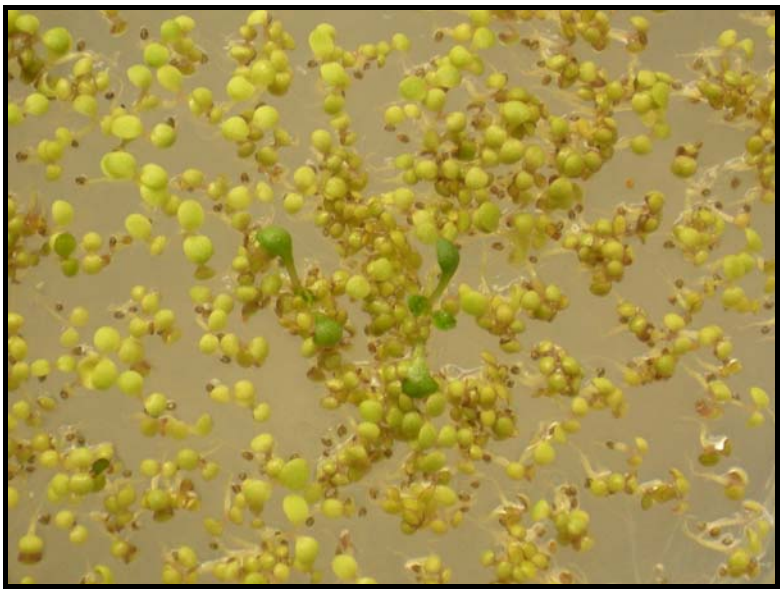


Figure 3. Kanamycin-susceptible (yellow) and kanamycin-resistant (green) Arabidopsis seedlings on MS medium containing 45 µg ml^{-1} kanamycin.

2.8 Nematode inoculation

Arabidopsis ecotype Col seeds were disinfected with 96% ethanol for 10 min and 6% sodium hypochlorite and 0.1% Tween 20 for 15 min and rinsed twice with sterile deionised water and dried. 10 dry seeds were placed in 9 cm Petri dishes containing a modified Knop medium with 2% sucrose (Sijmons *et al*., 1991). Seeds were incubated at 25°C with a photoperiod of 16 h light and 8 h dark. 12 days old plantlets were inoculated with 40 sterile *Heterodera schachtii* J2 per plant, kept for 24 h in the dark, and then incubated at 25°C with a photoperiod of 16 h light and 8 h dark.

3 Results

3.1 The gene *At1g64110*

A transcriptome analysis of syncytia revealed that among many other genes the gene *At1g64110* is highly expressed (Szakasits *et al*., 2009) and infection assays with *Heterodera schachtii At1g64110* T-DNA mutant Arabidopsis plants revealed a significant importance for nematode development of this gene (El Ashry and Bohlmann, unpublished results). The following chapter summarises the results of database analysis about this gene and two very closely related genes.

The sequence of the gene *At1g64110* consists of 2475 nucleotides and encodes a protein of 825 amino acids (listed in attachment). The predicted molecular weight is 91960.47 Da.

According to the TAIR database (2008) there are three different splice forms, *At1g64110.1*, *At1g64110.2*, and *At1g64110.3* (Figure 5). During this project the splice form *At1g64110.2* was used, as the differences between the different forms were found to be insignificant.

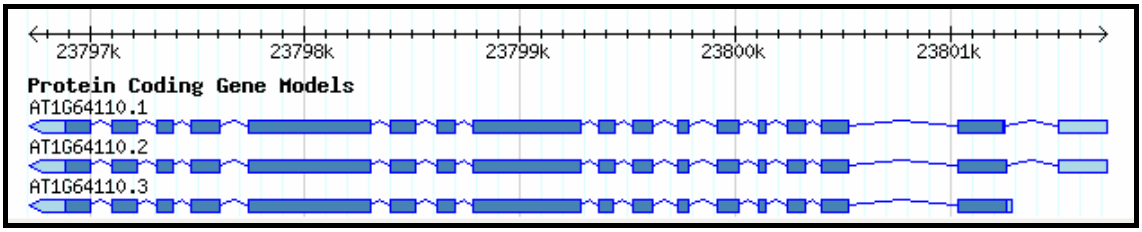


Figure 5. Three different splice forms of *At1g64110*. Start, Stop codon (light blue), exon (dark blue) and introns (blue line).

According to available microarray data at Genevestigator (Zimmermann *et al.*, 2004), the gene is highly expressed in pollen, seeds, imbibed seeds and mature siliques. Furthermore it is expressed in sperm cells (Bourge *et al.*, 2008). It is not expressed in seedlings, young and developed rosettes, flowers and immature siliques.

According to the Arabidopsis efP Browser (Winter *et al*., 2007) the expression of the gene is not inducible by any abiotic stress, except osmotic stress. 300mM mannitol in MS media induces a high expression of *At1g64110* (figure 6, Killian *et al*., 2007). This data might be interesting, considering the high amount of osmotic active substances and the high osmotic pressure in syncytia.

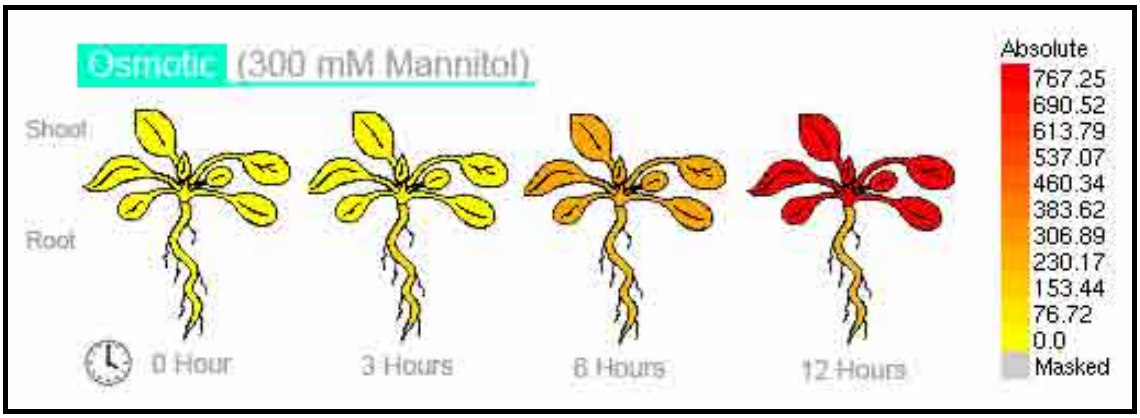
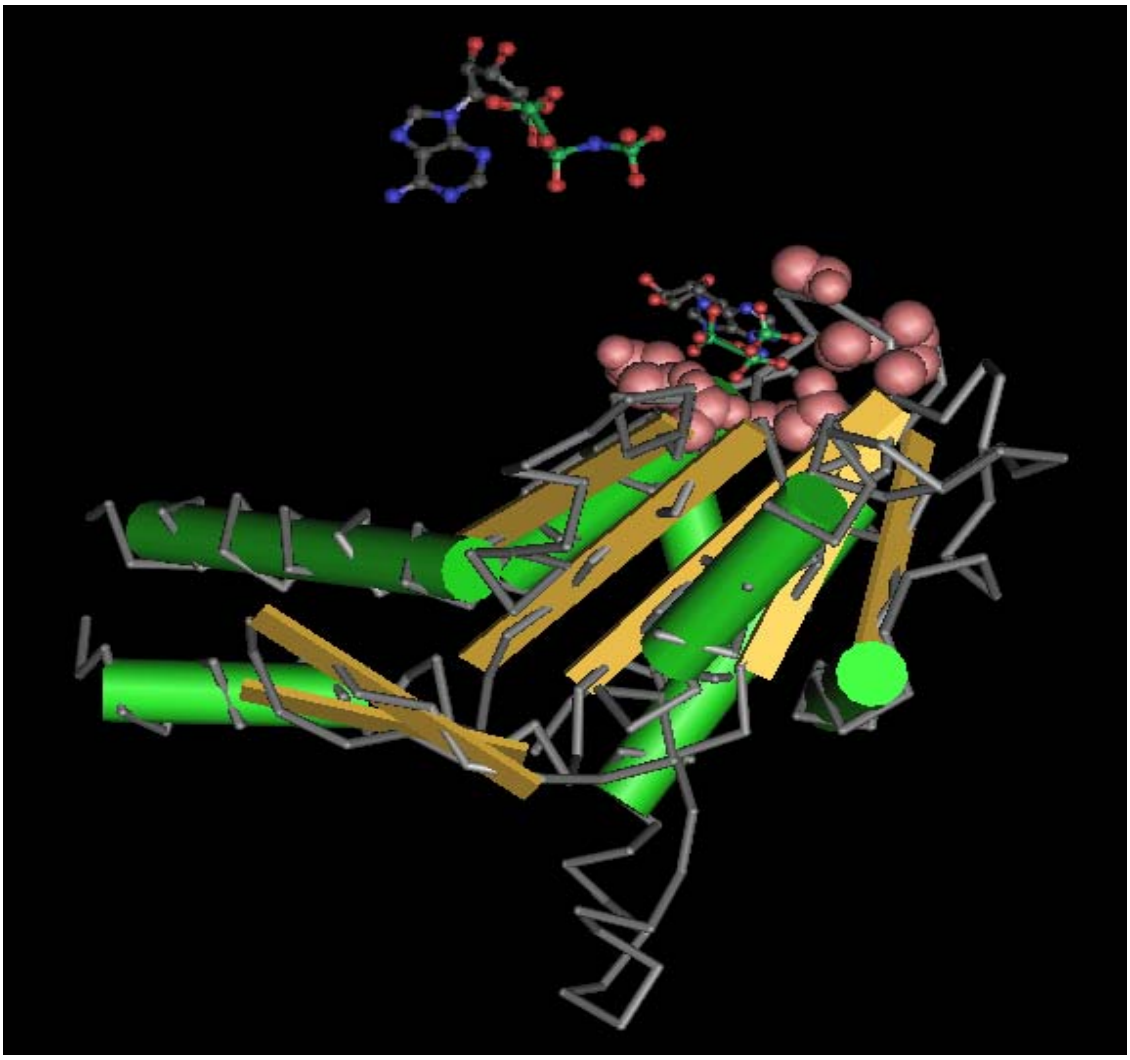


Figure 6. Expression of *At1g64110* is inducible by osmotic active substances like mannitol (300 mM), (Killian *et al.*, 2007).

