Norwegian University of
Science and Technology

Characterization of three novel genes encoding postulated peptides of the IDA family, and their possible function in plant defense in *Arabidopsis thaliana*

Karina Stensland Hornslien

Biology

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Supervisor: Atle M. Bones, IBI
Co-supervisor: Ane Kjersti Vie, IBI

Norwegian University of Science and Technology
Department of Biology

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Abstract

IDL6 and IDL7 are postulated peptides of the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) peptide family in the model organism *Arabidopsis thaliana*. The genes encoding the postulated peptides, *IDL6* and *IDL7,* were investigated to elucidate the function of the peptides and the possible relations to plant defense and stress tolerance in *A. thaliana.* A phenotypic characterization study of single and double knockout mutant lines*,* and over-expression lines of *IDL6* and *IDL7* was conducted to potentially find a phenotype linked to the genes by analyzing deviations in growth and development, compared to wild type (Wt) *A. thaliana.* Over-expression lines showed to have a higher amount of individuals reaching defined growth stages in seedling development, but this could not be concluded to be a phenotype linked to *IDL6* or *IDL7.*

The genes have been shown to be highly up-regulated in response to several stress treatments, both abiotic and biotic, in *in silico* data. Several different transgenic lines of *A. thaliana* were subjected to stress treatments to verify the *in silico* data. Knockout lines, double knockout lines, over-expression lines and *promoter:GUS* lines of *IDL6* and *IDL7* were treated with abiotic stress factors (NaCl, mannitol, UV-B light, H_2O_2 and paraquat) and biotic stress factors (aphids, *Pseudomonas syringae* and flagellin22) to investigate differences in tolerance, gene expression and promoter activity in the respective transgenic lines compared to Wt *A. thaliana.*

No absolute phenotype was detected in experiments related to stress tolerance, and great variations in promoter activity using *promoter:GUS* lines were observed. However, double knockout mutants of *idl6* and *idl7* showed a small trend to tolerate NaCl better than Wt *A. thaliana* and other transgenic lines. The great variation in *GUS* expression from GUS assay lead to a thorough screening and expression analyses of *promoter:GUS* lines. Results presented in this work, indicate that expression of the *IDL6* and *IDL7* genes may be subjected to extensive post transcriptional regulation through mRNA degradation, possibly governed by stress related environmental signals.

A novel member of the IDA peptide family, IDL8, was also analyzed. Segregation analyses of knock out lines were conducted and they were genotyped to verify that mutant lines of *idl8* were real knockouts. Results presented here show that one of the *idl8* mutant lines had the T-DNA inserted in the promoter region of the gene and is postulated to be a real knockout. However, further expression analyses should be conducted to verify that the gene is not transcribed. Over-expression lines of *IDL8* were successfully constructed through recombinant DNA technology and by T-DNA insertion using *Agrobacterium tumefaciens*.

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Abbriviations

ABA	**Abscisic acid**
ARE	**AUUUA repeat element**
Bp	**Base pair**
cDNA	**Complimentary DNA**
CFU	**Cell forming units**
CHX	**Cycloheximide**
CLE	**CLAVATA3-ENDOSPERM SURRONDING REGION (ESR)- RELATED**
Ct	**Crossing threshold**
DAB	**3,3'-diaminobenzidine**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
dsDNA	**Double-stranded DNA**
ETI	**Effector-triggered immunity**
Flg22	**Flagellin 22**
gDNA	**Genomic DNA**
GUS	**β-glucuronidase**
H_2O_2	**Hydrogen peroxide**
HR	**Hypersensitive respons**
HY5	***ELONGATED HYPOCOTYL 5*** **(gene)**
Hyg	**Hygromycin**
IDA	**INFLORESCENCE DEFICIENT IN ABSCISSION**
IDL	**IDA-Like**
JA	**Jasmonic acid**
LA	**Luria-Bertani agar**
LB	**Luria-Bertani**
LRR	**Leucine-rich repeat**
Kb	**Kilo base pair**
Km	**Kanamycin**
MACAW	**Multiple alignment construction and analysis workbench**
MAPK	**Mitogen associated protein kinase**
Mb	**Mega base pair**
MQ	**Milli-Q**
mRNA	**Messenger RNA**
MS	**Murashige and Skoogs**
NaCl	**Sodium chloride**
ndr	***Nonrace- specific disease resistance*** **(mutant)**
NRT	**Negative reverse transcription**
NTC	**No template control**
PAD4	***PHYTOALEXIN DEFICIENT 4*** **(gene)**
PAMP	**Pathogen associated molecular pattern**
PCR	**Polymerase chain reaction**
PDF	***Plant defensin*** **(gene)**
PRR	**Pattern recognizing receptor**
PR1	***PATHOGENESIS RELATED 1*** **(gene)**
Pst	***Psedomonas syringae*** **pv.** ***tomato***
PTGS	**Post-transcriptional gene silencing**

PTI	**PAMP triggered immunity**
qPCR	**Quantitative PCR**
REST	**Relative expression software tool**
RLK	**Receptor-like kinase**
ROS	**Reactive oxygen species**
RNA	**Ribonucleic acid**
RT	**Reverse transcription**
SA	**Salicylic acid**
SAR	**Systemic acquired resistance**
SD	**Standard deviation**
sos	***salt overly sensitive* (mutant)**
ssDNA	**Single-stranded DNA**
T-DNA	**Transfer DNA**
UidA	**β-glucuronidase (gene); *GUS***
UTR	**Untranslated region**
UV	**Ultra violet**
Wt	**Wild type**
X-Gal	**5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside**
X-Gluc	**5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid**

1 Introduction

1.1 *Arabidopsis thaliana*

Arabidopsis thaliana, also known as thale cress, is a small dicotyledonous plant of the *Brassicaceae* family. The *Brassicaceae* family includes several plants that are used as food crops such as broccoli, cauliflower and cabbage of the *Brassica oleracea* species, mustard (*Brassica* and *Sinapis* genus) and wasabi (*Wasabi japonica*) to mention a few. *A. thaliana* is a widely used model organism in plant biology research. It is especially valuable in the research field of molecular biology and genetics. Several features of *A. thaliana* make it an excellent model organism. It has a small genome of five chromosomes and a total of 125 mb which was sequenced in 2000 by the *Arabidopsis* Genome Initiative (Kaul et al., 2000; The Arabidopsis Information Resource, 2011).

In addition to its small genome, the plant itself is also relatively small. This makes it possible to cultivate numerous of plants in a restricted area. *A. thaliana* completes its life cycle in six weeks and produce a large quantity of seeds as a result of self-fertilization (The Arabidopsis Information Resource, 2011). Self-fertilization leads to a low rate of outcrossing (replacement of genetic material from a different individual) and this facilitates the analysis of progeny in later generations (Mitchell-Olds, 2001). The short life cycle makes it possible to investigate features of the plant through several generations in a relatively short period of time compared to other dicotyledonous plants. Efficient methods for genetic manipulation by transformation with *Agrobacterium tumefacience* also make *A. thaliana* a suitable organism when doing genetic research in molecular biology. Finally, *A. thaliana* is monoploid, which is a big advantage when performing genetic analyses (The Arabidopsis Information Resource, 2011).

1.2 Plant growth and development

The process of trying to elucidate the possible function of a gene and the possible process that a gene is involved in within an organism is complex and diverse. The approach chosen to investigate this is based upon which type of information initially available. If the plant individual shows a characteristic, aberrant phenotype and the aim is to try to find the gene that is responsible for this phenotype, the approach is called forward genetics. In the opposite case, if a plant with a specific known mutation within a gene is obtained and the aim is to elucidate the function of that gene, this can be done by searching for a visual phenotype. This approach is called reverse genetics.

In reverse genetics, it is important to have some general understanding of the plant's anatomy and the growth and development of the plant. This will ease the identification of phenotypic differences that might be related to a specific mutation. This chapter will give a brief introduction to the different

developmental stages and normal growth patterns of *A. thaliana*. A schematic presentation of a seedling and a fully grown *A. thaliana* is presented in Figure 1.1.

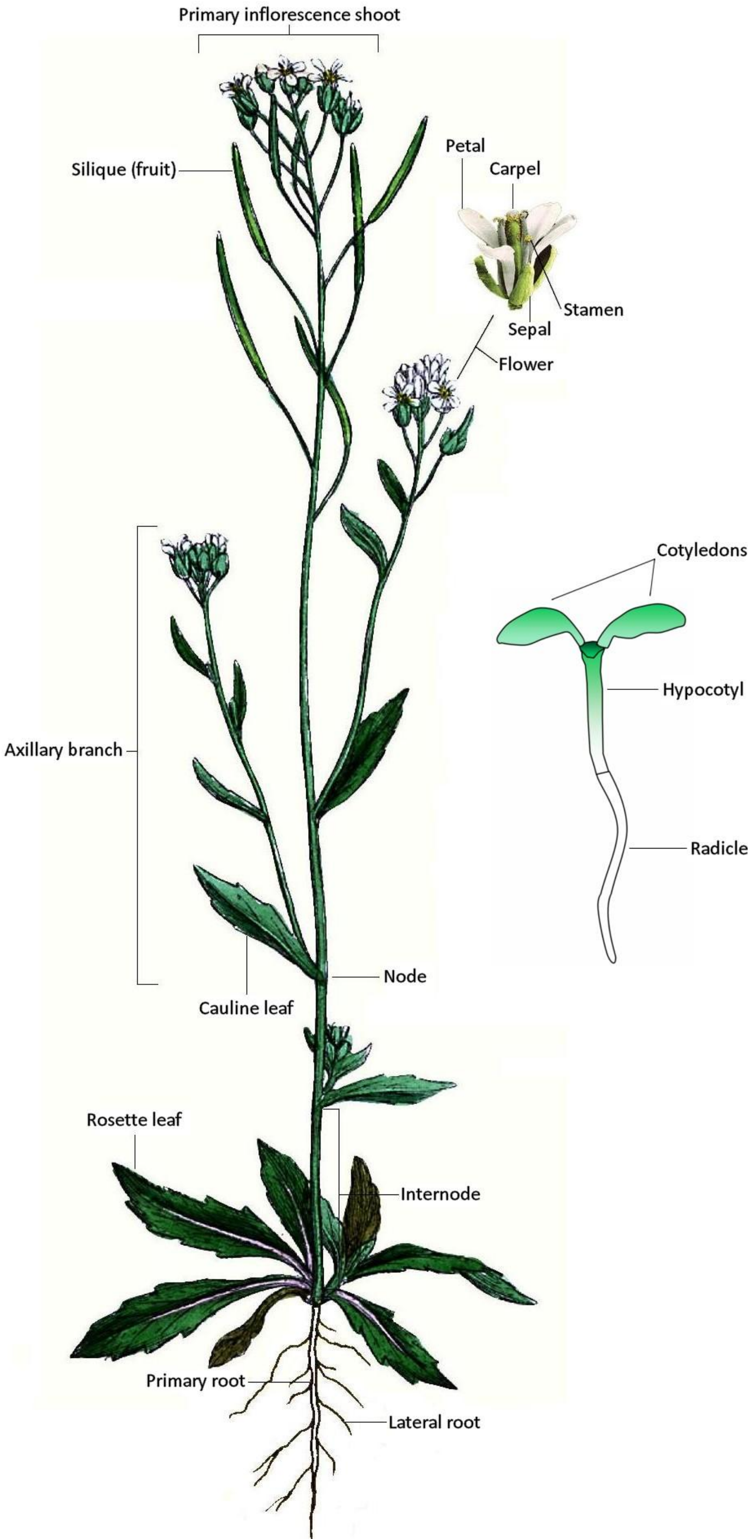

Figure 1.1 Schematic overview of plant organs of an adult individual of *Arabidopsis thaliana* and details of flower organ and major features of an *A. thaliana* seedling. Modified from the European Union Research Commission (1995-2011).

There are three different stages of development that all seed plants go through: the embryogenesis, vegetative development, and reproductive development (Taiz and Zeiger, 2006). The main events of the different developmental stages of *A. thaliana* are outlined in Figure 1.2.

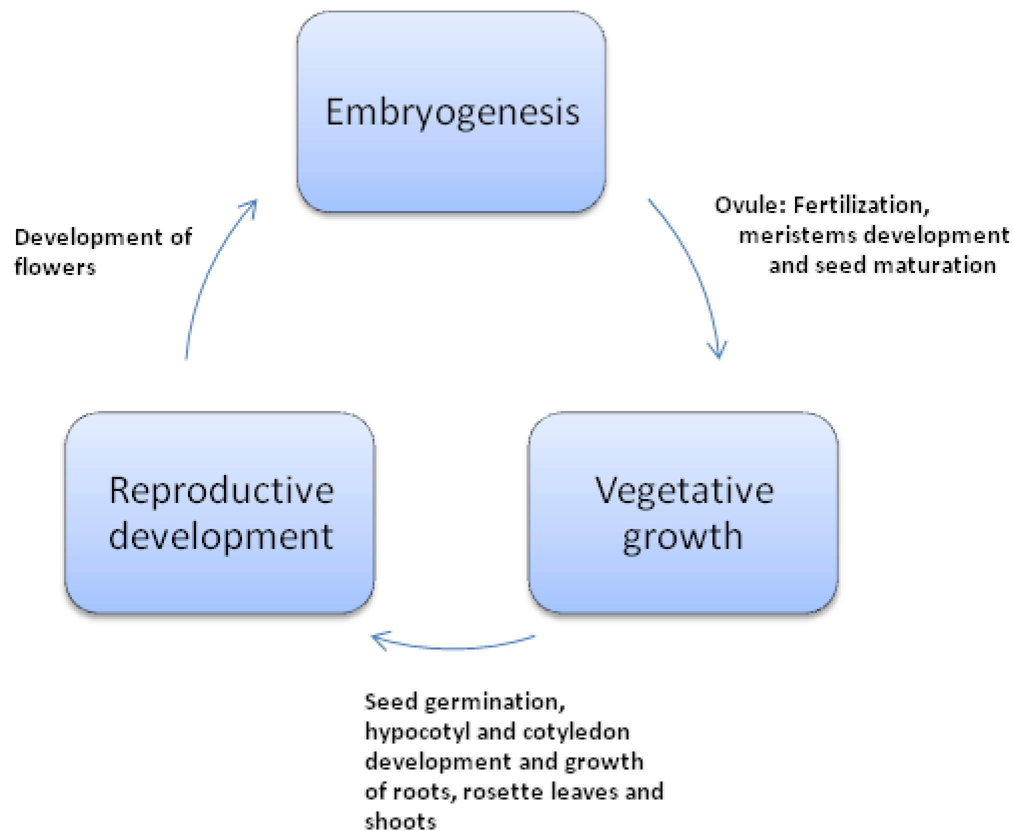

Figure 1.2 Developmental stages of *Arabidopsis thaliana*. Embryogenesis initiated after fertilization in the ovule to produce the embryo where the polarity of the plant is established and root and shoot meristems are formed according to polarity. Preferable conditions will ensure the offset of vegetative growth phase where the main tissue production is made possible by assimilation of energy through photosynthesis. When a preferable size is reached, and the environmental conditions are optimal, the vegetative plant will produce flowers in the reproductive state. Fertilization of an egg by a pollen will again lead to embryogenesis and production of new seeds.

1.2.1 Embryogenesis

The embryogenesis is the developmental stage where the plant evolves from a single cell to a mature multicellular seed. This process takes place in the ovule within the carpel of the flower. The egg cell is fertilized when pollen fuses with it. In the sequence of events that follows, the polarity of the plant is established and the cells will form epidermal, cortical and vascular tissue by assembling together in different groups. At this stage, the apical meristems are also formed at the growing point of the embryo (root and shoot). At the apical meristems, new tissue and organs will be developed during later developmental stages. When the embryogenesis is completed, the plant is considered as a mature seed.

This seed, however, does not develop further without preferable growing conditions (Taiz and Zeiger, 2006).

1.2.2 Vegetative development

When a seed starts to germinate, the vegetative development begins. The initiation of vegetative development normally depends on several different factors such as moisture levels, temperature and light. The radicle emerges from the root meristem and then the hypocotyl and the cotyledons develope from the shoot meristem. The formation of the cotyledons ables the plant to assimilate energy from the light and progress into photomorphogenesis and further development of the rosette, shoot and roots (Taiz and Zeiger, 2006).

1.2.3 Reproductive development

The offset of reproductive development starts after a period of vegetative growth in response to several signals, including plant size, photoperiod and temperature. Flowers develop from specialized floral meristems. *A. thaliana* are able to be fertilized by different means, both by self-fertilization and fertilization by a different individual (Taiz and Zeiger, 2006).

1.2.4 Growth and development analyses

A specific mutation in a gene may be linked to a specific phenotype. To investigate differences in phenotype amongst different mutants, a good knowledge of the plant growth and development is required. Boyes et al. (2001) published a standardized approach for analyzing growth and developmental stages in *A. thaliana*. This method is based on straight-forward measurements of emergence of radicle, hypocotyls and cotyledons, and further development of the rosette. It also includes analyses of size of the different plant organs and development of shoot, primary inflorescence and number of flowers. The analysis procedure is divided in two separate parts; i) analysis of plants grown on solid, nutritional medium in petri dishes (plate-based), and ii) analysis of plants grown on soil (soil-based). An overview of the different measurements recommended by Boyes et al. (2001) is presented in Table 1.1 and 1.2 for the plate-based and soil-based analysis respectively.

Table 1.1 Growth stages for plate-based phenotypic analysis of *A. thaliana* (Boyes et al., 2001).

Growth stage	Description	Days[a]
Principle growth stage 0	Seed germination	
0.10	Seed imbibition	3.0
0.50	Radicle emergence	4.3
0.70	Hypocotyl and cotyledon emergence	5.5
Principle growth stage 1	Leaf development	
1.00	Cotyledons fully opened	6.0
1.02	2 rosette leaves > 1 mm	10.3
1.04	4 rosette leaves > 1 mm	14.4

[a]: Average day from date of sowing (including 3 days of stratification).

Table 1.2 Growth stages for soil-based phenotypic analysis of *A. thaliana* (Boyes et al., 2001).

Growth stage	**Description**	**Days**[a]
Principle growth stage 1	Leaf development	
1.02	2 rosette leaves > 1 mm	12.5
1.03	3 rosette leaves > 1 mm	15.9
1.04	4 rosette leaves > 1 mm	16.5
1.05	5 rosette leaves > 1 mm	17.7
1.06	6 rosette leaves > 1 mm	18.4
1.07	7 rosette leaves > 1 mm	19.4
1.08	8 rosette leaves > 1 mm	20.0
1.09	9 rosette leaves > 1 mm	21.1
1.10	10 rosette leaves > 1 mm	21.6
1.11	11 rosette leaves > 1 mm	22.2
1.12	12 rosette leaves > 1 mm	23.3
1.13	13 rosette leaves > 1 mm	24.8
1.14	14 rosette leaves > 1 mm	25.5
Principle growth stage 3	Rosette growth	
3.20	Rosette is 20% of final size	18.9
3.50	Rosette is 50% of final size	24.0
3.70	Rosette is 90% of final size	27.4
3.90	Rosette growth complete	29.3
Principle growth stage 5	Inflorescence emergence	
5.10	First flower buds visible	26.0
Principle growth stage 6	Flower production	
6.00	First flower open	31.8
6.10	10% of flowers to be produced have opened	35.9
6.30	30% of flowers to be produced have opened	40.1
6.50	50% of flowers to be produced have opened	43.5
6.90	Flowering complete	49.4
Principle growth stage 8	Silique ripening	
8.00	First silique shattered	48.0
Principle growth stage 9	Senescence	
9.70	Senescence complete; ready for seed harvest	

[a]: Average day from date of sowing (including 3 days of stratification).

However, only a few mutations will give an observable phenotype under normal conditions. This is especially the case for genes involved in stress tolerance within the plant (chapter 1.4). Many genes involved in stress tolerance will not be activated unless the plant is exposed to the stress factor in question. Additional assays are therefore needed to investigate the presence of any observable phenotypes in a mutant of a stress related gene. The pre-set developmental stages presented may also be used in such cases if sufficient positive and negative controls are included (Boyes et al., 2001).

1.3 Cellular signaling in plants

In all multicellular organisms there are a variety of different mechanisms for communication between cells, both for signaling between adjacent cells as well as mechanisms for long distance signaling. In addition, mechanisms exist for perception and further signaling of changes in environmental conditions (Alberts et al., 2008).

In plants, the most studied cell-to-cell communication mechanisms are those mediated by plant hormones; auxins, gibberellins, cytokinins, absisicic acids, ethylene, brassinosteroids, salicylic acid and jasmonates. The plant hormones are small compounds that participate in a wide array of processes throughout the lifespan of the plant. Figure 1.3 below shows the chemical structures of the seven major plant hormones. The plant hormones are crucial for normal growth and development, but they are also important in several defense mechanisms in the plant. They act by modulating signals in a different cell or tissue than the one they are produced in (Santner et al., 2009).

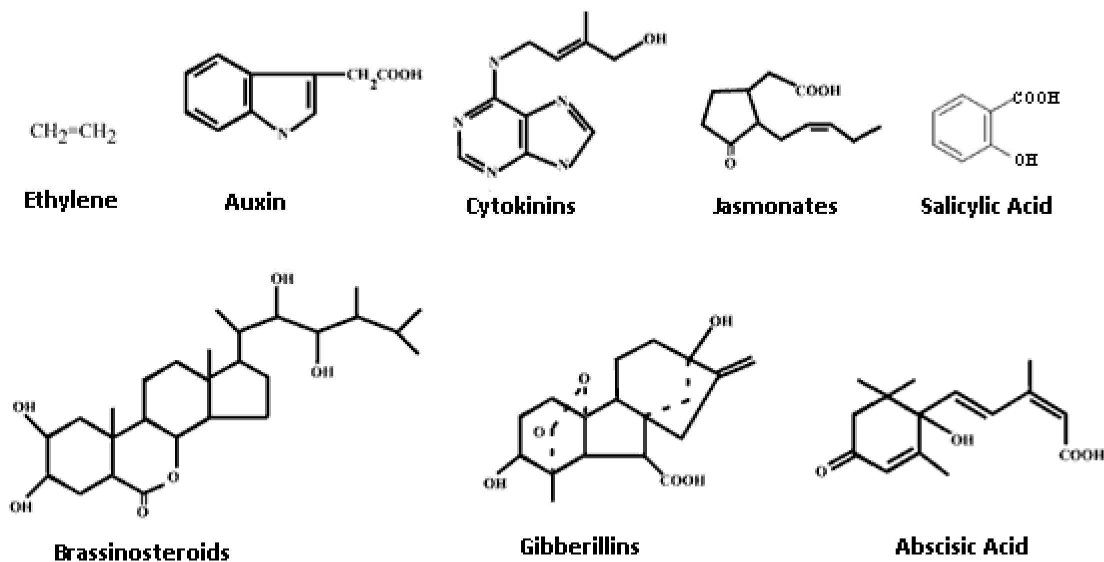

Figure 1.3 **Chemical structures of different plant hormones, modiefied from Gray (2004).**

However, in recent years, another group of signaling compounds has received a growing amount of attention, namely peptides and their signaling mechanisms. Interestingly, signaling through peptides is the most common way of cell-to-cell communication in animals, indicating that this mechanism evolved in an early stage of the evolutionary history of multi cellular organisms (Butenko et al., 2009; De Smet et al., 2009; Marshall et al., 2011).

1.3.1 Signal peptides in plants

A peptide is a small amino acid polymer. A peptide can be either bioactive in e.g. signaling processes, or they can be a result of degradation of larger proteins in relations to protein turnover. In most

bioactive processes, the peptide functions as a ligand which interacts with one or several other molecules (mostly cell surface receptors in cell-to-cell communication) in perception or processing of a signal (Farrokhi et al., 2008).

Plant peptides are small molecules ranging from about 60 to around 200 amino acids. In addition, the peptides are often processed from pro-peptides into smaller active peptides. The pro-peptides are often modified during processing in the Golgi apparatus. In general, these modifications may include addition of a biochemical functional group of various kinds, e.g. sulfur groups, hydroxyl groups, phosphate groups as well as lipids and carbohydrates. In addition, they may be cleaved by proteolytic enzymes and they can be as small as 5 amino acids and still be a functional peptide (Ytterberg and Jensen, 2010; Matsubayashi, 2011) .

There are several different well studied peptide signaling systems in plants, both secretory and non-secretory peptides, and they have been shown to be involved in a wide range of processes in the plant, including growth and development processes as well as defense responses (Matsubayashi and Sakagami, 2006).

The first peptide signaling system identified was the systemin from tomato (Pearce et al., 1991). This 18 amino acid peptide induces the expression of proteinase inhibitors and is an important component of the systemic defense mechanism upon wounding of plant tissue. Systemin was believed to act through the leucine-rich repeat receptor-like kinase (LRR-RLK) BRI1/SR160, but new investigations in 2009 showed that this may not be the case (Malinowski et al., 2009; Wheeler and Irving, 2010).

Although systemin was shown to not act through a LRR-RLK there are several other signal peptides which indeed work through known LRR-RLKs. CLAVATA3 (CLV3) is a peptide involved in regulation of shoot apical meristem growth. CLV3 acts through the LRR-RLKs CLV1 and CLV2. The peptide of this signaling system belongs to a protein family called the CLV3-ENDOSPERM SURROUNDING REGION (ESR) - RELATED (CLE)-family where the C-terminal end of the protein shows strong conservation (Wang and Fiers, 2010). Upon binding of CLV3 to the CLV1 and CLV2 receptors, an intracellular signaling pathway is activated which stimulates cell differentiation in the meristem. The offset of differentiation is mediated through inhibition of the gene regulatory protein that normally inhibits differentiation (Alberts et al., 2008).

1.3.2 The IDA peptide family

The INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) peptide family consists of six potential peptides in *A. thaliana* which show a highly conserved motif at the C-terminal end of the peptides (Butenko et al., 2003). The published sequence alignment of the IDA peptide family, consisting of IDA and the IDA-LIKE (IDLs) 1-5, is presented in Figure 1.4 together with homologues of the IDL1 peptide in 7 other different species. The *inflorescence deficient in abscission* (*ida*) mutant shows no

abscission of floral organs even after shedding of mature seeds, and studies of *ida* led to the discovery of the IDA signaling peptide. The IDA peptide consists of 77 amino acids and induces abscission in *A. thaliana* through interactions with the LRR-RLKs HAESA (HAE) and HAESA-LIKE 2 (HSL2). HAE and HSL2 then activate a mitogen activated protein (MAP) kinase phosphorylation cascade which eventually leads to abscission (Cho et al., 2008; Stenvik et al., 2008).

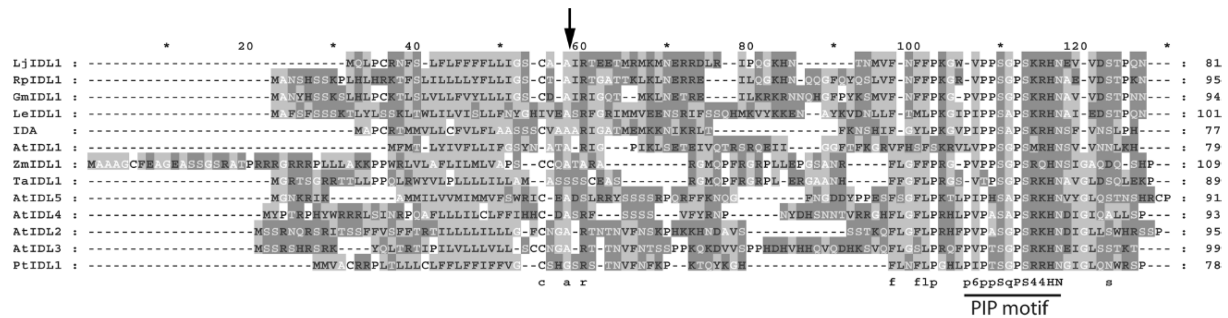

Figure 1.4 Peptide sequence alignment of IDA and IDL peptides in *A. thaliana*, tomato (LeIDL1), lotus (LjIDL1) soybean (GmIDL1), black locust (RpIDL1), maize (ZmIDL1), poplar (PtIDL1) and wheat (TaIDL1) (Butenko et al., 2003).

IDA and the IDL peptides 1-5 have predicted N-terminal secretion signals and all share a conserved motif at the C-terminal called a PIP motif. They also contain some additional conserved amino acids located N-terminally of the PIP motif and these, together with the PIP region, constitute the extended PIP (EPIP) motif. The conserved motifs of the IDA peptide family is highlighted in the sequence alignment in Figure 1.5 below. The PIP-motif is suggested to be the functional domain of IDA and the IDL proteins (Stenvik et al., 2008).

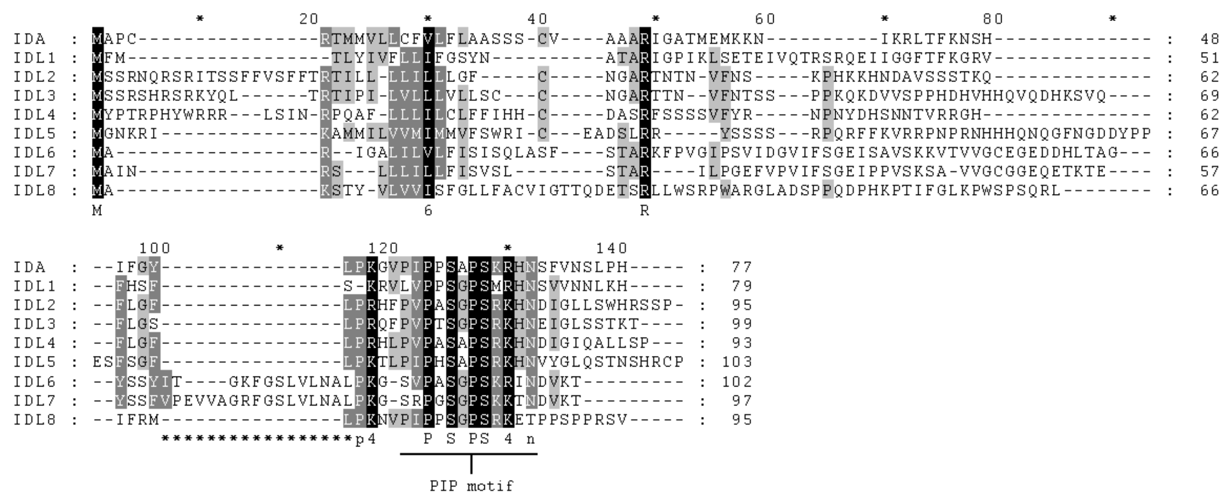

Figure 1.5 Peptide sequence alignment of IDA and the IDL peptides in *A. thaliana*. Highly conserved residues and the PIP motif is indicated (provided by Tore Brembu).

Recently, additional members of the IDA family in *A. thalina* (IDL6, IDL7 and IDL8) have been identified. The recent finding of the IDA-RELATED (IDR) family and more recently another family indicates that this might be a large family of potential peptide ligands in *A. thaliana*. The evolutionary

distance between IDA and IDLs is given in Figure 1.6. IDL6 and IDL7 show strong homology with the other IDLs, but also contain 5 additional amino acids within the EPIP peptide sequence.

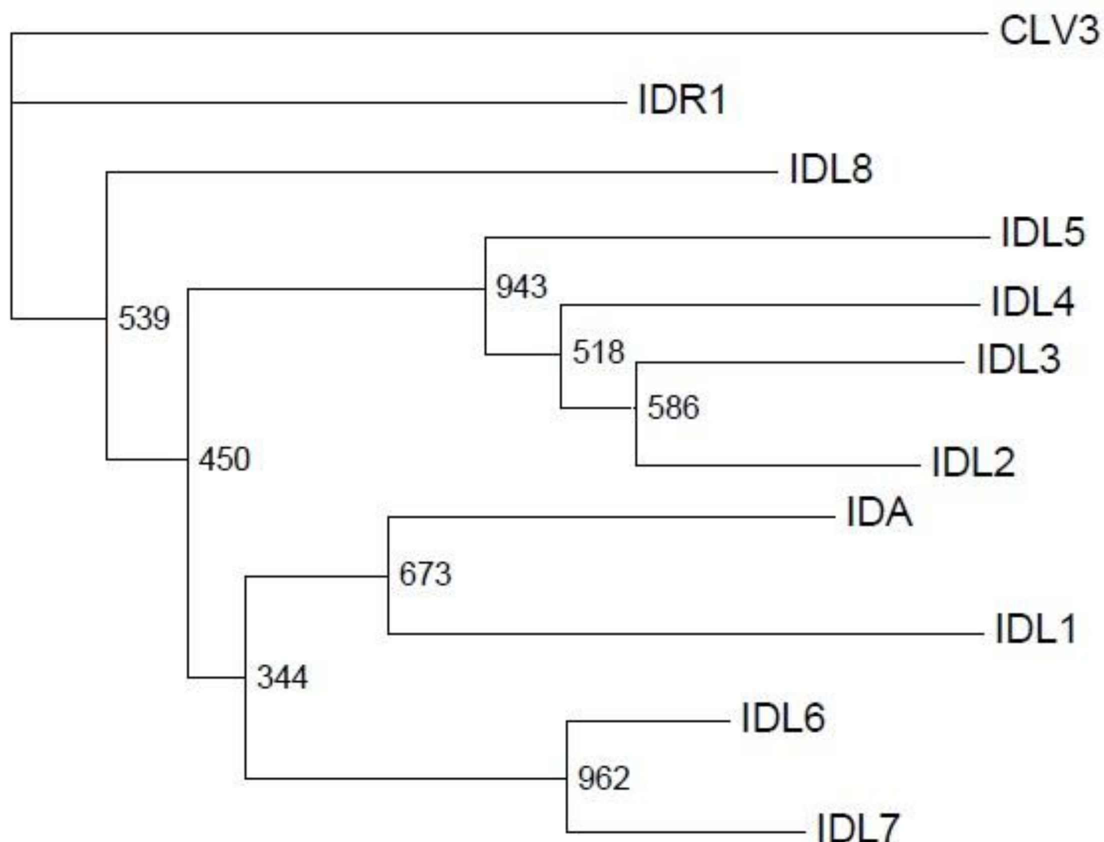

Figure 1.6 Phylogenetic tree of the IDA family (including IDR1). The tree is a bootstrapped neighbor joining tree based on the EPIP motif of the peptides and CLV3 was used as an out group. The numbers on branches indicate bootstrap values from 1000 bootstrap analyses (provided by Tore Brembu).

IDL6 and *IDL7* show low expression profiles under normal conditions but have been shown to be highly up-regulated in tissue treated with several different biotic and abiotic stress factors. Among the factors that induced the greatest up-regulation of *IDL6* and *IDL7* are ozone and ultra violet (UV) light. Biotic stress, such as treatment with certain pathogenic organisms or pathogen associated molecular patterns (PAMPs) from these (chapter 1.4.1), was also shown to up-regulate *IDL6* and *IDL7*. *IDL6* and *IDL7* are in addition co-expressed with several other genes that are defense related, such as several MAP kinases and WRKY transcription factors (unpublished data, Ane K. Vie).

IDL8 is an unannotated gene and is thus not available on any microarrays or in *in silico* data. Based on expressed sequence tag (EST) analyses, the open reading frame is postulated to be up-regulated during embryogenesis (unpublished data, Per Winge).

1.4 Plant defense and stress tolerance

Plants are exposed to different types of factors that may potentially be stressful or harmful for the plant. These factors may be of a biotic (living) character such as bacteria, fungi, insects or other herbivores attacking the plant. They may also be of an abiotic (non-living) character such as temperature changes, extreme light conditions and UV radiation, water deficiency or other nutritional deficiencies. Plants cannot escape these strains and have therefore evolved a large variety of defense mechanisms to try to avoid them or cope with them (Taiz and Zeiger, 2006).