The gene *At1g64110* codes for an AAA+ ATPase (TAIR, 2008). AAA+ ATPase proteins were first described by Erdmann *et al*., 1991, as a new family of "**A**TPases **A**ssociated with diverse cellular **A**ctivities". This large and diverse superfamily is found in all organisms and is characterized by a highly conserved P-loop NTPase domain of about 240 amino acids, which contains further regions of high sequence conservation. The conserved P-loop NTPase module includes an αβα nucleotide binding domain where the Walker A and Walker B motifs are found. Most members of the P-loop NTPase fold hydrolyse the β-γ phosphate bond of a bound nucleoside triphosphate, most often, ATP or GTP. The free energy of this hydrolysis reaction is typically utilized to induce conformational changes in other macromolecules. AAA+ proteins typically function as oligomeric rings, with a hexameric arrangement being most common. (Iyer *et al*. 2004, Snider *at al.,* 2008). Figure 7 shows a 3D model of the conserved P-loop domain with a bound and a free ATP molecule.



Figue 7. 3D model of the AAA+ ATPase P-loop modul with a bound and free ATP molecule (PLAZA, 2009).

AAA+ proteins are involved in a wide variety of different functions in which the energy extracted from ATP hydrolysis is used in molecular remodelling events. They are involved in processes as diverse as protein unfolding and degradation, peroxisome biogenesis, bacteriochlorophyll biosynthesis and DNA replication, recombination and repair. As a consequence of there diverse functions, AAA+ proteins can be found, beside eukaryotic cells, in archaea, bacteria and viruses (Snider *at al.*, 2008).

The protein encoded by the gene *At1g64110* shares common domains of AAA+ ATPases but it is unclear in which processes the protein is involved in the plant, and especially in syncytia.

A phylogenetic analysis of the gene *At1g64110* revealed that it is closely related to two genes in Arabidopsis and other genes in other plant species. In Arabidopsis the three related genes are the following: *At1g64110* (AAA+ ATPase family protein), *At4g28000* (nucleoside-triphosphatase activity), *At5g52882* (nucleoside-triphosphatase activity)

(TAIR, 2008). Figure 8 shows a phylogenetic tree with the three related genes in Arabidopsis and some related genes in other plant species (*Populus trichocampa* (*ptr*), *Carica papaya* (*cpa*), *Oryza sativa* (*osa*), *Sorghum bicolour* (*sbi*) and *Vitis vinifera* (*vvi*)), (PLAZA, 2009).

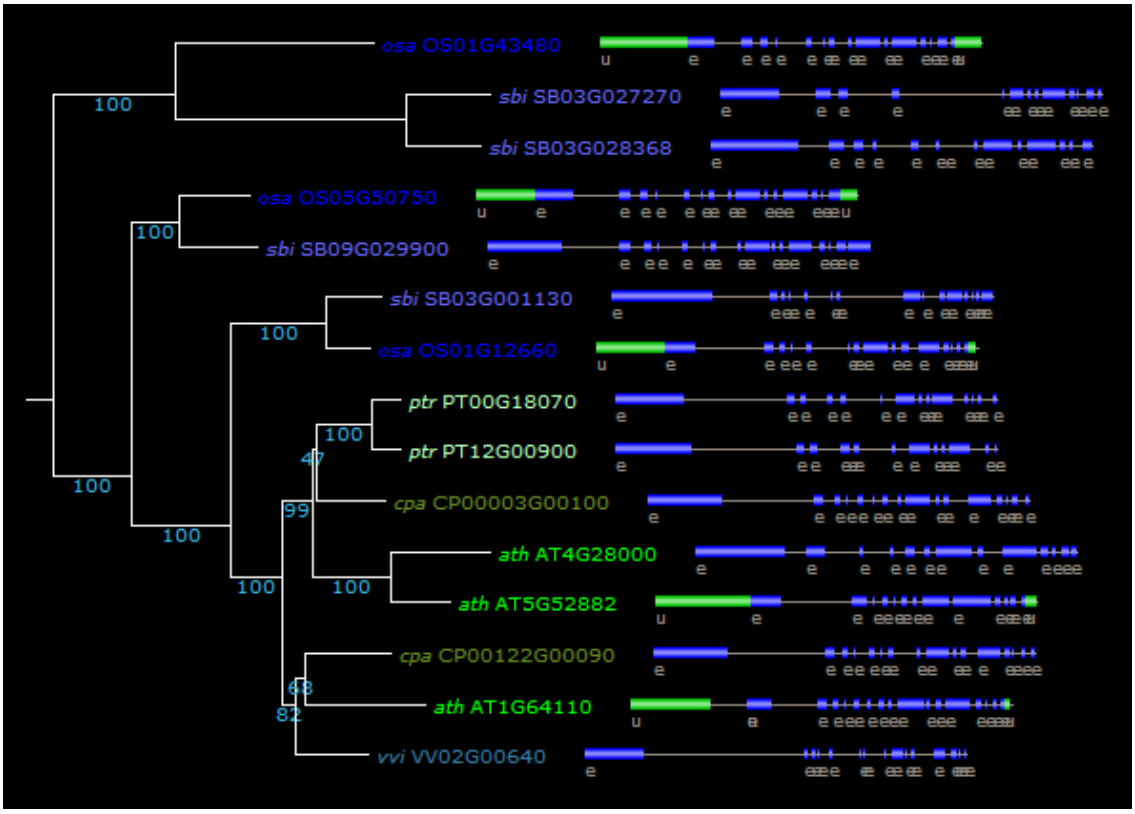


Figure 8. Phylogenetic tree with associated gene structures (green: UTR, blue: Exon) of the Arabidopsis genes *At1g64110, At5g52882, At4g28000* and related genes in other plants (PLAZA, 2009).

The three related genes in Arabidopsis share the highly conserved P-loop NTPase module but differ in gene structure and sequence, although they are very similar. Table 2 shows numbers of nucleotides and amino acids for the genes *At1g64110*, *At4g28000* and *At5g52882*. Figure 9 shows the gene structure including splice forms for *At1g64110, At4g28000* and *At5g52882*. Full sequence of genes are listened in the attachment (TAIR, 2009).

Gene	nucleotides total	nucleotides introns	nucleotides exons	amino acids
At1g64110	4369	1879	2490	829
At4g28000	3825	1332	2493	830
At5g52882	4208	1718	2490	829

Table 2. Numbers of nucleotides and amino acids for the genes *At1g64110, At4g28000* and *At5g52882*.

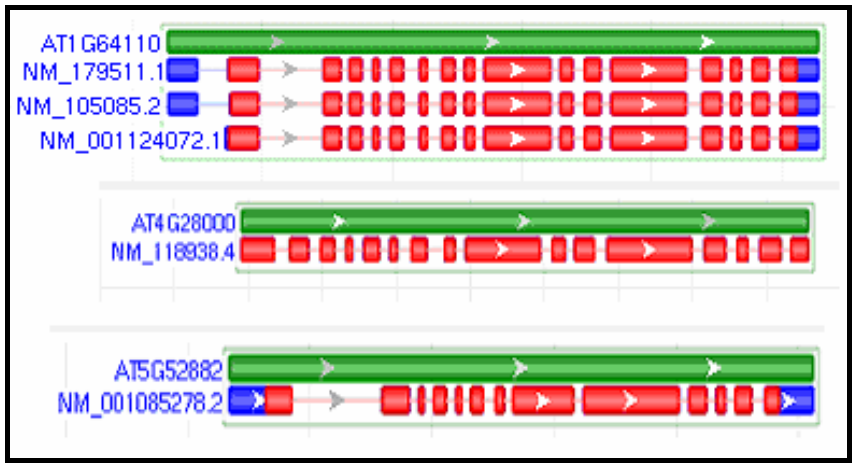


Figure 9. Gene structure of *At1g64110.1*, *At1g64110.2*, *At1g64110.3*, *At4g28000* and *At5g52882*. Green: complete gene, red: conding regions. (http://www.ncbi.nlm.nih.gov)

3.1.1 Promoter::GUS constructs

A core element of this thesis was to prepare transgenic promoter::GUS Arabidopsis lines with different length for a expressional analysis of the promoter for the gene *At1g64110*. The sequence of the promoter was taken from the TAIR database (TAIR, 2009). According to the sequence of the promoter three different primer pairs with restriction sites for EcoRI and NcoI were designed to amplify 907 bp, 1147 bp, and 1464 bp, respectively, downstream of the ATG start codon. Figure 10 shows the designed primers for the different fragments with the restriction sites for NcoI and EcoRI. The optimal annealing temperatures for the primer pairs were determined by gradient PCRs with genomic Arabidopsis DNA (table 3).