1.4.1 Biotic stress defense response

Defense against biotic stress factors is divided into two main mechanisms; constitutive defense response and induced defense response. The constitutive defense responses (also described as preformed defense mechanisms) are always present in the plant. The cuticle which coats the outer cell walls of the epidermis of the plant is one such constitutive defense mechanism. In addition to reducing the water loss to the environment, the cuticle also serves as a barrier for defense against pathogens like virus, bacteria and fungi. Constitutive defense can also exist as stored compounds in the plant that repel attackers by being toxic, or giving the plant a bad taste that may repel herbivores. These compounds are secondary metabolites, or precursors of secondary metabolites, and they are often specific for different plant species (Anderson et al., 2010).

Inducible defense mechanisms are responses that are only initiated after the plant has been attacked by a potential enemy. These defense responses may act locally in the infected tissue, but signals can also be transferred throughout the plant to activate defense responses in plant tissues not yet infected. This is called a systemic defense response. If a plant survives a pathogen attack at one site it often develops resistance in the whole plant, and will be capable to easier fight off pathogens in late encounters. This is known as systemic acquired resistance (SAR) (Taiz and Zeiger, 2006).

The plant can recognize a pathogen attack through signaling pathways which are activated by insect derived compounds, e.g. from insect saliva. Plants are also able to recognize signaling compounds related to bacteria or fungi, e.g. flagellin and glycoproteins respectivly. These compounds are called pathogen-associated molecular patterns (PAMPs), and are specific for different types of pathogens (Ryan et al., 2007).

PAMPs are thought to interact with receptors on the cell surface or in the cytoplasm and the signal derived from PAMPs can eventually initiate a defense response. The interaction between PAMPs and receptors in the plant which leads to a defense response is described as the plants innate immunity (Janeway and Medzhitov, 2002). The receptors are called pattern recognizing receptors (PRRs), and are transmembrane proteins containing a leucine rich repeat (LRR) involved in the recognition of PAMPs. The PRRs are divided into two main classes: receptor-like kinases (RLKs) which possess an

intracellular kinase domain, and receptor-like proteins (RLPs) which lack an intracellular domain (Vakhrusheva and Nedospasov, 2011). Binding and activation of the receptors leads to activation of an intracellular MAP kinase phosphorylation cascade. This cascade enhances and transmits the signal that may eventually lead to altered gene expression through transcription factors (Colcombet and Hirt, 2008). An immunity response caused by recognition of PAMPs is defined as PAMP-triggered immunity (PTI) (Vakhrusheva and Nedospasov, 2011) and is often related to MAPK cascade signalling (Pitzschke et al., 2009).

In some cases, the pathogen also encompasses specific effector proteins which suppress the first line of defense in the plant, the PTI. This can lead to a successful invasion of the pathogen, or it can trigger a second line of defense in the plant. These effector proteins can be recognized by yet another set of receptors in the plant, called resistance (R) proteins. This response is called effector-triggered immunity (ETI). The R proteins belong to a class of proteins which in addition to a LRR domain have a nucleotide-binding domain (Vakhrusheva and Nedospasov, 2011). Figure 1.4 shows an overview of PTI and ETI responses in the plant upon pathogen invasion.

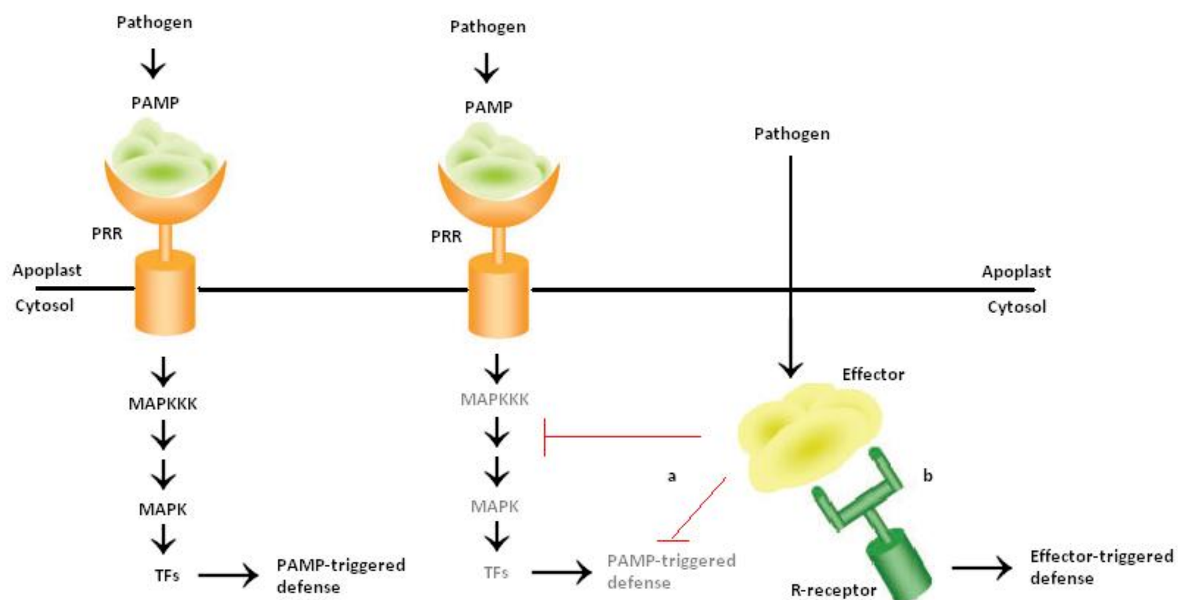

Figure 1.7 In the left part of the scheme, a transmembrane pattern recognition receptor (PRR) recognize pathogen associated molecular patterns (PAMPs) and a defense response is activated through intracellular signaling (mitogen activated protein kinases (MAPKs)) and activation of transcription factors (TFs). Pathogens that encompass effector proteins may suppress the PAMP triggered immunity (PTI) response (a), or another type of receptors, the resistance (R) receptor may recognize the effector molecule and initiate the effector-triggered immunity (ETI) response (b) (Modified from Pitzschke et al., 2009 and Anderson et al., 2010).

A common inducible defense mechanism in plants that may be activated by PAMP recognition or effector recognition is the hypersensitive response (HR). This mechanism creates unfavorable conditions for the pathogen in question and may inhibit further pathogen growth and spreading. The

HR mechanism also detoxifies harmful compounds secreted from the pathogen and limits spreading of these. HR eventually leads to a local programmed cell death. The pathway serves as a blocking mechanism for the pathogens availability of living plant tissue. The local necrosis will close of the infection and ultimately eliminate the pathogen infection (Heath, 2000; Lam et al., 2001).

1.4.2 Abiotic stress defense response

Abiotic stress factors can lead to morphological and anatomical changes and damages in the plant as well as cellular, biochemical and molecular changes and damages. Stress tolerance is associated with the ability of a plant to sustain in an unfavorable environment. Water deficiency, frost, heat and salinity all share some common features of cellular, biochemical and molecular responses upon exposure, and are often categorized as water deficit stress. Stress factors related to water deficiency is a large source of stress in nature. It causes an evolutionary pressure upon plant species and is maybe also the most important factor in limiting yield in agriculture worldwide today (Swindell, 2006).

Water deficit related stress factors have several common effects on growth and development in plants. Strategies to limit water loss include inhibition of leaf extension, leaf abscission, increased root growth and closure of stomata. Decreased leaf area and leaf expansion is a primary response to drought. Decreased leaf expansion is caused by a decreased turgor pressure in extending cells caused by the limited water availability and dehydration of cells. A decreased leaf area will limit water loss through transpiration and thus, the plant can sustain for a longer period if the water is limited. The decreased leaf expansion will reduce the consumption of energy in the leaves and enable the energy to be distributed to the root system. The energy will support further growth of the roots into lower soil layers that may contain more available water.

Several plant hormones are involved in the stress response caused by low water availability. Ethylene is an important signaling compound leading to leaf abscission to decrease total leaf area of the plant and thus decrease water loss by transpiration. Abscisic acid induces stomatal closure which reduces evaporation from the existing leaf area. Together these responses contribute to the sustainability of the plant in environments of low water availability.

Another group of abiotic stress is oxidative stress. This type of stress is induced by factors such as intense light (e.g. UV), wounding, herbicides and ozone. It may also be caused by drought or temperature stress as well as some biotic stress factors like pathogen attack (Gill and Tuteja, 2010).

Oxidative stress is defined as the production of reactive oxygen species (ROS). ROS are highly reactive and can cause damage to cell structures like DNA, proteins and lipid membranes if it is not removed quickly. Examples of ROS is hydrogen peroxide (H_2O_2), superoxide anion ($\cdot O_2^-$) and hydroxyl radical (·OH). Plants do not have the ability to escape UV irradiation or other factors that cause ROS production, and therefore have evolved several different defense mechanisms to cope with

oxidative stress. ROS can be removed from the plant by antioxidants as well as by enzymatic activity (Jansen et al., 1998).

The most effective enzyme that removes ROS from plants is superoxide dismutase (SOD). This enzyme is present in all aerobic organisms and catalyze the reduction of $\cdot O_2^-$ into H_2O_2 and oxidation into O_2. SOD activity has been shown to increase upon several different abiotic stress treatments in plants. Yet other enzymes will catalyze different types of ROS, but will not be further discussed. A non-enzymatic antioxidant worth mentioning is ascorbic acid. Ascorbic acid is found in all plant tissues and is considered as the most powerful scavenger of ROS in plants because of its great ability to donate electrons in a wide range of reactions (Gill and Tuteja, 2010).

2 Materials and methods

2.1 Experimental work on *A. thaliana*

Arabidopsis thaliana of the Columbia ecotype was used for all the experiments described in this thesis. All transgenic lines of *A. thaliana* used in experiments described here, are presented in Table 2.3, chapter 2.9.3. *A. thaliana* is native to Asia, Africa, Northern America and Europe, and can cope with a range of different growth conditions. The optimal temperature for growing *A. thaliana* is between 22-23°C. The optimal light intensities are between 120-150 µmol/m^{2}sec with a photoperiod of 8-24 hours. Long days are defined as a photoperiod of > 12 hours, and short days as a photoperiod of < 12 hours (Weigel and Glazebrook, 2002). Long days speed up the reproductive cycle whilst short days favor growth of vegetative tissue.

In the experiments described in this thesis, several different growth rooms and chambers were used: If not otherwise indicated, all plants grown on half strength Murashige and Skoog (½ x MS) nutrition medium (see Appendix A1 for recipe) were grown under long day conditions with a photoperiod of 16/8 hour light/dark and light intensity of 80 µmol/m^2/sec and a temperature of 22°C.

If otherwise not indicated all plants grown on soil were grown with the following growth conditions: Long day conditions with a photoperiod of 16/8 hour light/dark period and light intensity of 150 µmol/m^2/sec and a temperature of 20°C.

2.1.1 Surface sterilization of plant seeds

All seeds were vapor phase sterilized or liquid sterilized, before they were spread out on ½ x MS (see Appendix A1 for recipe) plates, in the experiments described in this thesis.

Vapour phase sterilization

A. thaliana seeds were placed in eppendorf tubes and then into a gas chamber in a fume hood. 100 mL chlorine was poured into a beaker in the gas chamber and 3 mL concentrated HCl was carefully poured into the bleach. The gas chamber was sealed and the seeds were left for sterilization by chlorine fumes for three to five hours. After sterilization 0,1% agarose (see Appendix A1 for recipe) was immediately added to the seeds (Clough and Bent, 1998).

Liquid sterilization

A. thaliana seeds were placed in an eppendorf tube and sterilized with 1 mL 70% ethanol (EtOH) for 5 minutes. The ethanol was then replaced with 1 mL of bleach solution (10 mL chlorine, 40 mL Milli-Q (MQ)-water and 1 mL 5% Tween) for 5 minutes. Finally, the seeds were washed for 5 minutes in washing buffer (50 mL MQ-water and 10 mL 5% tween) before they were resuspended in 0,1% agarose (see Appendix A1) (Clough and Bent, 1998).

2.1.2 Phenotypic characterization

The phenotypic analyses conducted in these experiments were based on the analytic platform published by Boyes et al. (2001). One part of the analyses was performed on seedlings grown on plates containing ½ x MS nutrition medium (see Appendix A1). The second part of the phenotypic measurements was conducted on plants grown on soil. All the measurements conducted were related to defined developmental stages previously presented in Table 1.1 and Table 1.2 (chapter 1.2.4).

In the plate-based analysis seeds were, in addition to analyses on regular petri dishes, sown on square plates on ½ x MS without sugar (see Appendix A1 for recipe) and grown vertically for root growth measurements. On sugar depleted medium, roots form less lateral roots and tend to grow more vertically (Mishra et al., 2009). This is convenient when measuring root growth. The plates were photographed every day for 12 days and ImageJ (Rasband, 1997) was used to calculate the length of the roots.

Measurements of plants grown on soil were conducted by registering development of different tissue and organs every day and by measuring height of primary shoot with a ruler.

2.2 Stress treatments of *A. thaliana*

To identify a potential involvement of the *IDL* genes in plant stress response, knockout lines, double knockout lines and over-expression lines of the gens *IDL6* and *IDL7* were subjected to different types of stress treatments in the search of a phenotype or any visual differences between the mutant lines and wild type (Wt) *A. thaliana.* All knockout lines and over-expression lines used in experiments described here, are presented in Table 2.4, chapter 2.9.3.

Promoter:GUS lines (see chapter 2.3) of the *IDL6* promoter and the *IDL7* promoter in *A. thaliana* were also subjected to a variety of stress treatments to analyze activity of the genes and the potential localization of the gene products. The specific transgenic lines are here on named: *promoterGENE OF INTEREST:GUS* (*pGOI:GUS*). *Promoter:GUS* lines used in experiments described here, are presented in Table 2.5, chapter 2.9.4.

2.2.1 NaCl assay

In the NaCl assay, seeds were sown out in large petri dishes (14 cm in diameter) containing ½ x MS (see Appendix A1 for recipe) and different concentrations of NaCl; 0 mM, 100 mM, 110 mM, 120 mM, 130 mM and 150 mM. One plate was divided into four sectors and 20 seeds from each line and Wt were sown out in defined sectors on each plate. The seeds were stratified for three days (for synchronizing of germination) before they were placed in the growth room with growth conditions as previously described, but with a light intensity of 140 µmol/m^2/sec. Three biological replicates were analyzed for each transgenic line and concentration. Each replica consisted of 3-5 parallel plates of

each transgenic line and concentration. Radicle emergence, cotyledon and rosette leaf development and green tissue development were registered every day for a total of ten days.

2.2.2 Mannitol assay

The same procedure as described for the NaCl assay was used in the mannitol assay. Mannitol was solved in ½ x MS following concentrations; 100 mM, 200 mM, 300 mM and 400 mM mannitol.

2.2.3 UV treatment for detection of ROS and qPCR

The *IDL6* and *IDL7* genes have shown to be up-regulated upon several different stress treatments in microarray experiments from *in silico* data (BAR, 2007), the genes could possibly be related to defense against oxidative stress (chapter 1.4.2).

ROS production in plants upon UV treatment was investigated in *idl6* and *idl7* knockout lines. Both knockout lines and a double knockout line were subjected to UV radiation and subsequently production of ROS in the plant tissue was analyzed by DAB-assay (described in chapter 2.4). This was done to investigate if the level of ROS production was altered in any visual manner in knockout lines compared to Wt.

Plants were treated with different doses of UV light (wavelength 365 nm) before harvesting of tissue for ROS detection. Plants were grown on ½ x MS and treated with UV by placing the petri dishes upside down on a UV table. Plants grown on ½ x MS in glass containers (diameter: 7.5 cm height: 9 cm) were also irradiated with UV light. Plants were irradiated for 5, 10 and 15 minutes before tissue, or whole plant was harvested for DAB assay.

Plants grown on soil were irradiated with UV for transcriptional analysis by quantitative (q) PCR (described in chapter 2.5.9) for confirmation of *in silico* data (BAR, 2007). The plants were placed in a box and the UV source was turned upside down to irradiate the plants from above. 14 days old Wt plants grown in growth cabinet were irradiated for 15 minutes with UV light of wavelength 365 nm. The cabinet had a 16 hour photoperiod with light intensity of 130 µmol/m^2/sec, temperature of 22/18°C (light/dark) and 60/70% (light/dark) relative humidity. Plants were replaced in the growth cabinet and plant tissue (excluding roots) was harvested after 0.5 h, 1 h, 3 h, 6 h, and 24 h for transcriptional analysis by qPCR.

2.2.4 Aphids

Several different insects from different orders are able to feed on *Brassicaceae* species. Aphids of the *Homoptera* order are amongst these. The generalist aphid *Myzus persicae* is a phloem-feeding insect which has been shown to activate several defense related genes in *A. thaliana* upon infestation (Kusnierczyk et al., 2007). Both *IDL6* and *IDL7* have previously been shown to be up-regulated upon infestation with aphids (Kusnierczyk et al., 2008) To investigate the potential role of *IDL6* and *IDL7*

in defense, *A. thaliana promoter:GUS* lines, *pIDL6:GUS* and *pIDL7:GUS*, were infested with *M. persicae* to potentially induce *GUS* expression.

Plants at growth stage 1.08 (Boyes et al., 2001) 21-25 days old, were infested with *M. persicae*. Aphids were transferred by a fine paint brush as described by Kuśnierczyk et al. (2007). The infested plants were kept in plexi glass cylinders in growth cabinets with a photoperiod of 16 hours with light intensity of 70 µmol/m^2/sec. The temperatures in the cabinet were 22/18°C (light/dark) and the relative humidity was 40/70% (light/dark). The aphids were bred on cabbage (*Brassica oleracea*), under the same conditions, upon transferring to *A. thaliana*.

Leaves from plants infested with aphids for 24 h, 48 h and 72 h were harvested for GUS assay. Leaves from plants not infested with aphids were harvested as negative controls. The GUS reaction was performed by vacuum infiltration as described in chapter 2.3.

2.2.5 *Pseudomonas* infiltration

5 weeks old vegetative *A. thaliana* GUS lines, *pIDL6:GUS* and *pIDL7:GUS*, grown in growth cabinets with short day conditions to develop large rosette leaves (Weigel and Glazebrook, 2002), were infiltrated with the gram negative bacteria *Pseudomonas syringae* pv. *tomato* (Pst) to analyze promoter activity for the given genes. The growth cabinet for short day conditions had an 8 hour photoperiod of 80 µmol/m^2/sec light intensity and a temperature of 22/18°C (light/dark). The relative humidity was 80% to ensure high humidity for bacterial growth (Weigel and Glazebrook, 2002).