	Promoter 1	**Promoter 2**	**Promoter 3**
Primer reverse	tggacagcaaacccatggtgttgtcgg		
Primer forward	tctctgaacgaatt cattaggaagtaac	gtatatgaattca aaatttcgaatgg	agccacgaattcc agatctaacacag
Annealing temp.	46°C	52°C	55°C

Table 3. Primer pairs and their optimal annealing temperatures of the different promoter fragments

Figure 10. Genomic sequence of the promoter for the gene *At1g64110* and the designed primers for the different fragments with the restriction sites for NcoI and EcoRI.

Promoter fragments were amplified from genomic DNA by PCR, digested with NcoI and EcoRI, purified and ligated to the GUS reporter gene in the vector pPZP3425 (Szakasits *et al*., 2007). The constructs were sequenced (AGOWA, Berlin; sequences are listed in the attachment) and transformed into *Agrobacterium tumefaciens* and then transformed into *Arabidopsis thaliana*. Transformed Arabidopsis seeds were selected for kanamycin resistance on MS media with 45 µg ml^{-1} kanamycin. About 15

kanamycin resistant plantlets per construct were transferred into soil and grown up to produce seeds. Tissue was taken from leaves and extracted DNA was tested by PCR for positive transformation (figure 11). Seeds were harvested and stored at 4°C and are available for selection of homozygous lines and GUS expression studies.

Primers used to amplify the promoter of the construct from T1 Arabidopsis plants:

forward primer (-175 forward): 5´-CAAGCTGCTCTAGCCAATAC-3´

reverse primer (GUS reverse): 5´-ACAGTTTTCGCGATCCAGAC-3´

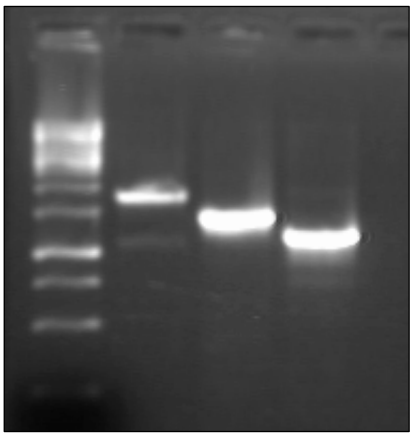


Figure 11. Amplification of Promoter 1, 2, 3 from transformed Arabidopsis plants (T1).
1: Ladder 100 bp
2: Promoter 1, expected size 1697 bp;
3: Promoter 2, expected size 1423 bp;
4: Promoter 3, expected size 1120 bp

3.1.2 Preliminary GUS expression analysis

Preliminary analysis of randomly selected T1 Arabidopsis plants showed transgenic promoter activity in trichomes (figure 12) and siliques.

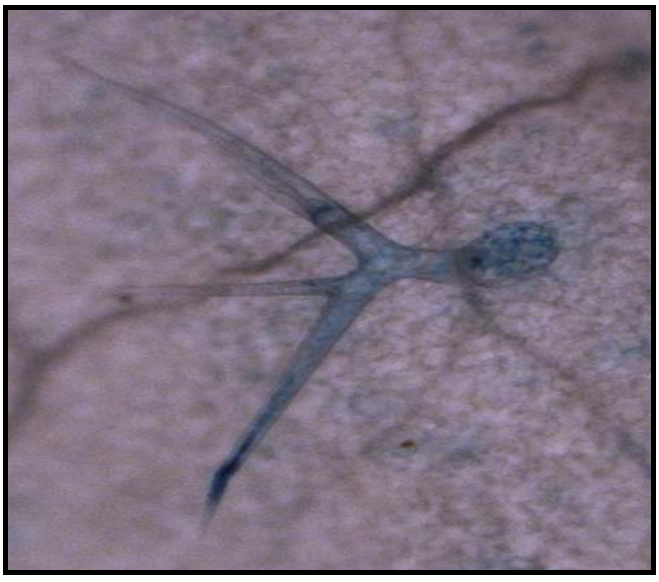


Figure 12. Transgenic promoter::GUS expression in trichomes.

3.1.3 Reverse transcriptase PCR for the gene *At1g64110*

To confirm the Affymetrix GeneChip data (Szakasits *at al*., 2009) for the gene *At1g64110*, a reverse transcriptase PCR and real time PCR were performed.
For that purpose the following primers were designed:

forward primer: 5´-GTGGGTTTAGGCTTGGCTTCT-3´
reverse primer: 5´-TGTTGGTAAAGCTCGGCAGG-3´
expected product size with introns: 890 bp
expected product size without introns: 302 bp
Primer positions and gene sequence are shown in the attachment.

I tested the primer pairs for there optimum annealing temperature with a gradient PCR from 45°C to 60°C with genomic DNA as template (figure 13). The optimum annealing temperature was 54°C.

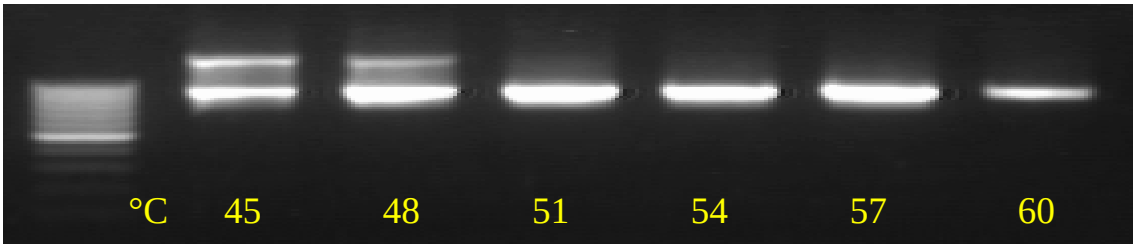


Figure 13. Annealing temperature gradient for primer pair *At1g64110* from 45°C to 60°C (slot 2-7) with genomic DNA. Optimum annealing temperature at 54°C, expected fragment size: 890 bp, left: 100 bp ladder

To test the expression of the gene in syncytia in comparison to normal, uninfected roots a RT-PCR was done. I tested 5 day old syncytia, 15 day old syncytia, uninfected older and younger root tissue and uninfected root tissue from the elongation zone. The results are shown in figure 14. The RT-PCR revealed a clear up regulation of the gene *At1g64110* in syncytia, with no differences between 5 day old syncytia and 15 old syncytia. Furthermore, the gene is higher expressed in older uninfected root tissue than in young uninfected root tissue (elongation zone).

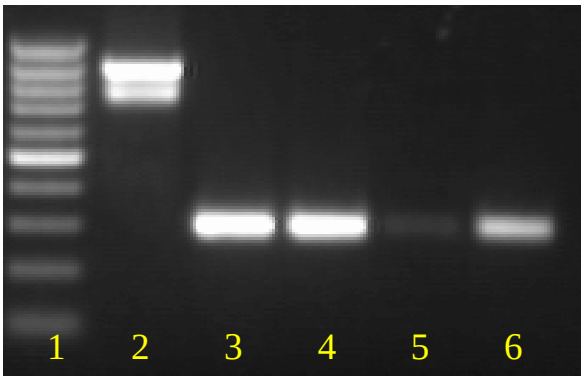


Figure 14. Expression of the gene *At1g64110*. 1: 100 bp ladder, 2: Genomic DNA (expected size: 890 bp), 3: syncytia 5 dpi, 4: syncytia 15 dpi, 5: control; root elongation zone without root tip from 13 day old plantlets, 6: control; older and younger root parts without root tips from 13 days old plantlets (expected size: 302 bp).

3.1.4 qRT-PCR for the gene *At1g64110*

To gain a more accurate insight about the syncytial expression of *At1g64110* a quantitative real time PCR was performed comparing syncytia and non infected root tissue from the elongation zone. The qRT-PCR revealed a 97.2-fold up regulation of the gene in syncytia compared to the uninfected control root (elongation zone), (Table 4).

ΔΔCt (log2)	fold-change
7.255	97.2

Table 4. qRT-PCR for *At1g64110*
(15 dpi syncytium vs. elongation zone root segments)

A second qRT-PCR was performed comparing the expression of *At1g64110* in syncytia versus the expression of the gene in younger and older root segments.

ΔΔCt (log2)	fold-change
2.4	5.5

Table 5. qRT-PCR for *At1g64110*
(15 dpi syncytium vs. younger and older root segments)

3.2 The gene *At5g52882*

The gene *At5g52882* is highly related to the gene *At1g64110*. As no Affymetix GeneChip data for the expression of *At5g52882* in syncytia is availably a RT-PCR and a qRT-PCR was performed. I designed primers to amplify the gene *At5g52882*. The sequence of the gene is listed in the attachment.

Primers for the gene *At5g52882*
forward primer: 5´-GAGCTTGGGCAGATAACAGA-3´
reverse primer: 5´-TTTTCCTTGAGCCTCCTTCT-3´
expected product size with introns: 617 bp
expected product size without introns: 334 bp
Primer positions and gene sequence are shown in attachment.