P. syringae DC3000 is a widely studied strain of the *P. syringae* pv. *tomato* strains. This strain has been shown to be pathogenic to both tomato and *A. thaliana.* The complete sequence of the DC3000 strain was obtained in 2003 (Buell et al., 2003). These properties make it an excellent “pathogen of choice” when studying plant host defense in *A. thaliana.* Pst naturally enters the plant through stomata openings and proliferates in the intercellular space where it is nourished by plant nutrients (Preston, 2000).

A liquid culture of *P. syringae* in Kings B medium (see Appendix A1 for recipe) was centrifuged for ten minutes at 4000 rpm and the pellet was resuspended and washed in 10 mM $MgCl_2$. The washing treatment was repeated and finally the pellet was resuspended in 10 mM $MgCl_2$. A dilution series was made to make a cell concentration of 10^4 cell forming units/mL (CFU/mL). A syringe without a needle was used to introduce the bacterial cells to the plant tissue (Lee et al., 2001; Katagiri et al., 2002). $MgCl_2$ was used as a negative control. Tissue was harvested for GUS assay and subsequently cleared by chloral hydrate (described in chapter 2.3).

2.2.6 H_2O_2 infiltration

5 weeks old vegetative *A. thaliana promoter:GUS* lines, *pIDL6:GUS* and *pIDL7:GUS*, grown under short day conditions as described in 2.2.5, were infiltrated with a 10 mM solution of the ROS;

hydrogen peroxide (H_2O_2). A syringe without a needle was used to introduce H_2O_2 to the plant tissue (Lee et al., 2001). MQ-water was used as a negative control. Tissue was harvested for GUS assay and subsequently cleared by chloral hydrate (described in chapter 2.3).

2.2.7 Stress treatments of plants cutured in liquid ½ x MS

8 days old *A. thaliana promoter:GUS* lines, *pIDL6:GUS* and *pIDL7:GUS,* grown on ½ x MS plates were transferred to liquid ½ x MS (see Appendix A1 for recipes) in 24 well trays, acclimatizeised for two days, and treated with either $NaCl_2$ (120 mM), mannitol (300 mM), H_2O_2 (10 mM), paraquat (4 µM) or flagellin22 (10 nM). MQ-water was used as a negative control. Treated tissue was harvested for GUS assay (described in chapter 2.3) after 0.5 h, 1 h and 2 h.

2.3 GUS staining

β-glucuronidase (GUS) is a bacterial enzyme (originally derived from *Escherichia coli*) which hydrolyzes 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) in to a blue, insoluble product. The gene that codes for β-glucuronidase (*UidA*) is commonly used as a reporter gene for promoter studies. The β-glucuronidase gene is transformed adjacent to the target promoter instead of the gene sequence of the target gene. In this way, wherever the target gene will be expressed (i.e. has an active promoter) there will be a blue staining in the plant tissue. This assay is extremely valuable when localization of a gene is an aim in research, or to analyze the effect of a treatment on gene expression versus differential treated controls. The amount of blue color that appears in the tissue after staining will also give some clues of how extensive the expression of the target gene actually is (Jefferson et al., 1987). The detection of GUS activity was performed by vacuum infiltration and recipes for phosphate buffer and GUS reaction buffer are provided in Appendix A2.

Procedure:

- Plant tissue was incubated in 90% acetone for one hour at -20°C.
- Material was then washed in 100 mM sodium phosphate buffer (pH 7.0-7.7) (Chambers et al., 1993) for 5 minutes, twice.
- GUS reaction buffer was then added to the tissue and the samples were vacuum infiltrated for 3 minutes. Samples were incubated for 1-2 hours at 37°C.
- The reaction was terminated by fixation in ethanol:acetate (3:1) for 1-16 hours at room temperature (Scarpella et al., 2003; Scarpella et al., 2004).

Clearing of plant tissue

Chloral hydrate solution was used for clearing of plant tissue before mounting on a microscope slide for imaging if tissue samples were too large to be imaged in a light microscope. 4 g of chloral hydrate were solved per mL MQ-water at 37°C. 1 mL glycerol was added per 10 g of chloral hydrate (Hamada et al., 2000).

2.4 DAB staining

3,3'-diaminobenzidine (DAB) was used to detect and localize H_2O_2 cytochemically. DAB is taken up in the plant tissue and polymerize instantly and locally to a brown/reddish compound where H_2O_2 is present (Thordal-Christensen et al., 1997). DAB assay was performed by vacuum infiltration, recipe for the DAB reaction buffer is provided in Appendix A2.

Procedure:

- Harvested tissue was covered with DAB reaction buffer in 6-wells trays and vacuum infiltrated for 3 minutes before the trays were covered with aluminum foil and incubated for 3-4 hours at room temperature.
- After incubation the tissue was destained in 96% EtOH over night (Thordal-Christensen et al., 1997).

2.5 Experimental work on nucleic acids

Nucleic acids are universal polymeric molecule chains which make up the basis of transferring genetic information from parent to offspring. The genetic information is stored as deoxyribonucleic acid (DNA) and in some cases ribonucleic acid (RNA). DNA and RNA make up the basis of the field of molecular biology and an understanding of some basic DNA and RNA characteristics are necessary to understand the methods and techniques frequently in use.

DNA and RNA consist of long chains of nucleotides. The DNA chain consists of a base (Adenine (A), Guanine (G), Cytocine (C) or Thymine (T)), a sugar molecule (deoxyribose) and a phosphate group. New copies of DNA are made by a process called replication, and the information stored in DNA is decoded to form RNA through the process of transcription. RNA also consists of a sugar group (ribose), a phosphate group and a base (Adenine (A), Guanine (G), Cytocine (C) or Uracil (U)).

Through processing of the RNA molecule, a messengerRNA (mRNA) molecule is formed. The information in the mRNA molecule directs the synthesis of amino acid chains through the process called translation.

2.5.1 DNA isolation and genotyping

Defining the specific genotype of an individual is called genotyping. This involves determining if an individual is homozygous or heterozygous for a given genotype, and is easily done by PCR (chapter 2.5.4). It also includes determination of the genotype in transgenic individuals to check if the desired mutation or insertion holds for a specific individual or specific transgenic line.

DNA isolation for genotyping was conducted using Extract-N-amp™ Plant PCR Kit (Sigma-Aldrich, 2010) for DNA extraction and PCR amplification.

Procedure:

- A 0.5 cm disk of plant tissue was harvested into an eppendorf tube prior to the DNA extraction. Only half of the reaction volumes outlined in the Extract-N-amp™ Plant PCR Kit (Sigma-Aldrich, 2010) were used for the DNA extraction and PCR reaction.
- 50 µL extraction solution was added to the plant tissue in the eppendorf tube, vortexed briefly and incubated at 95°C for 20 minutes.
- 50 µL dilution solution was added to the tube and vortexed to mix. Samples were kept at 2-8°C.

PCR reagents were mixed in PCR tubes as following:

Reagent	Volume
Extract-N-amp PCR ready mix	5 µL
Forward Primer	1.5 µL
Reverse Primer	1.5 µL
Leaf disk extract	2 µL
Total	10 µL

The sequence was amplified in a thermal cycler at the following conditions with an additional step for final elongation of PCR products for 10 minutes at 72°C. Chapter 2.5.4 describes the reaction in greater detail.

1	94°C, 3 min	
2	94°C, 30 sec 45-60°C, 30 sec 72°C, 60 sec	35 cycles
3	72°C, 10 min	
4	4°C, Hold	

The PCR products were separated on 1% agarose gel (see Appendix A3) by gel electrophoresis (described in chapter 2.5.5) to identify the genotype of the different plant individuals.

2.5.2 Quantification of nucleic acids

Nanodrop is a spectrophotometric method for defining the quality and concentration of nucleic acids in micro volumes. It is possible to measure concentrations of samples in volumes of 0.5-2.0 µL. It measures the absorbance of DNA and RNA in sample and determines the level of contaminants. The software that is included with the Nanodrop instrument gives the absorbance spectrum of the sample in addition to several other parameters. The 260/280 absorbance ratio defines the level of purity of the nucleic acid. Pure DNA and RNA have 260/280 ratios of about 1.8 and 2.0, respectively. Ratios below

these may be an indication of contaminants (e.g. of proteins or phenols) in the sample. The 260/230 absorbance ratio gives the degree of potential co-purified contaminants in the sample. Pure nucleic acids commonly have a 260/230 ratio of 1.8-2.2. Lower values indicate co-purified contaminants. The software also gives the concentration of nucleic acids in the sample as ng/μL (Thermo-Scientific, 2001). Only nucleic acid samples of good quality was used for further experiments.

2.5.3 Sequencing of DNA

Sequencing is the precise determination of the nucleotide bases that make up a DNA molecule. The process was first introduced in the late 1970s by Fred Sanger and colleagues. The method utilizes the properties of DNA replication from a template using DNA polymerase and freely available nucleotides. The technique further relies on the incorporation of a dideoxynucleotide (ddNTP) in the newly synthesized strand. This will terminate the replication process and give DNA fragments of different lengths (Reece, 2004).

In automated sequencing this determination is done by using a set of ddNTPs that is labeled with different fluorescent dyes. The addition of a fluorescent ddNTP will terminate the synthesis of a growing DNA sequence. This will give DNA fragments of different lengths which can be separated in a gel. Each fragment ending in a different ddNTP will give different wavelengths of fluorescence which can be detected by a fluorescence detector (Reece, 2004).

Big-Dye® Terminator v 3.1 sequencing protocol (Applied Biosystems) was used to amplify a sequence of interest for automated sequencing at the University of Tromsø. Sequencing can verify that no mutations or shifts have occurred in the sequence during cloning process and that the gene was cloned in the correct orientation. The protocol can also be used to analyze knockout mutants to investigate the structure of a DNA sequence to verify that a gene is knocked out or mutated.

Procedure:

The reaction mixture was prepared according to the protocol distributed by the university in Tromsø (UNN, 2010):

Reagent	Volume
Big-Dye v 3.1	2 μL
Sequencing buffer	3 μL
Template	200 ng
Primer	1 μL
MQ-water	q.s.
Total	20 μL

A thermal cycler was used to amplify the sequence by PCR as following (chapter 2.5.4 describes PCR in more detail):

1	96°C, 5 min	
2	96°C, 10 sec 50°C, 5 sec 60°C, 4 min	25 cycles
3	4°C, Hold	

Analysis of DNA sequences

The Chromas Lite software was used to analyze DNA sequences in chromatograms (Technelysium, 2005).

The Multiple Alignment Construction & Analysis Workbench (MACAW) software was used for analyzing sequence similarities between multiple sequences by aligning and linking blocks (stretches of homologous runs in sequences) together (Biology-Software-List, 1999-2011).

2.5.4 PCR- amplification of DNA fragments

The polymerase chain reaction (PCR) method was developed in the mid-1980s (Saiki et al., 1985). PCR utilizes the properties of DNA replication and is a method for amplifying specific DNA fragments *in vitro*. It is extremely effective because it works in an exponential manner, and will amplify billions of copies of DNA strands from a single strand in a very short time through repeating cycles (Baumforth et al., 1999).

For the reaction to take place there are several components that need to be present: The template to be amplified, specific primers to recognize the desired fragment, and a polymerase to synthesize DNA from primers. The polymerase should be a thermo stable enzyme which can tolerate heating for denaturing the DNA strands. The *Taq* polymerase from the thermophilic bacterium *Thermus aquaticus* is often used (Lawyer et al., 1989). In addition the reaction requires oligonucleotides to build the new DNA molecules and the correct reaction conditions are obtained by appropriate buffers.

The buffer ensures optimal pH environment for the Taq polymerase by the tris (hydroxymethyl) aminomethane (Tris) buffering agent, but it also provides the Taq polymerase with appropriate concentration of magnesium. Magnesium is required for the polymerase to function. Salt (i.e. KCl or NaCl) is also added to the buffer. Salt will facilitate annealing of primers to the template (Baumforth et al., 1999).

The reaction is conducted in a thermo cycler that regulates the temperature for the different steps in the reaction. Typically, a PCR program starts with a pre denaturing step at 94°C, to ensure the complete

denaturation of double-stranded DNA (dsDNA), for 2-5 minutes before continuing into repetition of the cycles (Reece, 2004).

One cycle in the thermo cycler consists of three different temperature stages. First step is called the denaturation step and is conducted at 94°C for 20-30 seconds. In this step the dsDNA molecules are separated into single-stranded DNA (ssDNA). Template specific primers can then hybridize to the ssDNA molecule in the next step called the annealing step. The annealing is usually conducted at 45-60°C for 20-60 seconds. This temperature is dependent on primer properties (Reece, 2004). Primers used for PCR in experiments presented here are listed in Table 2.1, chapter 2.9.1.

The last step is the extension step where the *Taq* polymerase binds to the 3'-end of the primers and catalyzes the extension of the new DNA strand by assembling free oligunucleotides to the 3'-end. The extension step is conducted at 72°C which is the temperature optima for the *Taq* polymerase enzyme. If the desired PCR products are large, the extension step may be continued for a longer period. One minute per kb DNA is usually sufficient, but a final extension step can be added after the completion of all the cycles in the PCR. The steps are often repeated 25 to 30 times (Reece, 2004).

The *Taq* DNA polymerase provided by TaKaRa Ex Taq™ kit has a terminal transferase activity that provides the PCR product with a poly-A 3'-end overhang (TaKaRa, 2011). This makes the PCR products suitable for cloning directly into vectors with a poly-T 3'-end overhang (discussed in chapter 2.6.2). One disadvantage of the *Taq* polymerase is the low replication accuracy and lack of proof reading activity. This can be compensated by using other thermo stable polymerases with a proofreading activity at the cost of poly-A 3'-end overhangs (Reece, 2004).

2.5.5 Separation of DNA fragments

Electrophoresis is a technique commonly used to separate nucleic acids based on their size. The negative charge of the phosphate groups in nucleic acids make it possible for the nucleic acids to travel through a porous gel in an electric field. The molecules will travel from the negative pole towards the positive pole in an electric field. The velocity a molecule can obtain in a porous gel is defined by the size of the molecule; larger molecules will obtain less speed due to more friction in the porous agarose gel than smaller molecules. In other words, small molecules will migrate longer than large molecules in the same period of time (Reece, 2004). Gel recipes are provided in Appendix A3.

To visualize the DNA in an agarose gel after gel electrophoresis it is necessary to stain the DNA fragments. This is commonly done by ethidium bromide staining. Ethidium bromide binds very efficiently to double stranded DNA fragments and will fluoresce when illuminated with UV-light (260-230 nm). The DNA will be visible as bands of fluorescence in the gel upon illumination. Ethidium bromide is an extremely strong DNA binder and is therefore also a strong carcinogenic agent (Reece, 2004).

Another possibility is the use of GelRed™. This is a less harmful method for visualizing DNA in an agarose gel. Gel Red™ was developed by Biotium and is incapable of crossing cell membranes and will therefore, not bind to DNA in living cells. It is easy to use and by adding 0.5 µL GelRed™ per 10 mL agarose gel solution before casting the gel, it will bind to DNA that later is to be separated in the gel and emit light when illuminated with ultraviolet light (Biotium, 2009).

2.5.6 Purification of DNA fragments from agarose gels

Promega's Wizard® SV PCR Purification kit is a rapid method for extraction and purification of DNA fragments from standard agarose gels. The Promega Wizard® method is a silica membrane based column technique (Promega, 2009).

Procedure:

- DNA band was sliced out from the gel by the use of a scalpel and placed in an eppendorf tube.
- Membrane binding solution was added to the gel slice in the eppendorf tube (10 µL/10 mg gel). The gel slice was vortexed and incubated at 50-65°C until the gel slice was completely dissolved.
- The dissolved gel was added to a spin column in a collection tube and incubated for 1 minute at room temperature before it was centrifuged for 1 minute at 14 000 rpm in a table top centrifuge. Flow-through was discarded.
- 700 µL membrane wash solution was added, and the column was centrifuged for 1 minute at 14 000 rpm. Flow-through was discarded.
- The column was washed once more with 500 µL membrane wash solution and centrifuged for 5 minutes at 14 000 rpm. Flow-through was discarded, and the column was centrifuged for one more minute to allow evaporation of any residual ethanol.
- The column was inserted to a clean eppendorf tube, and 30 µL nuclease free water was added to middle of the column. The DNA was eluted to the eppendorf tube by centrifuging for 1 minute at 14 000 rpm. DNA samples were stored at -20°C.

2.5.7 RNA isolation

Gene expression analysis can be conducted by analyzing the total mRNA content in a cell or an organism. A specific mRNA content is essentially the genes in a cell or an organism that is expressed at a given time point (Klug et al., 2009).

When isolating RNA from an organism it is essential that the RNA isolated represents the real gene expression pattern in the cell or tissue. Several factors play important roles in respect to this. One is the half-life of the mRNA molecules, which is very short because the mRNA is rapidly degraded by ribonucleases (RNases). RNases are very stable enzymes and they are present in large amounts in the

environment. They bind to RNA and cut them either in the middle (endonucleases) or from the end of the RNA molecule (exonucleases) (Klug et al., 2009).

Another problem that often can occur with respect to RNA isolation from plant tissue is the interference of secondary metabolites which can affect the isolation process or downstream applications. Sigma's Spectrum Plant Total RNA kit has taken the addressed problems above in to account and gives a high RNA yield from about 100 mg of plant material through a column based procedure. Further on, a factor that can affect the RNA content of a cell or tissue is the change in gene expression pattern upon harvesting tissue. Therefore it is important to merge the tissue directly in liquid nitrogen after harvesting and store the samples at low temperature (-80°C) to avoid any gene expression alteration caused by harvesting or storage and also to avoid RNase activity (Sigma-Aldrich, 2008).

Procedure:

- Tissue was harvested and embedded directly to liquid nitrogen and stored at -80°C.
- 90-110 mg of the frozen tissue was weighed out and grounded in the tissuelyser (Qiagen) for 2x1 minute. After grinding, the tissue was lysed with 500 µL lysis buffer and grinded 2x1 minute in the tissuelyser. The lysis buffer releases RNA and prevents ribonucleases and metabolites from interfering with further RNA isolation. The lysis mix was then incubated for 3-5 minutes at 56°C, and centrifuged for 3 minutes at 13 000 rpm.
- The supernatant was applied to a filtration column were the mRNA can be captured and purified, and centrifuged once more at 13 000 rpm for 1 minute.
- 500 µL binding solution was added to the flow-through and the flow-through was then added to a binding column and centrifuged at 13 000 rpm for 1 minute.
- 300 µL Wash solution 1 was added to the column and centrifuged for 1 minute, the flow-through was discarded and the column was treated with a DNase which removes any residual DNA in the column.
- The DNase mix applied to each column consisted of 70 µL RDD buffer and 10 µL DNase enzyme. The column was incubated for 15 minutes at room temperature before 500 µL of Wash Solution 1 was added and centrifuged for 1 minute.
- 500 µL of Wash Solution 2 was added to the column and centrifuged for 30 seconds at 13 000 rpm. This step was repeated and the column was then centrifuged an additional minute for drying. The RNA was collected to a collection tube by adding 50 µL Elution Solution to the column and centrifuged for 1 minute at 13 00 rpm.

The RNA quality and concentration was measured spectophotometric in a Nanodrop (described in chapter 2.5.2). In addition the samples were separated on an agarose gel in formaldehyde (FA) gel

elctrophoresis (See Appendix A2 and A3 for electrophoresis buffer and gel recipe, respectively). FA gel electrophoresis denatures RNA and gives clearer bands in the gel than do regular gel electrophoresis.

To further ensure the RNA quality, a ribonuclease inhibitor, RNasin (Promega, 2008) was added to each sample (1 µL RNasin per 40 µg RNA). RNA molecules were stored at -80°C.

2.5.8 cDNA synthesis

RNA is a very unstable molecule and to be further analyzed it needs to be converted to complimentary DNA (cDNA) by the process of reverse transcription (RT). Since cDNA is synthesized from mRNA it contains no introns in contrast to genomic DNA (gDNA). cDNA is a more stable molecule and is easier to work with in respect to molecular biology methods like qPCR (chapter 2.5.9).

In RT the enzyme reverse transcriptase, together with a primer mix and buffer, transcribes RNA to cDNA. The reverse transcription in these experiments is conducted using Qiagen’s QuantiTect Reverse Transcription kit. The Primer mix provided by the manufacturer contains an optimized mixture of nucleotides and random primers that enables cDNA synthesis from all regions of a RNA fragment (Qiagen, 2009a). The procedure consists of two main steps: 1) Elimination of genomic DNA and 2) the reverse transcription. A negative reverse transcription (NRT) reaction was also prepared to inspect the potential level of contamination of DNA in RNA samples (Roche-Applied-Science, 2008).

Procedure:

- RNA samples were diluted to produce a RNA concentration of about 500ng/µL. This makes the volumes in the rest of the procedure larger and thus easier to handle with the pipette.
- To each 1 µg of template RNA, 2 µL of gDNA wipeout buffer and RNase free water was added to a total volume of 14 µL. The reaction mix was then incubated for 2 minutes at 42°C. Negative reverse transcription reactions was prepared in the same manner but with half of the volume.
- Master Mix for the RT reaction was prepared by mixing 1 µL Quantiscript RT enzyme, 4 µL RT-buffer and 1 µL of RT-primer mix per reaction. The master mix for the negative RT (NRT) reaction was prepared with half of the volume, and the RT enzyme substituted with RNase free water.
- 6 µL (3 µL NRT) master mix was added to each of the samples and incubated for 30 minutes at 42°C.
- The reaction was finally terminated by incubation for 3 minutes at 95°C and samples were stored at -20°C.

2.5.9 RT-qPCR

Reverse transcription quantitative PCR (RT-qPCR) is a technique based on standard PCR and RT (as previously described in chapter 2.5.4 and 2.5.8 respectively) for gene expression analysis based on the level of mRNAs in a cell or a given tissue.

In the process of qPCR, cDNA is amplified for a defined number of cycles by PCR and the reaction is analyzed through an amplification plot to give information about the relative expression of a gene of interest compared to others. The amplification plot is obtained by measuring emitted light from a fluorescent reporter, such as SYBR® Green. SYBR® Green binds to dsDNA molecules and emits fluorescence upon binding. The light emitted can be detected continuously in each cycle throughout the reaction and the light will be proportional to the growing amount of cDNA in the sample (Roche-Applied-Science, 2008).

To analyze the relative expression of a gene from qPCR, a threshold value is set. This is called the Crossing threshold (Ct) and is the number of cycles it takes each reaction to emit enough fluorescence light to reach the threshold. The Ct should be set at the earliest cycle in the exponential phase as possible to avoid bias from degradation of products and to avoid less efficient PCR at later stages when the reagents or substrates of the reaction may be limiting (VanGuilder et al., 2008).

To define the relative expression of a gene the data should also be normalized to compensate for variations between biological and technical replicates. The normalization is done by including selected reference genes in the analysis. These genes should be expressed at a constant level throughout the organism. These genes are commonly called housekeeping genes because they are required in fundamental processes in the cell. The reference genes should also be resistant to the experimental conditions and not be affected by treatment of the tissue. Differences in amount and quality of the starting material as well as differences in reaction efficiency can be normalized if the proper reference genes are chosen (VanGuilder et al., 2008).

Several different factors may affect the PCR efficiency during RT-qPCR. Primer-dimers as well as PCR inhibitors will lower the PCR efficiency. Therefore, care should be taken when designing primers to avoid extensive primer dimerization, and samples should be diluted to reduce the effect of PCR inhibitors. An inappropriate volume of the cDNA sample compared to enzymes and other reagents can also affect the PCR efficiency if some of them becomes limiting e.g. dNTPs. A careful and precise pipetting technique is therefore also important to get high PCR efficiencies.

A no template control (NTC) is included to check if there are any contaminations in the reactions from other sources than the cDNA sample. The reaction from the NTC will not give any signal in qPCR if the reaction is non-contaminated (Roche-Applied-Science, 2008).

NRT (as previously described in chapter 2.5.8) will not give any signal in qPCR if the sample is not contaminated with gDNA (Roche-Applied-Science, 2008).

SYBR® Green has great advantages for the use in RT-qPCR: it has a low cost, is easy to use, and it is sensitive. However, it will bind to any dsDNA, including primer-dimers, and therefore, care should be taken when designing primers for RT-qPCR including the SYBR® Green reporter system (VanGuilder et al., 2008). Unbound SYBR® Green molecules will fluoresce weakly, but this can be subtracted during computer analysis (Roche-Applied-Science, 2008).

Procedure:

- cDNA samples and NRT reactions were diluted at least 1:5 to dilute possible PCR inhibitors that may disrupt the amplification of DNA fragments.
- Master mix for the qRT-PCR with the Light cycler 480 SYBR® Green I Master kit was prepared (Roche-Applied-Science, 2008). The master mix consisted of 3 μL PCR grade water, 1 μL PCR primer mix and 10 μL SYBR® Green per reaction for each designated primer pair. All primers used in qPCR presented in the experiments related to this thesis are presented in chapter 2.9.1.
- 5 μL template cDNA and 15 μL master mix was added to each well on a PCR plate. Template was substituted by PCR grade water in the NTC reaction wells.

In the Roche Light Cycler® the PCR conditions was as follows for the reaction cycles (2) and melting curve (3) (discussed in chapter 2.5.10):

1	95°C, 5 min	
2	95°C, 10 sec 55°C, 10 sec 72°C, 10 sec	45 cycles
3	95°C, 5 sec 65°C, 1 min	
4	40°C, 10 sec	

2.5.10 Analysis of qPCR data

qPCR generates data concerning the levels of relative expression of one or several target genes in a sample. The expression data of target genes produced are relative to one or several reference genes and as previously noted it is necessary to find the Ct-value to be able to define the relative amount of mRNA in the samples. The Ct-values were calculated using LinRegPCR (described below).

It will be desirable to investigate if the signal from fluorescence originates only from the desired PCR product, and not from other sources such as primer-dimers which frequently can occur using SYBR® Green. This is done by analyzing the melting points of the DNA in a melting curve analysis.

Mellting curve analysis

After PCR the reaction mixture is slowly heated to 97°C. This causes the dsDNA to separate and the bound SYBR® Green will emit less and less fluorescence as the dsDNA is degraded to ssDNA. The point where 50% of the DNA exists as ds-molecules and 50% exists as ss-molecules will be defined as the melting temperature (Tm) of a given sample. If a given reaction only amplified one fragment (amplicon) it will only show one peak in the melting curve. If there are e.g. primer-dimers present in the sample, these will show up as a separate peak in the curve. In qPCR experiments conducted here, melting curve analyses were performed for all amplicons and samples with deviating melting curves were not used for further analyses.

Calculating Ct-values

qPCR data consists of measurements of fluorescent light emission (e.g. from SYBR Green). As the reaction is proceeding in the qPCR machinery, the amount of SYBR Green emission from the sample will increase exponentially together with the production of new DNA fragments. In theory, the PCR product doubles itself every cycle, and by easy calculations the starting concentration of the sample could have been defined. However, the PCR efficiency is not 100% due to various reasons, as described in chapter 2.5.9.

In the exponential phase of the amplifying reaction the PCR efficiency is the highest. By defining this phase the PCR efficiency of each sample can be calculated and the Ct-value is determined. Thereafter, the starting concentration can be calculated based on the Ct-value and the mean PCR efficiency. Samples with low Ct-value will have reached the threshold faster, and thus have a higher starting concentration and thus are more expressed. High Ct-values indicate a low starting concentration, and the sample needs more time to reach the defined threshold (Ruijter et al., 2009).

These analyses can be performed by the freely available software LinRegPCR. This program defines a “window of linearity” in the exponential phase and use linear regression to define the starting concentration of a given sample (Ruijter et al., 2009).

REST

The Relative Expression Software Tool (REST) 2009 use statistical models to show the relative expression between several target genes and enable the comparison of several reference genes in the same data set. REST use information of reaction efficiency and Ct-values to define relative expression compared to a control group, e.g. Wt or untreated samples (Qiagen, 2009b).

2.6 Molecular cloning in *E. coli*

Molecular cloning, also known as recombinant DNA technology, is the process of moving a DNA fragment from its original position in a genome into a new position in a different source of DNA for amplification. The new position may be in a vector so that the desired DNA fragment can be incorporated to an organism (most commonly *E. coli*) and be multiplied or further manipulated (Clark, 2005). The daughter cells of the cell that first incorporated the DNA will also carry the new vector and will be clones of the mother cell (i.e. be genetically identical). In other words, recombinant DNA technology is the process of making many identical copies of DNA molecules.

A vector is a DNA molecule that is able to replicate independently within a host cell. Most vectors are based on naturally occurring DNA fragments and the most commonly used vectors are based upon plasmids (Tefferi, 2006). The different cloning vectors used in the work presented here will be presented when the cloning technique applied in the different procedures are presented.

The simplest means of inserting a DNA fragment to a vector is by cutting with restriction enzymes. Restriction enzymes recognize a specific cutting site in the DNA and cut the DNA fragment in the site creating specific patterns at the ends of the fragment. If you e.g. treat a DNA sequence and a vector with the same restriction enzymes, the vector and the DNA fragment will be able to bind together. The binding is mediated through the formation of hydrogen bonds between the compatible ends which have been cut with the same enzymes. The DNA backbone is further covalently sealed by the enzyme DNA ligase (Reece, 2004; Tefferi, 2006).

2.6.1 Gateway® cloning

The Gateway® Cloning system (Invitrogen, 2004) was used to make an over expression line in *A. thaliana*. This multiple vector system provides an efficient protocol for recombination of desired PCR product with the Gateway® vector pDONR as a primary vector. By cloning the PCR product into this vector, the vector provides an effective mechanism for further cloning into the destination vector. Further, correct transformed *E. coli* with pDONR will be resistant to the antibiotic Zeocin, which makes an easy way of excluding non-transformed cells on selection medium. A vector map of pDONR/Zeo is provided in Appendix B.

The system utilizes several different recombination enzymes. These enzymes are provided in a mixture from the manufacturer and recognize specific sites in the sequences to be recombined called attachment (*att)* sites. The pDONR vector contains *att*P sites, and desired PCR product of the sequence to be cloned in to the pDONR vector, should encompass *att*B sites. These are obtained by using *att*B primers during PCR amplification of the DNA fragment. The reaction of combining the PCR product with the primary vector is called the BP reaction (decribed below). The *att* sites in a

pDONR vector holding a PCR product produced by *att*B primers are called *att*L sites (Invitrogen, 2004).

The final destination vector should also contain *att* sites. This reaction is also mediated by several enzymes provided in a mixture from the manufacturer and it is called the LR reaction (described below) (Invitrogen, 2004).

Here the vector pEarlyGate (pEG) 100 (described below) was used as a destination vector and it contains *att*R sites to easy recombine the desired DNA fragment at *att*L sites in pDONR.

BP-reaction

The BP-reaction is the insertion of the PCR product to the pDONR vector. BP Clonase™ II from Invitrogen contains enzyme Integrase and Integration Host Factor proteins which ensures the correct recombination of a PCR product at *att* sites in the vector (Invitrogen, 2004). The reaction is shown schematically in Figure 2.1.

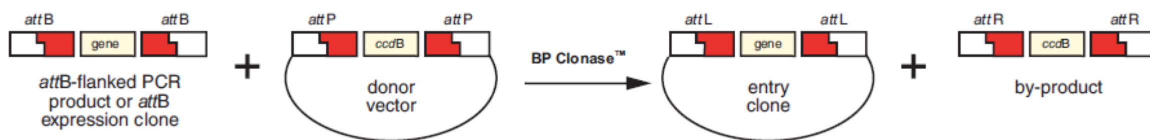

Figure 2.1 A recombination reaction aided by BP Clonase™ II, ensures that desired PCR product flanked by *att*B sites is inserted into appropriate donor vector with *att*P sites. An entry vector and a by-product is the result of correct recombination (Invitrogen, 2004).

The reaction was conducted in a PCR tube, only ¼ of the reaction volumes outlined in the Gateway protocol was used (Invitrogen, 2004).

Procedure:

- 1.5 µL attB PCR product was mixed with 0.5 µL pDONR (150 ng/µL) vector solution together with 0.5 µL BP clonase™ II enzyme and incubated at room temperature over night.
- The reaction was terminated by adding 0.5 µL Proteinase K to the mixture and incubated at 37°C for 10 minutes.
- *E. coli* were heat shock transformed (chapter 2.6.3) with the vector solution. Transformed *E. coli* was plated out on Luria Agar (LA) plates (see Appendix A1 for recipe) containing Zeocin (50 µg/mL) for subsequently plasmid proliferation and isolation (chapter 2.6.4).

LR reaction

The LR reaction is the recombination of the desired sequence from the pDONR vector into a desired expression system, the Gateway LR Clonase™ II was used. The LR Clonase contains the enzymes Integrase and Excisionase, as well as Integration Host Factor proteins, which ensures the correct

recombination of a DNA sequence flanked with *att*L sites from the primary vector (pDONR) to *att*R sites of the new vector (Invitrogen, 2004). The LR reaction is shown in Figure 2.2.

Figure 2.2 A recombination reaction aided by LR clonase™ II, ensures that desired PCR product flanked by *att*L sites in entry clone is inserted into desired destination vector with *att*R sites (Invitrogen, 2004).

In this case, the over expression system vector pEarlyGate (pEG) 100 was chosen as a suitable vector (see Appendix B for vector map) (Earley et al., 2006). This vector has a highly active promoter, called 35S, derived from the cauliflower mosaic virus which constitutively drives the expression of the gene placed under its control. In addition it carries selection markers for bacteria as well as for plants (kanamycin and basta respectively).

Procedure:

- 1.5 µL pDONR vector was mixed with 0.5 µL of the pEG100 (150 ng/µL) vector together with the 0.5 µL LR clonase™ II and incubated at room temperature over night.
- The reaction was terminated by adding 0.5 µL Proteinase K to the reaction mixture and incubated at 37°C for 10 minutes.
- *E. coli* were heat shock transformed (chapter 2.6.3) with the reaction mixture. Transformed *E. coli* was plated out on LA plates (see Appendix A1 for recipe) containing kanamycin (50 µg/mL) for subsequently plasmid proliferation and isolation (chapter 2.6.4).

2.6.2 TOPO TA cloning

Invitrogens TOPO® TA cloning provides an easy method for the insertion of a PCR product into desired plasmid vector. The plasmid vector that is provided from Invitrogen has a single 3'-end T overhang that will efficiently bind to PCR products encompassing single 3'-end A in the cloning site. PCR products with 3'-end A overhang is readily made by terminal transferase activity of a *Taq* polymerase in PCR (Invitrogen, 2006).

The TOPO® vector contains genes that ensure resistance against the antibiotics kanamycin and ampicillin in cells that has been transformed with the vector. Cells that do not contain the vector will not grow on medium containing one of these two antibiotics (Invitrogen, 2006). A plasmid map of the TOPO® vector is provided in Appendix B.

The TOPO® cloning site of the TOPO® vector is positioned within the *lac* operon. This is a cluster of several genes involved in the metabolism of sugar by the enzyme β-galactosidase. When the *lac*

operon is intact and harbors no additional sequences (i.e. a PCR product) it will be functional and break down sugars. In the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, a galactose sugar) and isopropyl-beta-D-thiogalactopyranoside (IPTG, inducer of the *lac* operon), the *lac* operon will be activated in transformed cells and β-galactosidase will metabolize X-gal to form 5-bromo-4-chloro-indoxyl. This compound will spontaneously be oxidized and form a blue insoluble product (5,5'-dibromo-4,4'-dichloro-indigo).

This is an easy method for screening transformed cells. Cells with an empty TOPO® vector will be able to metabolize X-gal and form blue colonies on agar plates. Cells that encompasses TOPO® vector with a PCR product in the cloning site, will not have a functional *lac* operon and therefore will not be able to metabolize X-gal. These cells will form white colonies in the agar plate. This is called a blue-white screen and gives a rapid indication of cells that are correctly transformed with the vector (Reece, 2004).