I tested the primer pairs for there optimum annealing temperature with a gradient from 45°C to 60°C with genomic DNA as template (figure 15). The optimum annealing temperature was determined at 54°C. This temperature works for the primers for the gene *At1g64110* as well, if they are used in the same PCR reaction as a control.

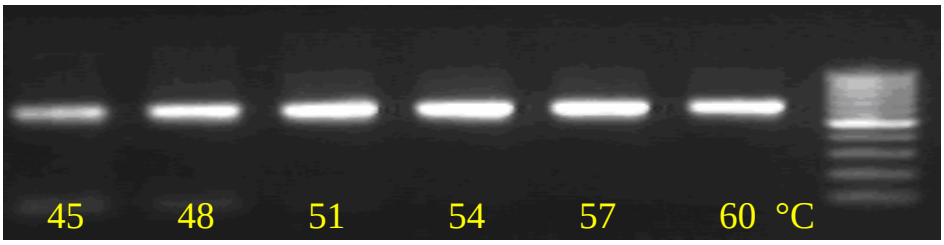


Figure 15. Annealing temperature gradient for Primer pair *At1g64110* from 45°C to 60°C (slot 1-6) with genomic DNA; slot 7: ladder 100 bp. The optimum annealing temperature was 54°C, expected fragment size 637 bp.

3.2.1 Reverse transcriptase PCR for the gene *At5g52882*

To analyse the expression of *At5g52882* in syncytia a reverse transcriptase PCR was performed. I tested RNA from 5 day old syncytia, 15 day old syncytia, uninfected older and younger root tissue and uninfected root tissue from the elongation zone. The results are shown in figure 16. The RT-PCR revealed a slight up regulation of the gene *At5g52882* in syncytia, especially in 5 day old syncytia. Furthermore, the gene is higher expressed in older uninfected root tissue than in young uninfected root tissue (root elongation zone).

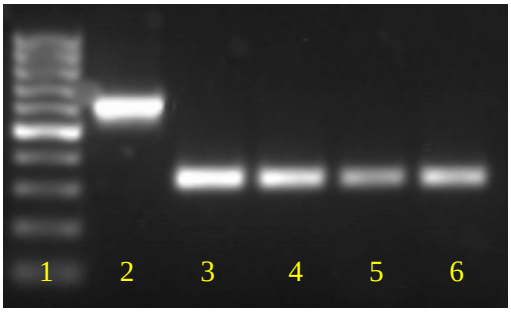


Figure 16. RT-PCR. Expression of the gene *At1g52882*. 1: ladder 100 bp, 2: genomic DNA (expected product size: 617 bp), 3: syncytia 5 dai, 4: syncytia 15 dai, 5: control root elongation zone without root tip (13 day old plantlets), 6: control different root parts without root tips (13 days old plantlets) (expected product size: 334 bp).

3.2.2 qRT-PCR for the gene *At5g52882*

To gain a more accurate insight about the syncytial expression of *At5g52882* a quantitative real time PCR was performed comparing the expression in syncytia and in non infected root tissue from the elongation zone. The qRT-PCR revealed neither a up regulation nor a down regulation of the gene in syncytia compared to the control root (Table 6).

ΔΔCt (log2)	fold-change
0.08	0.95

Table 6. qRT-PCR for *At5g52882*
(15 dpi syncytium vs. root, elongation zone)

3.3 Artificial micro RNA to target *At1g64110*

To down regulate the *At1g64110* in the whole plant and in syncytia a artificial miRNA was combined with different tissues specific promoters. The *CaMV* promoter should express the amiRNA in whole organism, the promoters *MIOX5* and *PDF2.1* only in syncytia.

Engineering of the precursor containing the artificial microRNA was performed according to the procedure described by Schwab *et al.* in 2006. The required primer sequences to create the precursor of the artificial miRNA were generated by the web based tool "WMD, Web microRNA Designer", Schwab *et al.* in 2006, (http://wmd.weigelworld.org/cgi-bin/mirnatools.pl). The designer created the 21 base pair sequence of the artificial microRNA and the sequences of four oligonucleotides (I to IV), which were used to engineer the artificial microRNA inside the endogenous miR319a precursor by site-directed mutagenesis. The sequences are listed in table 7.

Artificial microRNA:
5´-TAAACGTTTATGAAACTCGCC-3´
Oligo Primer I:
5´-gaTAAACGTTTATGAAACTCGCCtctctctttgtattcc-3´
Oligo Primer II:
5´-gaGGCGAGTTTCATAAACGTTTAtcaaagagaatcaatga-3´
Oligo Primer III:
5´-gaGGAGAGTTTCATATACGTTTTtcacaggtcgtgatatg-3´
Oligo Primer IV:
5´-gaAAAACGTATATGAAACTCTCCtctacatatatattcct-3´
Oligonucleotide A
5´-CTGCAAGGCGATTAAGTTGGGTAAC-3´
Oligonucleotide B
5´-GCGGATAACAATTTCACACAGGAAACAG-3´

Table 7. Sequences for the artificial microRNA, oligo Primers I – V, Oligonucleodides A and B. Oligonucleotides A and B are based on the template plasmid sequence. They are located outside of the multiple cloning site to generate bigger PCR products.

The amiRNA containing precursor was generated by overlapping PCR (table 8). A first round amplified fragments (a) to (c). These were subsequently fused in PCR (d).

Oligonucleotide primers I to IV were used to replace miRNA regions with artificial sequences. Primers A and B were based on template plasmid sequence. Regeneration of functional miRNA precursors was achieved by combining PCR products A-IV, II-III, and I-B in a single reaction with primers A and B.

	forward oligo	reverse oligo	template
(a)	A	IV	pRS300
(b)	III	II	pRS300
(c)	I	B	pRS300
(d)	A	B	(a)+(b)+(c)

Table 8. Primer combinations for the overlapping PCR to create the amiRNA precursor. I: microRNA forward, II: microRNA reverse, III: microRNA* forward, IV: microRNA* reverse

The sequence of the precursor with the amiRNA to silence the gene *At1g64110* with the insertion of the restriction sites NcoI, BamHI, the amiRNA and the corresponding reverse complement of the amiRNA inside the precursor is shown in table 9.

CCATGGACAAACACACGCTCGGACGCATATTACACATGTTCATACACTTAATACTCGCTGTTTTGAATTG
ATGTTTTAGGAATATATATGTAGAGGAGAGTTTCATATACGTTTTTCACAGGTCGTGATATGATTCAATT
AGCTTCCGACTCATTCATCCAAATACCGAGTCGCCAAAATTCAAACTAGACTCGTTAAATGAATGAATGA
TGCGGTAGACAAATTGGATCATTGATTCTCTTTGATAAACGTTTATGAAACTCGCCTCTCTCTTTTGTAT
TCCAATTTTCTTGATTAATCTTTCCTGCACAAAAACATGCTTGATCCACTAAGTGACATATATGCTGCCT
TCGTATATATAGTTCTGGTAAAATTAACATTTTGGGTTTATCTTTATTTAAGGCATCGGCATGGATCC

NcoI: CCATGG
BamHI: GGATCC
amiRNA: TAAACGTTTATGAAACTCGCC
amiRNA reverse completement: GGAGAGTTTCATATACGTT

Table 9. Sequence of the precursor with the amiRNA for the gene *At1g64110*, with insertion of restriction sites NcoI, EcoRI, with the amiRNA and the corresponding reverse complement.

The PCR product was digested with NcoI and BamHI, purified and inserted by ligation into three vectors (pPZP3425) with different specific promoters *CaMV*, *MIOX5* and *PDF2.1* (Szakasits *et al*., 2007). The constructs were sequenced (AGOWA, Berlin; sequences are listed in the attachment) to check the precursors for correct ligation and PCR amplification.

The constructs were transformed into *Agrobacterium tumefaciens* and then transformed into *Arabidopsis thaliana*. 15 lines per construct are available for further studies.

4 Discussion

4.1 The genes *At1g64110*, *At4g28000* and *At5g52882*

Previous transcriptome analysis has shown that many genes are up regulated and down regulated in syncytia (Szakasits *et al*., 2009). One of the strongly up regulated genes, *At1g64110*, codes for an ATPase. Infection tests of *At1g64110* T-DNA mutants with *Heterodera schachtii* indicated that this gene might be important for nematode development. (El Ashry and Bohlmann, unpublished results).

No data are available about the function of the protein, except that it shares a common domain of an ATPase. Structures and functions of ATPases were presented in detail under chapter 3.1, but even after intensive literature research it is was not possible to assign a clear function to this protein. However, further expression and functional analysis might give important hints to its function in the plant and in syncytia. In this context this thesis was meant to provide transgenic Arabidopsis lines for a expression analysis and a functional analysis. In Arabidopsis exist two highly related genes to the gene *At1g64110*, the gene *At4g28000* and the gene *At5g52882*. According to the transcriptome analysis the gene *At4g2800* is not expressed in syncytia. The gene *At5g52880* was not part of the transcriptome analysis as the gene is not on the Affymetrix ATH1 GeneCip. The expression of the gene *At1g64110* and the highly related gene *At5g52882* in syncytia were assessed by RT-PCR and qRT-PCR during this thesis.

According to the publicly availably micro array data at Genevestigator (Zimmermann *et al.*, 2004) the gene *At1g64110* is highly expressed in pollen, dry seeds, imbibed seeds and mature siliques.