Procedure:

- 4 µL fresh PCR product was mixed with 1 µL salt solution and 1 µL TOPO vector solution.
- Reagents were mixed carefully and incubated for 5 minutes at room temperature. The TOPO-vector is best stored at -20°C.
- Transformation of $RbCl_2$ competent *E. coli* cells was conducted as described in chapter 2.6.3 by carefully mixing 2 µL TOPO vector with the cells.

Transformed cells were plated out on selective medium containing 50 µg/ml kanamycin or ampicillin, IPTG (100 mM in water) and X-gal (40 mg/mL in dimethyl sulfoxide (DMSO)).

2.6.3 Heat shock transformation of $RbCl_2$ competent cells

Heat shock transformation will aid the uptake of foreign DNA into *E. coli*. The cells were made competent by $RbCl_2$ which disturbs the bacterial cell wall and makes the bacterial cell capable of taking up naked DNA from a solution. By heating competent cells shortly to 42°C they will swell and absorb the foreign DNA contained in the solution they are suspended in (Reece, 2004).

Procedure:

- $RbCl_2$ competent cells were thawed carefully on ice.
- Desired amount of vector was added and carefully mixed by the pipette tip. The cells were incubated on ice for 30 minutes.
- Heat shock transformation was conducted in a 42°C water bath for 45-55 seconds.
- The cells were then incubated on ice for additional 2 minutes before 1 mL Luria-Bertani (LB) (see Appendix A1 for recipe) preheated to 37°C was added to the cells.

- The cells was incubated 1-2 hours with shaking (225 rpm) at 37°C and eventually plated out on LA (see Appendix A1 for recipe) with antibiotics which selects for *E. coli* that successfully has incorporated the vector with the DNA fragment of interest. The plates were incubated over night (37°C) for growing of transformed *E. coli* cells.

2.6.4 Plasmid isolation by miniprep

Plasmids from host cells (e.g. *E. coli*) can be purified from a liquid culture if a concentrate of the plasmid is needed for further cloning work or analysis.

For plasmid isolation QIAgen Miniprep kit was used. This system is a column based procedure utilizing a silica based membrane that specifically binds DNA before it is eluted as purified plasmid. The unique conditions this kit provides will ensure that RNA, cellular proteins and metabolites will be cleared from the final plasmid solution. In addition, it prevents degradation of nucleic acids by removing endonucleases. By lysing the bacterial cells under alkaline conditions chromosomal DNA will be denatured and can be separated from the plasmid DNA (Qiagen, 2006).

Procedure:

- Colonies from LA plates with appropriate antibiotics for selection were picked and transferred to LB with antibiotics in 13 mL tubes and incubated, with shaking (225 rpm), at 37°C over night.
- The 13 mL tubes were centrifuged at 4000 rpm for 10 minutes after incubation over night.
- The pellet was resuspended in 250 µL resuspension buffer (Buffer P1) with RNase A, and the solution was transferred to microcentrifuge tubes.
- 250 µL Buffer P2 was added and the tubes were inverted several times. Buffer P2 contains NaOH that ensures alkaline conditions and sodium dodecyl sulphate (SDS) which denatures chromosomal and plasmid DNA as well as proteins and cell membranes.
- 350 µL neutralizing buffer (Buffer N3) was added and the tubes were inverted 4-6 times before tubes were centrifuged for 10 minutes at 13000 rpm. Buffer N3 gives the lysate high salt concentrations and neutralizes the lysate. This causes denatured chromosomal DNA, proteins and cellular debris to precipitate while plasmid DNA renatures and stays solved in solution (Qiagen, 2006).
- The supernatant was transferred to the spin column and centrifuged for 60 seconds.
- The flow-through was discarded and the columns were washed by adding 500 µL PB buffer and centrifuged once more for 60 seconds.
- Finally, the column was washed with 750 µL PE buffer and centrifuged for 60 seconds twice before the DNA from the column was eluted in 30 µL EB buffer by centrifuging one last time for 60 seconds (Qiagen, 2006). Plasmids were stored at -20°C.

Nanodrop (see chapter 2.5.2) was used to measure the concentration and quality of the isolated DNA molecules.

2.6.5 Control of cloning

To verify the incorporation of a plasmid to a host cell is easily done by selection markers in the plasmid. Cells carrying a specific plasmid with an antibiotic selection marker will be able to grow and proliferate in the presence of the antibiotic, while cells not carrying the plasmid will not be able to grow with the antibiotic present. However, the presence of a gene or other DNA fragment in a plasmid has to be detected by other means.

Bacterial PCR for efficient screening of bacterial colonies

The presence of desired DNA fragment in bacterial colonies grown on agar plates can be efficiently determined by using PCR. This was easily done by picking a colony with a pipette tip and dipping it into 5 µL of MQ-water in a PCR tube before dropping the pipette tip into LB for further culturing. The rest of the PCR reagents were further added to the PCR tube as described below. *Taq* polymerase and appropriate buffer from New England Biolabs (NEB) was applied.

Reagent	Volume
MQ-water and cells	5 µL
dNTP	1 µL
Buffer (NEB)	2 µL
Forward Primer	1 µL
Reverse Primer	1 µL
Taq polymerase (NEB)	0.2 µL
MQ-water	q.s.
Total	20 µL

The reaction was conducted in a thermal cycler as described in chapter 2.5.4 with an additional pre-step for disruption of the bacterial cells at 94°C for 10 minutes as follows:

1	94°C, 10 min	
2	94°C, 30 sec 50-60°C, 30 sec 72°C, 30 sec	35 cycles
3	4°C, Hold	

Restriction cutting

The correct insertion of a DNA fragment in a plasmid may also be analyzed by restriction cutting with restriction enzymes followed by analyzing the fragments in an agarose gel by gel electrophoresis. The restriction enzymes will recognize and cut the DNA strand in specific restriction sites. The size of the inserted DNA fragment and additional nucleotides flanking it can be determined when restriction sites

are known. The number and size of fragments from restriction cutting of a plasmid will reveal if the DNA has been incorporated in the plasmid by analysis in an agarose gel.

Procedure:

- The restriction reaction mixture was prepared as following and incubated for 2-3 hours at 37°C before the fragments was separated in a 1% agarose gel:

Reagent	Volume
Plasmid DNA	1 µL
Buffer (NEB)	1 µL
Restriction enzyme(s)	0.5 µL
MQ-water	q.s.
Total	10 µL

2.8 Transformation of *A. thaliana* with *Agrobacterium tumefaciens*

Transformation of *A. thaliana* can be conducted through using the microorganism *Agrobacterium tumefaciens* to introduce genes into the plant's genome. *A. tumefaciens* is a gram negative bacterium that naturally occurs in the soil and is responsible for Crown gall disease in plants. This disease causes a proliferation of the stem tissue making cancerous tumors on the infected plant's stem.

The bacterium carries a Tumor inducing (Ti) plasmid which encompasses virulence genes for infecting the plant tissue in addition to genes which stimulates cell growth and division. It also carries genes for opine metabolism which enable transformed plant cells to produce opines which *A. tumefaciens* can use as their only source of carbon and energy. Figure 2.3 display an overview of the different segments of a naturally occurring Ti-plasmid in *A. tumefaciens.* The genes which stimulate cell growth and division (auxin and cytokinin) and genes for opine synthesis, are positioned within a special region on the Ti-plasmid called the transfer DNA (T-DNA) region. The T-DNA is integrated to a random position in the plant by the virulence genes and will cause disease symptoms in nature. The T-DNA is flanked by a left and a right border which is necessary for the integration of the T-DNA to the plant genome (Reece, 2004; Tzfira et al., 2004; Meyers et al. 2010).

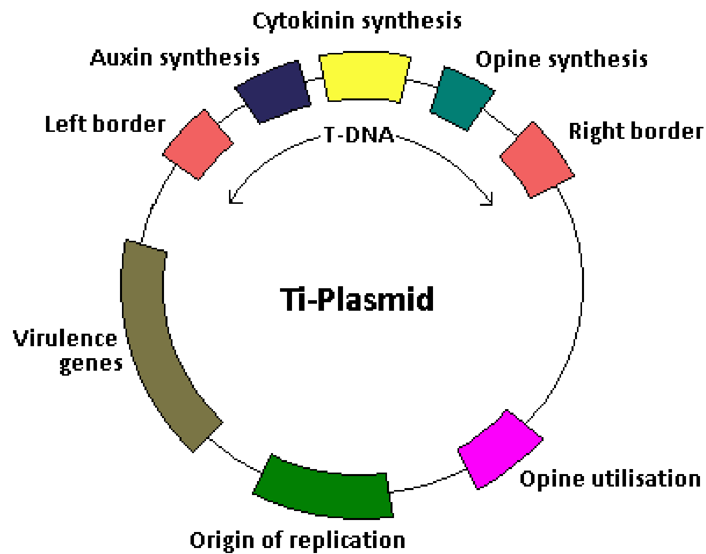

Figure 2.3 The naturally occurring Ti-plasmid of *Agrobacterium tumefaciens.* The plasmid includes genes for cell growth and cell division (auxin and cytokinins) and opine metabolism in addition to virulence genes needed for the infection process and gene transfer. DNA between the left and the right border are transferred to the plant genome upon infection (modified from Reece, 2004).

However, in *A. tumefaciens* used for controlled transformation of *A. thaliana*, the genes for cell proliferation and opine synthesis have been removed so that the bacterium do not cause any disease but still is capable of inserting the T-DNA into the plant's genome through the virulence genes on the Ti-plasmid (Clough and Bent, 1998). A gene of interest can be cloned into the T-DNA region and any DNA sequence located between the left and the right border will be transferred to the plant's genome.

The transformation of *A. tumefaciens* to contain the gene of interest and with the desired features was mediated through a co-integrated vector system, such as *E. coli*.

2.8.1 Electroporation of *A. tumefaciens*

Electroporation is the process of making microscopic pores in the cell membrane to enable the uptake of naked DNA from the surrounding solution. The pores form in approximately 1 µsec, but they reseal in a much lower rate, several minutes (Reece, 2004). For this experiment Genpulser II from Biorad was used to conduct the electroporation. In electroporation it is necessary that the cell mixture has as low conductivity as possible and that the plasmid is present in a solution with a low concentration of salts to prevent overflow in the cuvette.

Procedure:

- 40 µL electro competent *A. tumefaciens* cells were thawed in room temperature and kept on ice.
- 1-2 µL diluted plasmid (about 20 ng/µL) was added to the cell culture, mixed and then incubated for 1 minute on ice.

- The cell-plasmid mix was applied to the bottom of a cold electroporation cuvette and placed in the cuvette holder for electroporation at 25uFD and 2.5 kV. The pulse controller was set at 200 Ohm.
- 1mL preheated LB (30°C) was then added to the cuvette and the cells were resuspended and transferred to a 13 mL growth tube.
- The cells were incubated for one hour at 28°C with shaking (225 rpm) before they were plated out on LA plates containing appropriate antibiotics for selection of correct transformants (*A. tumefaciens* strain C58: Rifampicin (50 µg/mL), carbenicillin (50 µg/mL)). The plates were incubated for 2 days at 28°C.

2.8.2 Floral dip transformation of *A. thaliana*

Infection of plants was conducted by simply dipping plants with unopened floral buds into a solution containing transformed *A. tumefaciens*, sucrose and the surfactant Silwet L-77 (Clough and Bent, 1998). Floral dipping was conducted following Bents protocol (2003) with some minor adjustments as follows.

Procedure:

- One day prior to dipping, 100 mL culture of transformed *A. tumefaciens* was placed in the incubator at 28°C with shaking (225 rpm).
- The over-night cell culture was centrifuged in two 50 mL tubes at 4000 rpm for 10 minutes and kept on ice.
- Cells were resuspended in a 5% sucrose solution in a suitable container to an appropriate volume. Silwet L-77 was added to the bacterial-sucrose solution to a final concentration of 0.02% just before dipping the plants.
- All open flower and siliques were removed before dipping. The plants were laid on their side, and inflorescence parts of the plant were dipped in the solution for 2-3 seconds. The plants were then covered with aluminum foil and incubated in humid conditions over night.
- Plants were then wrapped in plastic bags to ensure high humidity and limited spreading of mature seeds. The plants were grown in growth cabinets until the seeds were ready to be harvested.

Seeds were harvested and surface sterilized by liquid sterilization and sown out on large ½ x MS (see Appendix A1 for recipe) plates containing basta (50 µg/mL) for selection of correct transformants and claforan (500 µg/mL) to restrict growth of leftover *A. tumefaciens*.

2.9 List of primers, vectors, mutants and constructs used in the work of this thesis

2.9.1 Primers for PCR and qPCR

All primers used for amplification of DNA in the work presented in this thesis are listed in Table 2.1 (for PCR) and Table 2.2 (for qPCR).

Table 2.1 **Oligonucleotide primers used for PCR in the work presented here.**

PCR Primers	Sequence	Orientation and specification
IDL6KO123F	5' TGG TCT TTT CAG ATA GGT GTG T 3'	forward primer *IDL6*
KO_209	5' AAT CAA AGC TCC AAT TCT AGC C 3'	reverse primer *IDL6*
IDL7KO1F	5' CGT TAG TTT TCT TGC TGG GTC C 3'	forward primer *IDL7*
KO_210	5' CCG GAG AGT TTG TTC CAG TCA T 3'	reverse primer *IDL7*
IDL8KO1F	5' TTG TGT CAT TGG AAC CAC TCA A 3'	forward primer *IDL8*
KO_211	5' ACA AGC TTT GCA TCA GAT TCA C 3'	reverse primer *IDL8*
LBN	5' CGG AAC CAC CAT CAA ACA GGA T 3'	left border, for T-DNA knockout-verification
LBpD991	5' CAT TTT ATA ATA ACG CTG CGG AC 3'	left border, for T-DNA knockout-verification
M13UNI	5' CGC CAG GGT TTT CCC AGT CAC GAC 3'	forward sequencing primer for pDONR and pTOPO vectors
M13REV	5' AGC GGA TAA CAA TTT CAC ACA GGA 3'	reverse sequencing primer for pDONR and pTOPO vectors

Table 2.2 **Oligonucleotide primers used for qPCR experiments in the work presented here.**

Gene	Primer sequence	Orientation
IDL6	5' TGC TTC GTT CTC AAC AGC TAG G 3'	forward
	5' GAA TAT CCA GCC GTC AAG TGA T 3'	reverse
IDL7	5' CGT TAG TTT TCT TGC TGG GTC C 3'	forward
	5' CCG GAG AGT TTG TTC CAG TCA T 3'	reverse
UidA	5' GTT GCA ACT GGA CAA GGC ACT A 3'	forward
	5' ATC CGG TTC GTT GGC AAT ACT C 3'	reverse
TIP41-like	5' GTG AAA ACT GTT GGA GAG AAG CAA 3'	forward
	5' TCA ACT GGA TAC CCT TTC GCA 3'	reverse
PP2A	5' TGG CTC CAG TCT TGG GTA AG 3'	forward
	5' ATC CGG GAA CTC ATC TTT CA 3'	reverse
HY5	5' AAG GCT TGC ATC AGC ATT AG 3'	forward
	5' TCT GAA CTT GAA GAG CGA CT 3'	reverse
PAD4	5' TGC CAT ACT CAA ACT CTT TCT 3'	forward
	5' CCA AAG TGC GGT GAA AGC 3'	reverse

2.9.2 Vectors and plasmid constructs

Vectors used for cloning work in this thesis are presented in Table 2.3 together with details on selection marker and relevant features. Vector maps are presented in Appendix B.

Table 2.3 Vectors, their size and features used for Gateway ® and TOPO TA Cloning in the work presented here. The specific constructs created are also indicated.

Vector name	Size	Selection marker	Features
pDONR/Zeo	4.3 Kb	kanamycin/ zeocin	Gateway cassette
pDONR-*IDL8*			*IDL8* gene in Gateway cassette
pEG100	11.6 Kb	kanamycin, basta	Gateway cassette, 35S promoter
pEG100-*IDL8*			*IDL8* gene in Gateway cassette
TOPO	3.9 Kb	kanamycin or ampicillin	*lac* operon, 3'-end T overhang
TOPO-*idl8*			*idl8* gene in *lac* operon

2.9.3 Knockout and over-expression lines

Mutant lines, double mutant lines and over expression lines of the *IDL6*, *IDL7* and *IDL8* in *A. thaliana* used for experiments in this thesis (excluding specific lines for segregation analysis) are listed in Table 2.4.

Table 2.4 Different transgenic lines of *A. thaliana* used for experiments described in this thesis.

Mutant lines	Features
idl6 1.2	Knockout of *IDL6*
idl6 3.5	Knockout of *IDL6*
idl7 1.4	Knockout of *IDL7*
idl8 1.1	Potential knockout of *IDL8*
idl8 1.2	Potential knockout of *IDL8*
idl6xidl7 1.9	Double knockout of *IDL6* and *IDL7*
idl6xidl7 1.10	Double knockout of *IDL6* and *IDL7*
idl6xidl7 2.20	Double knockout of *IDL6* and *IDL7*
idl6xidl7 5.14	Double knockout of *IDL6* and *IDL7*
35S:IDL6 1.3	Over-expressing *IDL6*
35S:IDL6 2.6	Over-expressing *IDL6*
35S:IDL7 3.1	Over-expressing *IDL7*
35S:IDL7 6.5	Over-expressing *IDL7*

2.9.4 *Promoter:GUS* lines

Promoter:GUS lines used in experiments in this thesis (excluding specific lines included in segregation analysis and general GUS screens on background promoter activity) are shown in Table 2.5.

Table 2.5 ***Promoter:GUS*** **lines of** ***A. thaliana*** **used in experiments described here.**

GUS lines	Experiments
pIDL6:GUS 4.1	Aphid infestation
pIDL6:GUS 4.4	Pst and H_2O_2 infiltration
pIDL6:GUS 4.5	Screening by qPCR
pIDL6:GUS 15.8	Screening by qPCR
pIDL6:GUS 18.4	Aphid infestation, liquid stress assay, screening by qPCR
pIDL6:GUS 25.1	Screening by qPCR
pIDL6:GUS 25.4	Aphid infestation
pIDL7:GUS 3.2	Screening by qPCR
pIDL7:GUS 3.5	Aphid infestation, Pst and H_2O_2 infiltration
pIDL7:GUS 5.5	Screening by qPCR
pIDL7:GUS 6.5	Liquid stress assay, Pst and H_2O_2 infiltration
pIDL7:GUS 16.3	Screening by qPCR
pIDL7:GUS 20.4	Screening by qPCR
pCYP79B2:GUS	Aphid infestation
pCYP79B3:GUS	Aphid infestation
pPDF:GUS	Pst and H_2O_2 infiltration
pPR1:GUS	Pst and H_2O_2 infiltration
pPR1:GUS/ndr1-1	Pst and H_2O_2 infiltration

3 Results

In this thesis, the *A. thaliana* genes *IDL6*, *IDL7* and *IDL8* have been examined to possibly find a potential function of these genes in the plant. The diagrams in Figure 3.1 a and 3.1 b give an overview of the project and indicate the composition of this chapter.

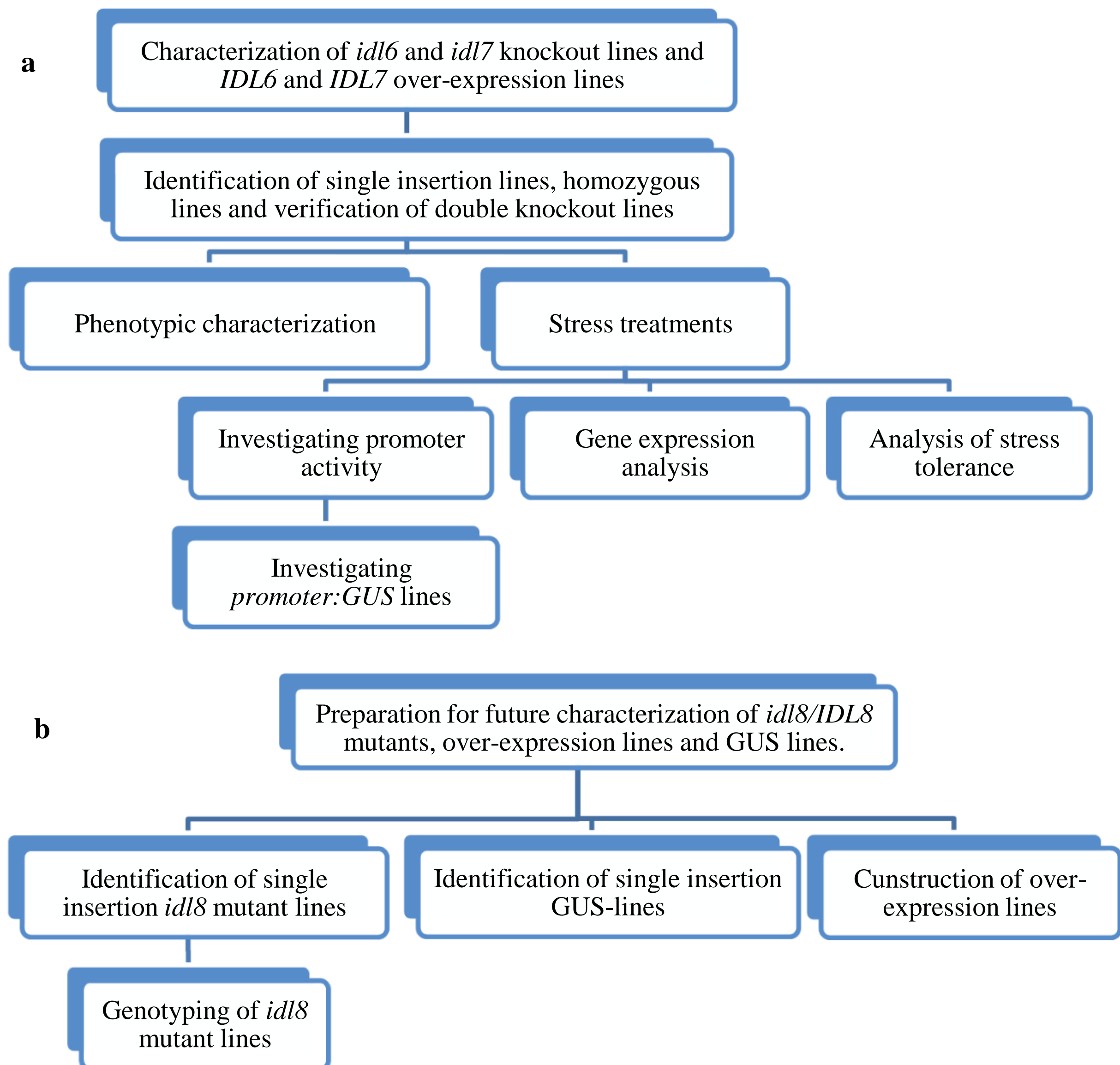

Figure 3.1 Flow diagram of the experiments conducted in the thesis presented here. a) Characterization of *idl6* and *idl7* single knockouts, double knockouts and over-expression lines to find a potential phenotype, and investigating possible differences between mutant lines and wild type *A. thaliana* related to stress treatment and tolerance. b) Verification of *idl8* knockout lines and molecular cloning work of *IDL8*.

Knockout lines of *idl6* and *idl7* have been analyzed to find potential phenotypes linked to mutation in the respective genes during normal conditions and upon various stress treatments. Double knockout

lines of *idl6* and *idl7* genes were also investigated. Plants over-expressing *IDL6* and *IDL7* genes have been analyzed in the same manner as the knockout lines.

Reporter lines of the *IDL6* or *IDL7* promoter have been screened to find stable transgenic lines. Reporter lines were further used to investigate promoter activity of the genes in *A. thaliana* upon various stress treatments.

idl8 mutant lines were analyzed by genotyping and by sequencing to verify knockout lines as a true knockout lines. In addition, over-expression lines of *IDL8* have been constructed.

3.1 Identification of single insertion lines of *IDL6* and *IDL7* transgenic lines

Agrobacterium-mediated T-DNA insertion is used to construct transgenic lines, and is an effective method used to manipulate the *A. thaliana* genome. T-DNA insertion in the host genome is random, and may distrupt gene activity if it is inserted into the coding sequence or regulatory elements of a gene. This property is exploited to generate T-DNA knockout lines, where the T-DNA disrupts the gene it is inserted in. In addition, the T-DNA harbors selection markers for subsequent identification of mutants (Radhamony et al., 2005). T-DNA insertion can also give the plant other features, like over-expression of a gene or enable the plant to metabolize X-Gluc in promoter studies.

When constructing transgenic lines by T-DNA insertion, whether it is knockout mutants, over-expression lines or *promoter:GUS* lines, it is preferable to use lines that only have one T-DNA insertion in the genome. This will ensure stability and predictability of future generations.

Since multiple insertions are common, segregation analyses will aid the identification of single copy T-DNA insertion lines. Lines carrying a single insertion of T-DNA are predicted to show a 3:1 mendelian distribution of resistant:sensitive individuals when analyzed in medium containing appropriate selection marker for selection of T-DNA insert. After transformation and generation of T_1 seeds, this should be the desired distribution of seedlings. In T_2 generation seeds, homozygous lines should be detected by further segregation analysis to ensure complete knockout of a gene in both alleles, or to ensure predictable expression patterns in respect to *promoter:GUS* lines.

Tables A1, A2 and A3 in Appendix D show segregation analysis of over-expression lines *35S:IDL6* and *35S:IDL7* grown on ½ x MS with hygromycin as a selection marker, made by Ane Kjersti Vie (2008, unpublished data). A χ^2-test (Appendix C) was used to evaluate the null hypothesis “3:1 distribution” of resistant:sensitive individuals and the null hypothesis “1:0 distribution” for heterozygous and homozygous lines respectively.

For analyses of promoter activity of *IDL6* and *IDL7*, segregation analyses of T_2 *pIDL6:GUS* lines and T_1 *pIDL7:GUS* lines were performed. Table A4 and A5, Appendix D, show segregation analyses of seedlings grown on ½ x MS with hygromycin for selection of lines containing T-DNA. A χ^2-test was used to evaluate the null hypothesis “3:1 distribution” of resistant:sensitive individuals (Appendix C).

3.2 Identification of true double knockout lines

Double knockout lines of *idl6* and *idl7* were analyzed by qPCR to verify if the transgenic lines were true knockouts of both *idl6* and *idl7*. Four different lines were analyzed by qPCR: *idl6xidl7 1.9*, *idl6xidl7 1.10, idl6xidl7 2.20* and *idl6xidl7 5.14.* Relative expression (Log2 ratios) of *IDL6* and *IDL7* in the different knockout lines are presented in Figure 3.2 and mean Ct-values and reaction efficiencies are presented in Table A22, Appendix D. 14 days old Wt and double knockout lines were treated with Flg22 to ideally induce expression of *IDL6* and *IDL7* prior to harvesting of tissue for qPCR.

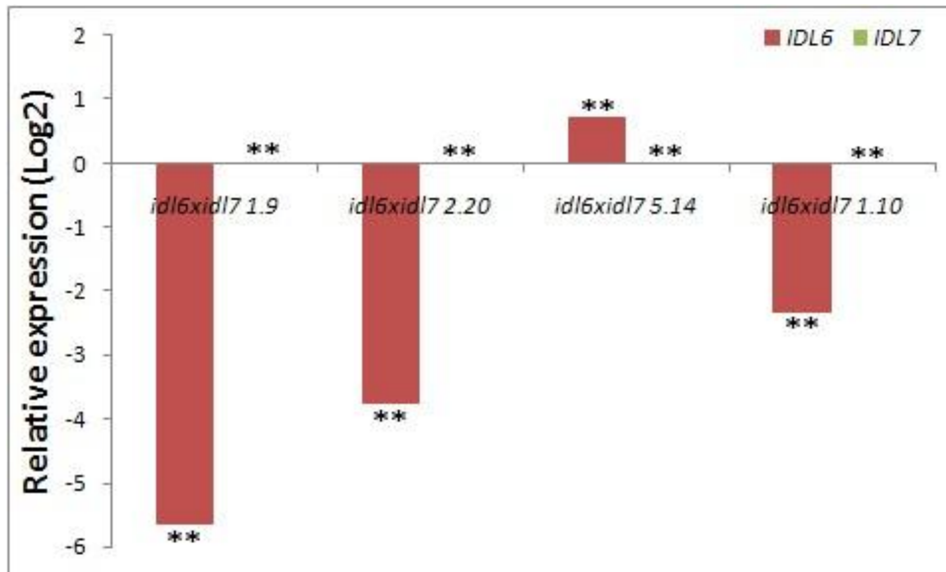

Figure 3.2 Relative expression (Log2 ratios) of *IDL6* and *IDL7* in 14 days old knockout lines: *idl6xidl7 1.9, idl6xidl7 1.10, idl6xidl7 2.20* and *idl6xidl7 5.14* compared to Wt *A. thaliana.* All plants were grown under normal conditions and treated with Flg22 to induce potential expression of *IDL6* and *IDL7* prior to tissue harvesting of tissue for qPCR.

Idl6xidl7 1.9, idl6xidl7 1.10 and *idl6xidl7 2.20* had higher Ct-values than Wt for both the *IDL6* and the *IDL7* amplicon. *idl6xidl7 5.14* had higher Ct-values than Wt only in respect to the *IDL7* amplicon. By analysis of Ct-values in REST2009, expression of *IDL6* was confirmed to be lower in *idl6xidl7 1.9, idl6xidl7 1.10* and *idl6xidl7 2.20* than in Wt with p-values ≤ 0.05 for both amplicons. REST analyses also confirmed that the *IDL7* amplicon was not expressed in any of the knockout lines. REST analyses of *idl6xidl7 5.14* showed that *IDL7* was not expressed compared to Wt ($p \leq 0.05$) but that *IDL6* was in fact up-regulated by a factor of 1.66 ($p \leq 0.05$). *idl6xidl7 5.14* was therefore excluded for further analyses.

3.3 Phenotypic characterization

Growth and development of *A. thaliana* was analyzed at different time points throughout the life cycle of the plant according to Boyes et al. (2001). The different analyses were separated into analyses of: plants grown on ½ x MS plates and plants grown on soil.

3.3.1 Plate-based analyses

Growth stages 0.50 (radicle emergence, i.e. germination), 0.70 (hypocotyls and cotyledon emergence), 1.0 (cotyledons fully opened), 1.02 (2 rosette leaves > 1 mm) and 1.04 (4 rosette leaves > 1 mm) (Boyes et al., 2001) were analyzed to investigate any differences in growth and developmental patterns between knockout lines, double knockout lines, over-expression lines and Wt *A. thaliana* plants. The number of individuals that reached a specific growth stage was noted every day. Figure 3.3 displays the percentage of individuals that reached a certain growth stage during the days of analyses.

The results represent the average between three different replicas; each replica contained at least 60 individuals of each transgenic line and Wt (except for data representing day 11, these are only one independent replica, and thus no statistics are performed in relations to these data). Student's t-test was used to investigate deviations in growth and development patterns between the different mutant lines, over-expression lines and Wt plants at 0.05 (**) or 0.1 (*) levels. Percentage of individuals reaching a certain growth stage and p-values derived from Student's t-test follows in Table A9, Appendix D.

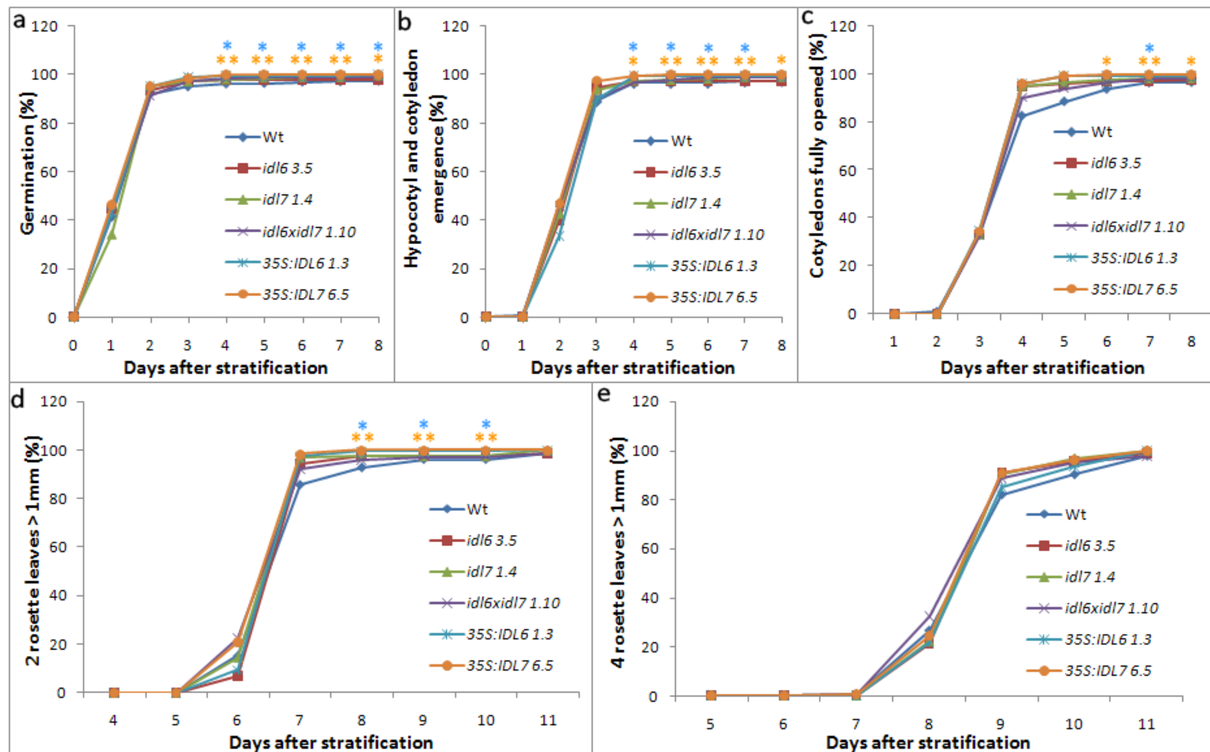

Figure 3.3 Plate-based phenotypic characterization of wild type (Wt) *A. thaliana*, *idl6 3.5* and *idl7 1.4* single mutant lines, *idl6xidl7 1.10* double knockout line, *35S:IDL6 1.3* and *35S:IDL7 6.5* over-expression lines on ½ x MS during normal conditions. (a) Percentage of seeds that germinated, (b) percentage of seedlings with emerged hypocotyls and cotyledons, (c) percentage of seedlings with cotyledons fully opened and percentage of seedlings with (d) 2 and (e) 4 rosette leaves > 1mm, respectively. Average percentages from three separate replicas containing at least 60 seeds of each transgenic line and Wt. Asterisks indicate significant deviations between transgenic lines and Wt. One asterisk (*) indicates p-values ≤ 0.1, two asterisks (**) indicate p-values ≤ 0.05. Colors of the asterisks are consistent with the colors of the graphs.

The only significant deviations at a 0.05 significance level, were observed for the *35S:IDL7 6.5* line and at a 0.1 significance level for the *35S:IDL6 1.*3 line. The over-expression lines seemed to have a slightly higher total percentage of germinating seeds (Figure 3.3 a), development of hypocotyls and cotyledons (Figure 3.3 b), proportion of individuals with cotyledons fully opened (Figure 3.3 c) and development of the 2 first rosette leaves (Figure 3.3 d). No significant differences were observed related to development of 4 rosette leaves.

Seeds were further sown out on square petri dishes containing ½ x MS with no additional sugar for analysis of root growth. Sugar depleted medium is convenient when analyzing root growth because plants grown in sugar depleted medium develop few lateral roots and grow more vertically than on medium containing sugar (Mishra et al., 2009). Photographs, like the one presented in Figure 3.4, were obtained every day for at least ten days, and subsequently analyzed in ImageJ for measurements of root growth.

Figure 3.4 8 days old Wt *A. thaliana* grown on ½ x MS depleted of sugar in vertical standing square petri dishes for root growth measurements.

Figure 3.5 shows average root growth of 3 replicas, each containing 60 seeds of each line distributed on three parallel square plates. A Student's t-test was used to evaluate the differences in root growth.