Furthermore it is expressed in sperm cells (Bourge *et al.*, 2008). According to the efP browser (Winter *et al*., 2007) the expression of the gene is not inducible by any biotic or abiotic stress, except by high osmotic active substances. A high expression of the gene is inducible by the osmotic active substance mannitol (300mM in MS media), figure 6, a lower expression is attained with salt (150mM $NaCl_2$ in MS media) (Killian *et al.*, 2007). In syncytia the osmotic pressure increases up to 9000 to 10000 hPa. (Jones and Northcote, 1972; Böckenhoff and Grundler, 1994). The high osmotic pressure in syncytia might therefore be one of the reasons for the up regulation of the gene. A direct induction of *At1g64110* by mannitol is unlikely as the gene is expressed

also under the influence of osmotic active $NaCl_2$ (Killian *et al.*, 2007). In this context it would be interesting to test *At1G64110* T-DNA Arabidopsis mutants for there capability to resist osmotic pressure. As the protein might be involved in active transport mechanisms in membranes to resist the osmotic pressure, the mutant might show a decelerated development compared to wild type plants.

The phylogenetic analysis of *At1g64110* revealed that beside the above mentioned two related genes in Arabidopsis there exist highly related genes in two other plants; one in *vitis vinifera* (VV02G00640) and one in *carica papaya* (CP00122G00090), (figure 8). It would be interesting to analyse the expression patterns of these genes, as they might have the same or similar function.

The transcriptome analysis (Szakasits *et al*., 2009) revealed that the related gene in Arabidopsis, *At4g28000,* is not up regulated in syncytia. According to the efP Browser the gene shares no common expression patterns with *At1g64110*, except the fact that it is highly expressed in pollen. The expression of *At4g28000* is not inducible by chemicals, hormones, biotic or abiotic stress. The fact that it is not inducible by osmotic active substances together with the data obtained by transcriptome analysis of syncytia separates it from the gene *At1g64110* in terms of expression, even if they show a high similarity in gene and protein structure. However, additionally *At4g28000* Promoter::GUS Arabidopsis lines together with qRT-PCRs should be performed and analysed to confirm these findings.

4.2 RT-PCR and qRT-PCR for *At1g64110* and *At5g52882*

To analyse the expression of the genes in syncytia a RT-PCR and a qRT-PCR of syncytial RNA were performed. For that purpose 12 days old Arabidopsis plantlets were inoculated with sterile *Heterodera schachtii* larvae. Syncytia were cut 5 days after inoculation and 15 days after inoculation and RNA was extracted. Two types of uninfected control roots were cut from 12 days old Arabidopsis plantlets. As the nematode *Heterodera schachtii* is known to invade the root preferentially at the elongation zone, uninfected root material from the elongation zone was harvested and RNA was extracted from that material. A second uninfected control consisted of older and younger root material. In both controls no root tip material was used as it is known that undifferentiated cells in the root tip and differentiated cells differ greatly in gene

expression patterns. The RT-PCR revealed that the gene *At1g64110* is higher expressed in older root tissue than in roots from the elongation zone (figure 14).
The RT-PCR indicated no difference in expression levels in syncytia between 5 days after infection and 15 days after infection. This results is in line with the transcriptome analysis done by Szakasits *et al.* in 2009, where little differences in expression levels between 5 days and 15 days after infection were found. According to the already mentioned transcriptome analysis, the gene *At1G64110* is 208-fold up regulated in syncytia. The RT-PCR gave similar results, but it was not possible to determine a exact fold value. Therefore qRT-PCR was used to quantify the expression levels of *At1g64110* in syncytia compared to controls. Compared to the expression in control roots (elongation zone material) qRT-PCR showed a 97.2-fold up regulation of the gene in syncytia. The difference to the previous transcriptome analysis (208-fold up regulation) could be attributed to the fact that syncytial content was not obtained through microaspiration, but was cut from the root with a razor blade, which lead inevitably to a dilution of syncytial material with surrounding root tissue.
When uninfected controls with older and younger root material (no pure elongation zone material) were used for qRT-PCR, there was only a moderate 5.2-fold up regulation of the gene in syncytia (table 5), which might be explained by the higher expression of *At1g64110* in older root tissue than in younger root tissue.

The gene *At5g52882* was found to be very similar to the gene *At1g64110* but, unfortunately, no GeneChip data were available. To analyse the expression in syncytia, a RT-PCR and a qRT-PCR were performed. The RT-PCR indicated a slight up regulation of the gene in syncytia (figure 14) and the qRT-PCR showed neither an up regulation nor a down regulation (figure 6). The data indicates that the gene *At5g52882* is not up regulated at the same high level in syncytia as it is the case for *At1g64110* and might therefore assume that the two related genes are not expressed in the same way.
It is important to mention that the performed experiments were not repeated and additional repetitions, including the nematode infection process, harvest of material and RNA extraction are necessary to confirm the data obtained during this thesis.
To verify the findings regarding the gene *At5g52882* additional promoter::GUS Arabidopsis lines should be made and syncytial GUS expression should be analysed.

4.2 At1g64110 promoter::GUS fusions

To analyse the promoter and the expression of *At1g64110* in syncytia and in the whole plant, different promoter::GUS constructs were made and transformed into Arabidopsis. The different deletions of the promoter region in combination with the reporter gene GUS might give additional insight into the expression patterns of the gene. The deletions were set in order to end up with three different promoter::GUS constructs of different length (figure 10). Preliminary analysis of randomly selected T1 Arabidopsis plants indicated a transgenic promoter activity in trichomes (figure 12) and siliques. A proper GUS expression analysis will be performed when homozygous transgenic Arabidopsis lines are selected and infection tests with *Heterodera schachtii* might give indications about the activity of the different promoter::GUS constructs in syncytia. However, these analysis did not fit into the time frame set for this thesis, but they will be performed later.

One might assume a high expression of the longest promoter::GUS construct and lower expression of the shorter versions, although it also might be possible that there are regions in the longer fragments, which may act as repressors for expression. In addition, the promoter of the gene *At1g64110* might also be used as a "syncytium specific" promoter beside the promoters for *MIOX5* and *PDF2.1*.

4.3 Artificial microRNA to target At1g64110

Infection tests of *At1g64110* T-DNA Arabidopsis mutants with *Heterodera schachtii* revealed that this gene is important for nematode development as more males than female individuals developed during the infection process (El Ashry and Bohlmann, unpublished results).

It is known that Arabidopsis T-DNA knock-out lines might contain more than one T-DNA of other secondary mutations. To confirm the importance of the gene *At1g64110* for nematode development, two possible approaches were taken in consideration. The first approach should give evidence by a complementation of the *At1g64110* T-DNA mutant genome with a over expressed intact version of the gene. By such complementation the normal development of *Heterodera schachtii* would be restored. The second approach should down regulate the gene by a specific artificial microRNA. We decided to confirm the linkage of *At1g64110* to nematode development

by a down regulation of the gene by an amiRNA and not by complementation. Using the amiRNA we had the possibility to down regulate the gene tissue specific in the whole plant or in syncytia by using tissue specific promoters. The *CaMV* promoter expresses the amiRNA in the whole plant, the promoters *PDF2.1* and *MIOX5* are promoting the expression in syncytia (Siddique *et al*., 2009).

Nevertheless, it is known that the efficacy for amiRNAs differ from construct to construct (Schwab *et al*., 2006) and the silencing of the target gene might be insufficient to reveal a decreased nematode development. If this should be the case a traditional complementation of the T-DNA mutant might be performed to demonstrate that the gene *At1g64110* is essential for optimal syncytia formation and nematode development.

At1g64110 is highly expressed in pollen, siliques and imbibed seeds (Genevestigator, Zimmermann *et al*., 2009). As a consequence of the silencing effect of the amiRNA, homozygous transgenic plants might become sterile or seeds might loose there capability to germinate, although the T-DNA mutant showed no such effects. This potential effect might be the case for the *CaMV*::amiRNA construct and at a less extend for the constructs with syncytia specific promoters *MIOX5*::amiRNA and *PDF2.1*::amiRNA. So far for T1 Arabidopsis lines no obvious effects on germination, flowering or seed production were found.

5 Conclusions

During this thesis the up regulation of *At1g64110* in syncytia was confirmed trough RT-PCR and qRT-PCR. Research on the highly related genes *At5g52882* and *At4g28000* indicated different expression patterns. As a basic for further studies on the gene *At1g64110*, different promoter::GUS constructs and tissue specific artificial microRNAs to target the gene *At1g64110* were created and successfully transformed into Arabidopsis.

Future work will be focused on selecting homozygous transgenic promoter::GUS and amiRNA Arabidopsis lines, analyse expression and function and linking the gene to developmental pathways in syncytia.

To confirm the different expression patterns for the genes *At4g28000* and *At5g52882*, promoter::GUS fusions together with qRT-PCR should be performed and analysed.

6 Summary

Previous transcriptome analysis has shown that many genes are up regulated and down regulated in syncytia. One of the strongly up regulated genes, *At1g64110*, codes for an ATPase. Infection tests of *At1g64110* T-DNA mutants with *Heterodera schachtii* revealed that this gene is important for nematode development.

The aim of this thesis was to prepare transgenic Arabidopsis lines for a functional analysis and for a expression analysis of *At1g64110*. For the functional analysis, artificial microRNA constructs with tissue specific promoters were created to silence the gene in the whole plant (promoter *CaMV*) and in syncytia (Promoter *MIOX5*, Promoter *PDF2.1*). The constructs were successfully transformed into Arabidopsis. 15 T1 Arabidopsis lines for each construct were isolated for further studies.

For the expression analysis three promoter::GUS constructs with different length were created and also transformed into Arabidopsis. Preliminary analysis of randomly selected T1 Arabidopsis plants showed transgenic promoter activity in trichomes and siliques. 15 T1 Arabidopsis lines for each construct were isolated for further GUS expression analysis.

Moreover, the previous transcriptome analysis for the gene *At1g64110* was confirmed by qRT-PCR during this project. The qRT-PCR showed a 97.2-fold up regulation of the gene in 15 dpi (days post infection) syncytia in comparison to uninfected roots which is in line with the previous GeneChip data. A qRT-PCR comparing the expression of *At1g64110* in syncytia with younger and older roots showed a 5.2-fold up regulation of the gene in syncytia.

The gene *At5g52882* was found to be highly similar to the gene *At1g64110*, but as no transcriptome expression data were available for this gene a RT-PCR and a qRT-PCR were done. The analysis showed no change in expression levels of this gene in 15 dpi syncytia in comparison to normal roots from the elongation zone.

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8 Attachment

8.1 Gene sequence for *At1g64110.2*

Primer for RT-PCR and qRT-PCR

Forward primer: GTGGGTTTAGGCTTGGCTTCT

Reverse primer: TGTTGGTAAAGCTCGGCAGG

Exon Intron

ATGGACAGCAAACAGATGTTGTTGTCGGCGCTTGGCGTCGGAGTTGGAGTAGGTGTGGGTTTAGGCTTGG
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8.2 Amino acid sequence for *At1g64110*

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       1 mllsalgvgv gvgvglglas gqavgkwagg nsssnnavta dkmekeilrq vvdgreskit
      61 fdefpyylse qtrvlltsaa yvhlkhfdas kytrnlspas raillsgpae lyqqmlakal
     121 ahffdaklll ldvndfalki qskygsgnte sssfkrspse saleqlsglf ssfsilpqre
     181 eskaggtlrr qssgvdikss smegssnppk lrrnssaaan isnlasssnq vsaplkrsss
     241 wsfdekllvq slykvlayvs kanpivlylr dvenflfrsq rtynlfqkll qklsgpvlil
     301 gsrivdlsse daqeidekls avfpynidir ppedethlvs wksqlerdmn miqtqdnrnh
     361 imevlsendl icddlesisf edtkvlsnyi eeivvsalsy hlmnnkdpey rngklvissi
     421 slshgfslfr egkaggrekl kqktkeessk evkaesikpe tktesvttvs skeepekeak
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     601 lftlaskvsp tiifvdevds mlgqrtrvge heamrkikne fmshwdglmt kpgerilvla
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     721 gsdlknlctt aayrpvreli qqerikdtek kkqreptkag eedegkeerv itlrplnrqd
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Table xy: Amino acid sequence of *At1g64110*

8.3 Gene sequence for *At5g52882*

Primer for RT-PCR and qRT-PCR

Forward primer: TCTGTTATCTGCCCAAGCTC

Reverse primer: TTTTCCTTGAGCCTCCTTCT

Exon Intron

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CTTAAGAAATACGGAAACTCTTCAAAAGTGACAGTACTTTCTCTACCATCAACAATTTGTCTAATCAACT
CTTGCTCAATCTGTTCTCCGGTGAGTCCATCTTCAACAGAACCTGACCCGTTTGCCCATCTTCCCAAACT
TTGACCTGATGCTAACCCAATCCCTAACCCAACACCAACACCTAAAGCTGACAACAACACGCTCTTCTGC
TC

8.4 Gene sequence for *At4g28000*

Exon Intron

ATGGAGCAGAAGAGCGTGTTGTTTTCCGCGTTAGGGGTTGGTGTTGGGTTAGGGATTGGTCTAGCGTCGG
GTCAGAGTTTGGGTAAATGGGCAAACGGGTCTATTTCTGCGGAGGATGGACTAACCGGAGAAAAGATTGA
GCAAGAGTTGGTTAGGCAGATCGTTGATGGCAGAGAGAGTAGTGTCACTTTCGACGAGTTTCCTTATTAC
CTAAGgtacattgctcgacatatctttcttgcattttattactaaaagattgataacgttgcctaaatgg
gacagtgttgtgttatatactaatgtttcttttccatgttgcagTGAGAAAACTCGGCTTTTATTGACAA
GTGCAGCTTATGTTCACCTAAAGCAATCTGATATCTCGAAGCACACGCGTAATCTTGCACCTGGAAGTAA
GGCCATTCTTCTGTCTGGACCTGCTGgtaaaaatctcctacccttttgtcttccaacctcaatcttttc
taattcagactttggtgtaatttttgttgcctcttttactctgcagAATTCTATCAGCAAATGCTTGCAAA
AGCATTGGCTCATTACTTTGAATCGAAATTACTGTTACTAGATATAACCGACTTCTCTATCAAGgtgatt
cattgcatacaaatttagtatctttgtttgctaaagaacagcttgtaatgttcctctttcatattaattg
ttacagATACAGAGCAAGTATGGATGCGTCAAGAAAGAACCTgtaagttacttgtgatcttatgttgtgt
agcttaattttgtgtgttgaccatgattatgttttctttcatctctaatgttttgatagTCTCACAAGAG
GTCTATTTCTGAGTTGACAATGGACAAAATGTCTAATTTGATGGGTTCTATCTCTGTGCTCTCCCAAAAG
GAAGCTACAAGAGgtacaatatcaaaacactggtctttgtttatgtattgccctcaaaagatctcaagtg
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ttgtgttggttttgatatcttgtttgttttgtcatctggaatatagctttatatgttttatgctagctca
ctaattgctctcacgtaatacgtcagGGGCTTTGACGTTACAAGTCAGCCTCCACGACTCAAGCGGAATG
CTTCTGCTGCTTCTGATATGAGTAGCATATCATCCCGTTCTGCAACTTCTGTTTCAGgtgtgtgaaagca
ttgaaatgaataatttttcttacgtttaacaaggtctagatcctctttaagtcttattctagtttagattt
ctaaaccctttttgttcatttgtggctgtttatatctttctagCTTCAAGCAAACGCAGTGCTAACTTGT
GCTTTGATGAGAGACTTTTCCTTCAGTCACTTTACAAGgtaacacttaaaatgaaatcagcagggaaaga
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CAATCCTATAATAATATACCTGAGGGATGTTGAGAAGCTTTGTCAGTCAGAAAGGTTCTACAAGTTGTTC
CAGAGGCTCTTGACGAAGCTTTCTGGCCCTGTTTTGGTTCTCGGCTCAAGACTATTGGAACCCGAAGATG
ACTGTCAAGAAGTAGGAGAGGGCATTTCTGCTTTGTTTCCTTACAACATCGAGATCAGACCGCCAGAGGA
TGAAAATCAACTCATGAGCTGGAAAACGAGATTTGAAGATGACATGAAAGTGATACAGTTTCAAGACAAC
AAGAACCACATAGCCGAGGTTCTAGCGGCTAATGATCTTGAGTGTGATGACTTAGGTTCCATATGCCATG
CAGATACAATGTTCCTAAGCAGTCACATTGAGGAAATTGTTGTTTCTGCAATCTCCTACCATCTGATGAA
CAACAAAGAACCCGAATACAAAAACGGGAGGCTTGTAATATCTTCTAATAGgtactaagaaccacagtta
tctcttcaacaaaactaagtaattattattttcttatagctaactaaataaatggttctgcaacagCTTG
TCCCATGGACTGAATATATTACAGGAAGGTCAAGGATGCTTTGAGGACTCCTTGAAGCTGGACACAAACA
TCGACTCCAAGgtataatacagttttgaccatgtactttaaaatttaaagtgagtaaaatgtgaatggc
tctggtgcagGTAGAAGAAGGTGAAGGGATAACAAAGAGCGAGTCAAAATCTGAGACAACTGTTCCGGAA
AACAAAAATGAATCGGATACATCAATCCCGGCAGCAAAAAATGAATGCCCCCTGCCTCCGAAAGCACCTg
tacatgtaaactaatacatatgattcagcattgataccttgatgtgtttgcctgaacataggtttgattt
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CCCAGCAAATGAGATTGGTGTGACTTTTGCTGATATAGGTTCACTGGATGAAACCAAAGAATCACTTCAA
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TCCTCCTCTTTGGACCACCTGGTACTGGAAAAACTATGATGGCAAAAGCCATTGCGAACGAAGCTGGGGC
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GCTTTGTTCACCCTCGCGGCCAAAGTGTCTCCAACTATTATATTTGTGGATGAAGTGGATAGTATGCTGG
GACAGAGGACAAGAGTTGGCGAGCATGAAGCTATGAGGAAGATCAAAAATGAGTTCATGACACACTGGGA
TGGGCTTATGTCAAATGCGGGTGACCGTATTCTCGTTCTTGCAGCAACAAACAGACCATTTGATCTCGAT
GAAGCTATCATCAGGAGGTTTGAGCGAAGgtaagcaggcccggtacgccttttcacttgaaaccctgaac
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AGCTTGCGCAGATGACAGATGGCTATAGTGGAAGTGATCTCAAGgtttgtcacagttcccatctcgcttt
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AGCAGCATATCGACCAGTGAGGGAGCTGATAAAGCAAGAGTGCTTGAAAGACCAGgtaagaactcctaaa
actcgtccttcctttatttctctactttctagcgctagaatgagtattttaatggtggtttctcataaaa
tccagGAGAGGAGGAAGAGAGAGGAAGCAGAGAAGAATAGTGAAGAAGGATCTGAAGCAAAAGAAGAAGT
ATCTGAAGAGAGAGGCATAACGTTGAGACCTTTGAGCATGGAAGACATGAAAGTAGCTAAAAGCCAGgta
aagttaaaaaaagattagttgcggtgttgatgatgatcatgcgcaaaacactaactgtttcgcgtttaca
gGTTGCTGCTAGTTTTGCAGCAGAAGGAGCAGGAATGAACGAACTCAAGCAGTGGAATGATTTGTATGGA
GAAGGAGGCTCCAGGAAAAAGGAACAGCTCTCTTACTTCCTTTAA

8.5 Sequence of the mirna precursor template pRS300

T7 SP6 amp resistance oligo A oligo B miRNA miRNA*

GGAAATTGTAAACGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAAC
CAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTC
CAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCA
GGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTA
AATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGG
AAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCAC
CACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCGCGCCATTCGCCATTCAGGCTGCGCAACTGT
TGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGC
GATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTGTAATA
CGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATC
GAATTCCTGCAGCCCcaaacacacgctcggacgcatattacacatgttcatacacttaatactcgctgtt
ttgaattgatgttttaggaatatatatgt**aga**gagagcttccttgagtccat**tcacaggtcgtgatatga**
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tgaatgatgcggtagacaaattggatcattgattctctttgattggactgaagggagctccctctctctt
ttgtattccaattttcttgattaatctttcctgcacaaaaacatgcttgatccactaagtgacatatatg
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GGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGT
TAATTCCGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC
ACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTA
ATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCC
AACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTC
GGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGG
GATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGC
TGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCG
AAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCG
ACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCAC
GCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCA
GCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCA
CTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGT
GGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTT
CGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGC
AAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACG
CTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGAT
CCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTAC
CAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCC
CCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGA
CCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGT
CCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAG
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TTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTT
AGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAG
CACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAA
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CCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCT
TACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTT
CACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGG
AAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGA
GCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGT
GCCACCTG

8.6 Sequence of the *At5G56640.1* (MIOX5) promotor

CATggaggaagatgagactgatgcgccactcaaaatatatatatgagactgaaataagtccactaagccc
gatttttaatgggccataaaagcccatatgttttatgaaaggcctaagttgtaatgttctgtttttagcc
gttgcaacaaacttcatcatctcataatgtttttctctagctttgatgtcgtaatcttcttccatcata
atttaaaggaaataagaaaatgatgtcgaaggaaaacttatattacgaaaactgttatgccattgtagta
tttgtaacaaactaataacaacgagatatttttgagttgtcttttaacaatgcaaaaatatttgttttca
ctcaaaagtcgaaagcatgggccgtggaaactcacacgaattgtcttctccacttggctctaaagttagt
tagtcaattagttacgaacataatcaaaagaaaaagaaaaaaaacgtaaaaagacaaccaatttattaca
atttacacatgttcaaaatccacaatactccaaaatttgaaggagaatatactgtaaatagcataaatgg
gggagaaactaataagtgtgaatgttataaatatgaaaaatgggggaaagtgaaaaacactgataaaaa
ggtaagaaaatggagaatctttttttttttgaataagaaaatggagaatctaagtataattaactctctt
ggacatatgtgtctatgataattcataagaatatcacatagatgttaatagtctactatatctattttag
aacatgacacatgtcttcctaaatcttaaaaggaagaaaatatgccaaaaagttgaccaatcaagaagag
agtaaatttaaggatttaggatgtttggtatatttcaaaacataaacaaacgcagtagagtaaataaact
aaaaaaaacgaaaacaaaaaaattaactcttgtatataaatgttaagagatctttatcatgccacagttt
ttattttcttggacaaagtttatttttcgtggtcaaaagatttggatttctctctaaatatagaggaaag
tacctatcgtctcctaaaccatcaccactacaaataccctttttgtgtctccatcaaattacatcttttc
gtagacattctctctcgatctttagttttcataactcatcttctttttttactttgttttttttttggaagA
TG

8.7 Sequences by AGOWA sequencing service

Promoter 1

Primer: GUS reverse 5´-ACAGTTTTCGCGATCCAGAC-3´
Template: pPZP3425

GGTTGGGGTTTCTACAGGACGGACCATGGGTTTGCTGTCCATTTGATCTTCCAAAAGCAAAATATGTATT
AAAAAAAAAACAAAAAAAAACAAAGTATTAGTATTATTAATATATAGATCAAGATCTAAAACGTTGTTAC
TAATCGTAACAACAATAAAAATTGAATGATAACAAACAAAAGGTATCGATTTATCTATAATTCTATATTG
ACTATTTAACCTAACTTATTCGAGAATCCAACCGAAACTTTGAAAGTATTTTCAAAGATTTAACAAGAGA
AATATATACCTTGTTTCGTAAGATATCTCTCTCAAATCTTTTCTTCTGCAGTTTCTTTGTACTTGTTTTG
GTCCTAACGATTCAATAGTCTGAAAAATCACAGAGAGTAGTGAGAACCGTGAAAACCAGATCTATTTACA
CGTCATCACCGGTGTTGAAAATGAGGCAAAGACGGCGGCGGATATGAGAGATTTAGGGTTCTTTTAATGA
AGATTTTTTTGATCGCTTCTCTGCTTTTTTATTTCATGGGGGTAATATGTTTGCACATGAAAACTGATGA
GTCATTACCGAGACTTTGCGTACGTGTTGTCATATAAACGGCTACTAGGTTGGTTTTTCGGCAGTTTTTT
AATTCTGATGTTGCCGCTTCTTTACTCTAATGCCCTTTAGTTTTTTAGTTTCTTTAATTTATTCCTTGAT
AGTTTCGGAATTGTCATTATAGTCCAGCTTTAATTAAATTATTCCGGTAAATTTTGTTTTCGATTGTGGG
TATTCTGTGATAATTGAATATTTTTATAGGGGTAGTTTATAGATGTTGGCACAAAATTAAACACAAAGGA
AAAAATGTAGATAAAACACTAAAAAAAAAGGGGTGCAGACTGTAGAAAGCATGACTGTGT

Promoter 2

Primer: -175 forward 5´-CAAGCTGCTCTAGCCAATAC-3´
Template: pPZP3425

CGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAA
TGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGA
ATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACATGATTACGAATTCAAAATTTCGAATGG
TTAGTTTTTCTTTCGAGGACCAAAACAAAAAGCCCATTCAATCACTAGAAAAATATCACTAGTCAATCAA
TAGACCAAAAGATTGAAAGTAGGATATATTTGTTTAATAATGCCTACGATTCTGCGAAGACAGGAGAAGC
CATACCTTTCAATCTAAGCCGTCAACTTGTTCCCTTACGTGGGATCCTATTATACAATCCAACGGTTCTA
AATGAGCCACGCCTTCCAGATCTAACACAGTCATGCTTTCTACAGTCTGCACCCCCTTTTTTTTTAGTGT
TTTATCTACATTTTTTCCTTTGTGTTTAATTTTGTGCCAACATCTATAACTTACCCCTATAAAAATATTC
AATTATCACAGAATACCCACAATCGAAAACAAAATTTACCGGAATAATTTAATTAAAGCTGGACTATAAT
GACAATTCCGAAACTATCAAGGAATAAATTAAAGAAACTAAAAAACTAAAGGACATTAGAGTAAAGAAGC
GGCAACATCAGAATTAAAAAACTGCCGAAAAACCAACCTAGTAGCCGTTTATATGACAACACGTACGCAA
AGTCTCGGTAATGACTCATCAGTTTTCATGTGCAAACATATTACCCCCATGAAATAAAAAAGCAGAGAAG
CGATCAAAAAAATCTTCATTAAAAGAACCCTAAATCTCTCATATCCGCCGCCGTCTTTGCCTCATTTTCA
CACCGGTGATGACGTGTAAATAGATCTGGTTTTC

Promoter 3

Primer: GUS reverse 5´-ACAGTTTTCGCGATCCAGAC-3´
Template: pPZP3425

TTTGATTTCCGGGTTGGGGTTTCTACAGGACGGACCATGGGTTTGCTGTCCATTTGATCTTCCAAAAGCA
AAATATGTATTAAAAAAAAAACAAAAAAAAACAAAGTATTAGTATTATTAATATATAGATCAAGATCTAA
AACGTTGTTACTAATCGTAACAACAATAAAAATTGAATGATAACAAACAAAAGGTATCGATTTATCTATA
ATTCTATATTGACTATTTAACCTAACTTATTCGAGAATCCAACCGAAACTTTGAAAGTATTTTCAAAGAT
TTAACAAGAGAAATATATACCTTGTTTCGTAAGATATCTCTCTCAAATCTTTTCTTCTGCAGTTTCTTTG
TACTTGTTTTGGTCCTAACGATTCAATAGTCTGAAAAATCACAGAGAGTAGTGAGAACCGTGAAAACCAG
ATCTATTTACACGTCATCACCGGTGTTGAAAATGAGGCAAAGACGGCGGCGGATATGAGAGATTTAGGGT
TCTTTTAATGAAGATTTTTTTGATCGCTTCTCTGCTTTTTTATTTCATGGGGGTAATATGTTTGCACATG
AAAACTGATGAGTCATTACCGAGACTTTGCGTACGTGTTGTCATATAAACGGCTACTAGGTTGGTTTTTC
GGCAGTTTTTTAATTCTGATGTTGCCGCTTCTTTACTCTAATGCCCTTTAGTTTTTTAGTTTCTTTAATT
TATTCCTTGATAGTTTCGGAATTGTCATTATAGTCCAGCTTTAATTAAATTATTCCGGTAAATTTTGTTT
TCGATTGTGGGTATTCTGTGATAATTGAATATTTTTATAGGGGTAGTTTATAGATGTTGGCACAAATTAA
ACACAAAGGAAAAAATGTAGATAAACACTAAAAAAAAAGGGGTGCACCCCTTTTTTTTTAGTGTTTATCT
ACATTTTTTCCTTTGTGTTTAATTTGTGCCAACATCTATAAACTACCCCTATAAAAATATTCAATTATCA
CAGAATACCCACAATCGAAAACAAAATTTACCGGAATAATTTAATTAAAGCTGGACTATAATGACAATTC
CGAAACTATCAAGGAATAAATTAAAGAAACTAAAAAACTAAAGGGCATTAGAGTAAAGAAGCGGCAACAT
CAGAATTAAAAAACTGCCGAAAAACCAACCTAGTAGCCGTTTATATGACAACACGTACGCAAAGTCTCGG
TAATGACTCATCAGTTTTCATGTGCAAACATATTACCCCCATGAAATAAAAAAGCAGAGAAGCGATCAAA
AAAATCTTCATTAAAAGAACCCTAAATCTCTCATATCCGCCGCCGTCTTTGCCTCATTTTCAACACCGGT
GATGACGTGTAAATAGATCTGGTTTTCACGGTTCTCACTACTCTCTGTGATTTTTCAGACTATTGAATCG
TTAGGACCAAAACAAGTACAAAGAAACTGCAGAAGAAAAGATTTGAGAGAGATATCTTACGAAACAAGGT
ATATATTTCTCTTGTTAAATCTTTGAAAATACTTTCAAAGTTTCGGTTGGATTCTCGAATAAGTTAGGTT
AAATAGTCAATATAGAATTATAGATAAATCGATACCTTTTGTTTGTTATCATTCAATTTTTATTGTTGTT
ACGATTAGTAACAACGTTTTAGATCTTGATCTATATATTAATAATACTAATACTTTGTTTTTTTTTGTTT
TTTTTTTAATACATATTTTGCTTTTGGAAGATCAAATGGACAGCAAACCCATGGTCCGTCCTGTAGAAAC
CCCAACCCGGAAATCAAA

***CaMV*::amiRNA**

Primer: mirna forward
Template: pPZP3425

ACTCGCTGTTTTGAATTGATGTTTTAGGAATATATATGTAGAGGAGAGTTTCATATACGTTTTTCACAGG
TCGTGATATGATTCAATTAGCTTCCGACTCATTCATCCAAATACCGAGTCGCCAAAATTCAAACTAGACT
CGTTAAATGAATGAATGATGCGGTAGACAAATTGGATCATTGATTCTCTTTGATAAACGTTTATGAAACT
CGCCTCTCTCTTTTGTATTCCAATTTTCTTGATTAATCTTTCCTGCACAAAAACATGCTTGATCCACTAA
GTGACATATATGCTGCCTTCGTATATATAGTTCTGGTAAAATTAACATTTTGGGTTTATCTTTATTTAAG
GCATCGGCATGGATCCTCTAGAGTCCGCAAAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTATT
TTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCTTATAGGGTTTCGCTCATGT
GTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAAT
TCCTAAAACCAAAATCCAGTGACCTGCAGGCATGCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTG
ACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAA
TAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGCTAGAGCAGC
TTGAGCTTGGATCAGATTGTCGTTTCCCGCCTTCAGTTTAAACTATCAGTGTTTGACAGGATATATTGGC
GGGTAAACCTAAGAGAAAAGAGCGTTTATTAGAATAATCGGATATTTAAAAGGGCGTGAAAGGTTTATCC
GTTCGTCCATTTGTATGTGCATG

Primer: mirna reverse
Template: pPZP3425

ACTATATATACGAAGGCAGCATATATGTCACTTAGTGGATCAAGCATGTTTTTGTGCAGGAAAGATTAAT
CAAGAAAATTGGAATACAAAAGAGAGAGGCGAGTTTCATAAACGTTTATCAAAGAGAATCAATGATCCAA
TTTGTCTACCGCATCATTCATTCATTTAACGAGTCTAGTTTGAATTTTGGCGACTCGGTATTTGGATGAA
TGAGTCGGAAGCTAATTGAATCATATCACGACCTGTGAAAAACGTATATGAAACTCTCCTCTACATATAT
ATTCCTAAAACATCAATTCAAAACAGCGAGTATTAAGTGTATGAACATGTGTAATATGCGTCCGAGCGTG
TGTTTGTCCATGGTGTAATTGTAAATAGTAATTGTAATGTTGTTTGTTGTTTGTTGTTGTTGGTAATTGT
TGAAGGATC

***PDF2.1*::amiRNA**

Primer: SP-47
Template: pPZP3425

GCCAGCTTGCATGCCTGCAGGTCACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAGAAGTATT
TTACAAATACAAATACATACTAAGGGTTTCTTATATGCTCAACACATGAGCGAAACCCTATAAGAACCCT
AATTCCCTTATCTGGGAACTACTCACACATTATTCTGGAGAAAAATAGAGAGAGATAGATTTGTAGAGAG
AGACTGGTGATTTTTGCGGACTCTAGAGGATCCATGCCGATGCCTTAAATAAAGATAAACCCAAAATGTT
AATTTTACCAGAACTATATATACGAAGGCAGCATATATGTCACTTAGTGGATCAAGCATGTTTTTGTGCA
GGAAAGATTAATCAAGAAAATTGGAATACAAAAGAGAGAGGCGAGTTTCATAAACGTTTATCAAAGAGAA
TCAATGATCCAATTTGTCTACCGCATCATTCATTCATTTAACGAGTCTAGTTTGAATTTTGGCGACTCGG
TATTTGGATGAATGAGTCGGAAGCTAATTGAATCATATCACGACCTGTGAAAAACGTATATGAAACTCTC
CTCTACATATATATTCCTAAAACATCAATTCAAAACAGCGAGTATTAAGTGTATGAACATGTGTAATATG
CGTCCGAGCGTGTGTTTGTCCATGGTTGGAGAAAGAGAAAGAAAATTGAGAGAGACAGAGTTGGAAATAA
GTGTTATGTGTGTGTGTGTACATTACTAAGGATGAAGATGGCACTATATAAAGAGGTATCGGGATATTTG
ATAGTCTATTAGAGAATTGCATTTTTAAGTATTTGTTTAGATATTTTGGGGGTAAATATCATCTTTCTCG
GATAATTCACTTTTGATTTAAACCAAATTCGTAACATTTGTCAACATTTTTCTTCTTTTGACTGCTTTTT
CAACATTTTCTTTGTTTTCTAGTTTGTCGATTTATTTGCTTTACCGACCC
TA

***MIOX5*::amiRNA**

Primer: mirna reverse
Template: pPZP3425

ATATATACGAAGGCAGCATATATGTCACTTAGTGGATCAAGCATGTTTTTGTGCAGGAAAGATTAATCAA
GAAAATTGGAATACAAAAGAGAGAGGCGAGTTTCATAAACGTTTATCAAAGAGAATCAATGATCCAATTT
GTCTACCGCATCATTCATTCATTTAACGAGTCTAGTTTGAATTTTGGCGACTCGGTATTTGGATGAATGA
GTCGGAAGCTAATTGAATCATATCACGACCTGTGAAAAACGTATATGAAACTCTCCTCTACATATATATT
CCTAAAACATCAATTCAAAACAGCGAGTATTAAGTGTATGAACATGTGTAATATGCGTCCGAGCGTGTGT
TTGTCCATGGTCCAAAAAAAAACAAAGTAAAAAAGAAGATGAGTTATGAAAACTAAAGATCGAGAGAGAA
TGTCTACGAAAAGATGTAATTTGATGGAGACACAAAAAGGGTATTTGTAGTGGTGATGGTTTAGGAGACG
ATAGGTACTTTCCTCTATATTTAGAGAGAAATCCAAATCTTTTGACCACGAAAAATAAACTTTGTCCAAG
AAAATAAAAACTGTGGCATGATAAAGATCTCTTAACATTTATATACAAGAGTTAATTTTTTTGTTTTCGT
TTTTTTTAGTTTATTTACTCTACTGCGTTTGTTTATGTTTTGAAATATACCAAACATCCTAAATCCTTAA
ATTTACTCTCTTCTTGATTGGTCAACTTTTTGGCATATTTTCTTCCTTTTAAGATTTAGGAAGACATGTG
TCATGTTCTAAAATAGATATAGTAGACTATTAACATCTATGTGATATTCTTATGAATTATCATAGACACA
TATGTCCAAGAGAGTTAATTATACTTAGATTCTCCATTTTCTTATTCAAAAAAAAAAAGATTCTCCATTT
TCTTACCTTTTTTATCAGTGTTTTTCACTTTCCCCCCATTTTTCATATTTATA