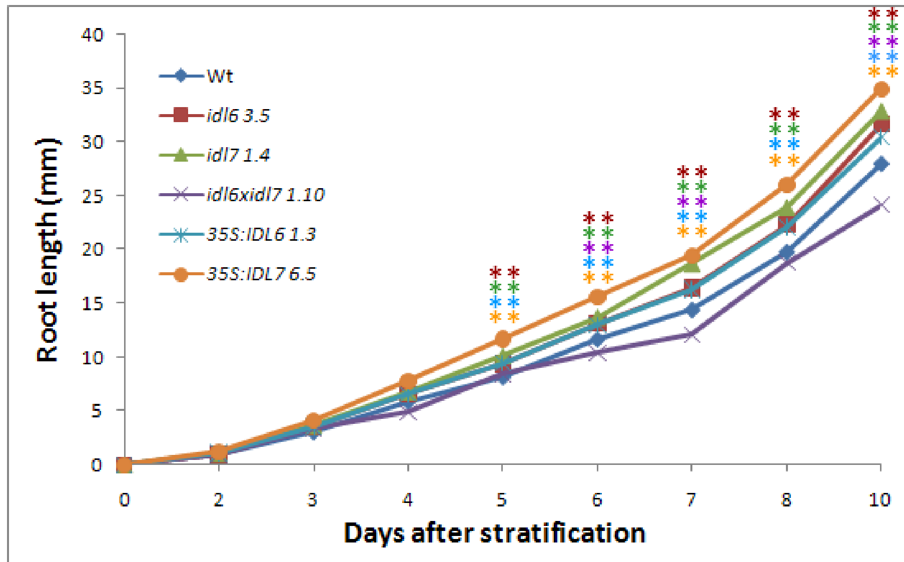

Figure 3.5 Average root growth of different transgenic lines of *A. thaliana* grown vertically on sugar depleted ½ x MS medium. Average percentages from three separate replicas containing at least 60 seeds of each transgenic line and Wt. Root length was measured in mm by analyses in Image J. Asterisks indicate significant deviations (p-values ≤ 0.05) between transgenic lines and wild type (Wt) *A. thaliana.* Colors of the asterisks are consistent with the colors of the graphs.

All lines deviated significantly from Wt at day 10. Over-expression lines and single knockout lines seemed to grow significantly longer roots than Wt *A. thaliana*. These analyses were conducted using a significance level at 0.05 (p-values ranging from the highest at 0.035 to the lowest at $1.06x10^{-8}$ at day 10). Table A10 in Appendix D shows p-values from Student's t-test of comparison between Wt and transgenic lines. The double knockout line *idl6*x*dl71.10* showed a tendency to grow shorter roots than Wt, and was also significantly shorter at day 10.

T-tests also revealed that the double knockout line *idl6*x*dl71.10* grew significantly shorter roots than all other lines (asterisks not shown) at day 10. P-values from Student's t-tests between all the different transgenic lines and Wt at day 10 are given in Table A11, Appendix D. The *idl6 3.5* mutant grew significantly shorter roots than *35S:IDL7 6.5* at day 10, and significantly longer roots than Wt and *idl6xidl7 1.10,* but no significant deviation in comparison to *idl7 1.4* or *35S:IDL6 1.3* was found. The *idl7 1.4* single knockout also developed shorter roots than *35S:IDL7 6.5,* but longer roots than *35S:IDL6 1.3, idl6xidl7 1.10* and Wt. In addition, *35S:IDL7 6.5* had significantly longer roots than all other lines, whereas *35S:IDL6 1.3* only had significantly longer roots than Wt and the *idl6xidl7 1.10* line.

3.3.2 Soil-based analyses

Growth stages 1.04 (4 rosette leaves > 1 mm), 1.08 (8 rosette leaves > 1 mm), 5.10 (first flower buds visible) and 6.0 (first flower opened) were analyzed according to Boyes et al. (2001). The number of individuals that reached a specific growth stage was noted every day during the period each of the traits developed. In addition, analyses of primary shoot growth were performed by measuring height

every day for 10 days after bolting. Development of secondary shoots was also analyzed by counting number of individuals that reached this specific growth stage at different time points. The diagram in Figure 3.6 shows the average number of days each transgenic line and Wt *A. thaliana* used to reach the respective growth stages. For measurements of number of rosette leaves and first flower buds visible, the average represent two biological replicas consisting of 10 individuals of each line. The last two (first flower opened, and secondary shoots) only represent one biological replica of 10 individuals of each line.

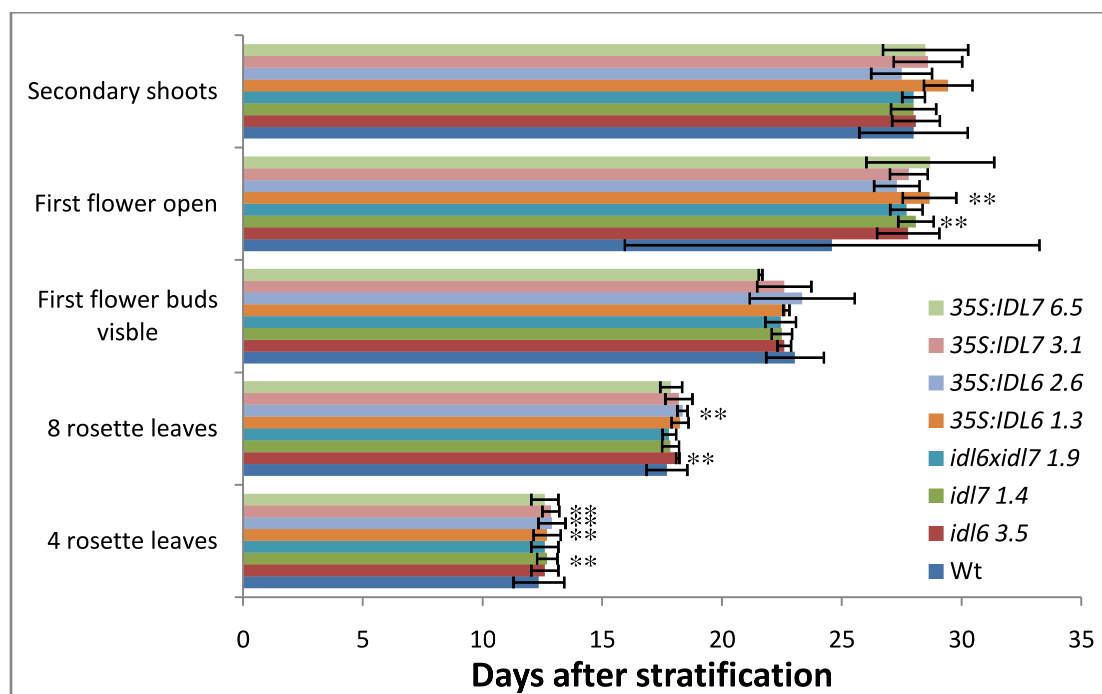

Figure 3.6 Soil-based phenotypic characterization of wild type (Wt) *Arabidopsis thaliana*, *idl6 3.5* and *idl7 1.4* mutant lines, *idl6xidl7 1.10* double mutant line, *35S:IDL6 1.3* and *2.6* over-expression lines, and *35S:IDL7 3.1* and *6.5* over-expression lines grown on soil during normal conditions. Bars represent average number of days to reach a defined developmental stage for the respective lines ± standard deviation derived from to biological replicas containing at least 10 individuals from each transgenic line and Wt. Asterisks indicate significant difference compared to Wt ($p \leq 0.05$).

Some significant deviations in growth and development were detected by Student's t-test during the analyses conducted in these experiments. The diagram in Figure 3.6 shows that development of rosette leaves was similar for all the lines without any large deviations. Deviations were supported by t-test in relations to development of 4 and 8 rosette leaves (p-values are given in Table A12 in Appendix D). Wt showed to develop 4 rosette leaves faster than some of the other lines. However, the variations in development of 4 and 8 rosette leaves were restricted to number of hours.

The number of days until the first flower bud opens showed larger average deviation between the lines. Wt developed faster than *Idl7 1.4* and *35S:IDL6 1.3* despite the large standard deviations.

The diagram in Figure 3.7 displays the average growth rate of primary shoot between two biological replicas of transgenic lines and Wt *A. thaliana* grown on soil. Each series contained 10 and 12 individuals for each of the different transgenic lines and Wt.

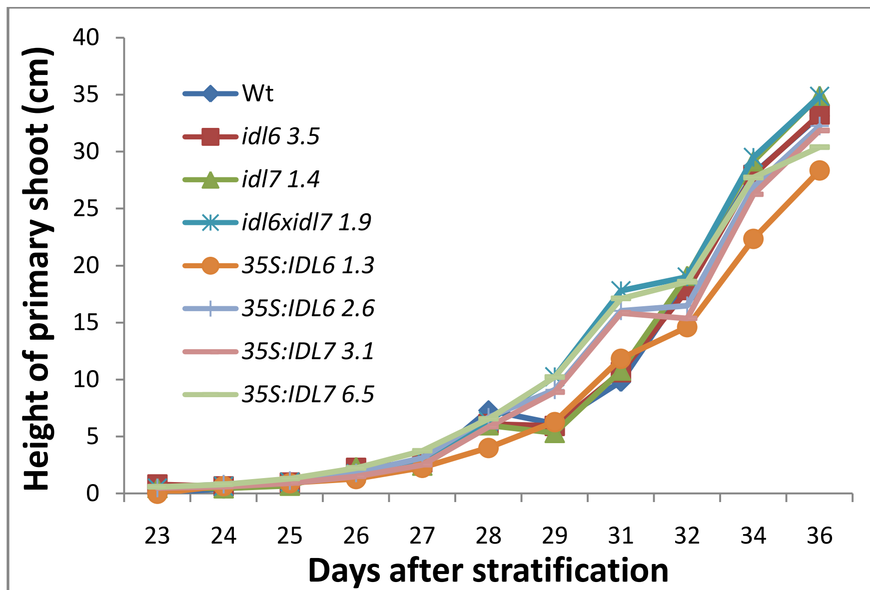

Figure 3.7 Height measurements of primary shoot of wild type (Wt) *Arabidopsis thaliana*, *idl6 3.5* and *idl7 1.4* mutant lines, *idl6xidl7 1.10* double knockout mutant line, *35S:IDL6 1.3* and *2.6* over-expression lines, and *35S:IDL7 3.1* and *6.5* over-expression lines grown on soil during normal conditions. Average height was derived from to biological replicas containing at least 10 individuals from each transgenic line and Wt.

T-test of height measurements showed no significant deviation between the different transgenic lines and Wt *A. thaliana*. A tendency of the over-expression line *35S:IDL6 1.3* to grow slower during later stages was visible. However, this was not the case of the other over-expression lines. Figure 3.8 exemplifies the large variation that was observed in the growth analyses. The shortest and the tallest individuals of Wt, *idl6 3.5* and *idl7 1.4* at day 35 are displayed side by side.

Figure 3.8 35 days old wild type (Wt) *A. thaliana* (left) and mutants idl*6 3.5* (middle) and *idl7 1.4* (right) grown under normal conditions. The shortest and the tallest individuals of all plants analyzed are displayed and show large deviation in growth pattern.

3.4 Stress tolerance treatments

To investigate stress response in Wt *A. thaliana* and different transgenic lines, the *Arabidopsis* eFP Browser (BAR, 2007) was used as a basis for choosing different stress responses. Experiments were designed to result in data that would be comparable with data found in the *Arabidopsis* eFP Browser or with other previously published literature. Table 3.1 and 3.2 show relative expression values (Log2 ratioes) of *IDL6* (At5g05300) and *IDL7* (At3g10930) upon selected biotic and abiotic stress treatments, respectively, obtained from the *Arabidopsis* eFP Browser.

Table 3.1 Relative expression of *IDL6* (At5g05300) and *IDL7* (At3g10930) in tissue harvested at different time points after treatment with different biotic stress factors: *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 and the bacterial derived elicitor flagellin (Flg22). Expression values are displayed as Log2 ratios and are relative to mock treatment with $MgCl_2$ and H_2O for Pst and Flg22, respectively (BAR, 2007).

	Relative expression		
Pst	**2 h**	**6 h**	**24 h**
IDL6	-0.07	-0.94	0.32
IDL7	0.80	-0.25	2.35
Flg22	**1 h**	**4 h**	
IDL6	2.14	1.04	
IDL7	0.57	2.17	

Table 3.2 Relative expression of *IDL6* (At5g05300) and *IDL7* (At3g10930) in tissue harvested at different time points after treatment with different abiotic stress factors and chemicals: NaCl, mannitol, UV-B and cycloheximide (CHX). Expression values for rosettes/roots are displayed as Log2 ratios and are relative to tissue harvested at time point 0 (BAR, 2007).

	Relative expression					
NaCl	**1 h**	**3 h**	**6 h**	**24 h**		
IDL6	2.45/0.92	2.34/-0.29	0.73/0.16	0.46/0.43		
IDL7	4.54/1.61	2.67/0.22	2.05/0.98	1.89/0.83		
Mannitol	**1 h**	**3 h**	**6 h**	**24 h**		
IDL6	0.42/0.46	0.74/-0.62	0.05/0.44	0.10/0.87		
IDL7	3.23/2.03	5.37/2.55	2.05/2.97	-3.09/1.14		
UV-B	**0.25 h**	**0.5 h**	**1 h**	**3 h**	**6 h**	**24 h**
IDL6	1.80/0.18	3.14/1.34	3.52/0.19	3.63/-0.78	1.91/0.42	-1.21/-0.14
IDL7	3.34/-0.53	3.51/0.32	2.57/1.05	1.92/-0.22	1.43/0.78	1.06/1.32
CHX	**3h**					
IDL6	5.78					
IDL7	5.67					

In addition to the experiments designed with the *Arabidopsis* eFP Browser as a starting point, other experiments were also conducted in relations to stress response, but the *Arabidopsis* eFP Browser did

not offer any *in silico* data in respect to these. Tolerance of salt stress and osmotic stress was evaluated using NaCl assay and mannitol assay, respectively.

3.4.1 NaCl assay

Plants grown under salty conditions generally tend to show delayed germination in addition to delayed development of hypocotyls and cotyledons. This is an effect of retarded plant growth and inhibition of germination, which are common effects of salt stress in plants (Weigel and Glazebrook, 2002). They also lose pigmentation in cotyledons and rosette leaves, rendering them white. At 150 mM concentration of NaCl, plants rarely develop green tissue or rosette leaves and cotyledons are seldom fully opened. Figure 3.9 a shows examples of how the white tissue appears at different concentrations, and also how seedlings grown in the presence of high concentration NaCl appear to not develop cotyledons or other leaf tissue. Seeds of different transgenic lines were spread out in defined sectors on large petri dishes together with Wt *A. thaliana* as exemplified in Figure 3.9 b.

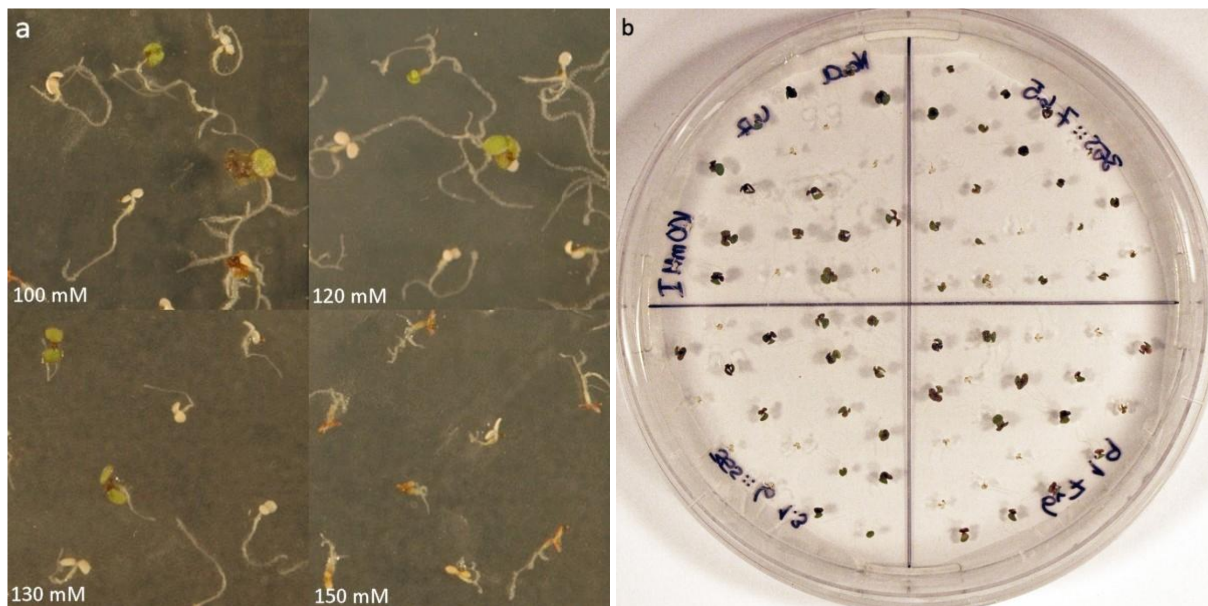

Figure 3.9 a) Cotyledons and rosette leaves of wild type (Wt) *A. thaliana* loose pigmentation when grown on moderate NaCl concentrations (100 mM, 120 mM and 130 mM) on ½ x MS. On 150 mM NaCl, Wt plants do not develop rosette leaves or green tissue and the cotyledons are rarely fully opened. b) Example of seedlings grown on large petri dishes containing 100 mM NaCl.

However, concentrations employed in these experiments were not lethal. When plants were transferred to regular ½ x MS without NaCl, plants grew normally although they were clearly stressed from the time spent on NaCl rich medium. Plants grown on 150 mM NaCl did not develop rosette leaves, but did so when transferred to medium without NaCl. Seedlings with only white cotyledons and rosette leaves did, in addition, regain their pigmentation when transferred from NaCl-plates to regular ½ x MS. Seedlings grown in the presence of 130 mM NaCl and below continued to develop on ½ x MS with NaCl, at a slow rate. Plants with e.g. two white cotyledons would eventually develop rosette

leaves. The new rosette leaves was green before they rapidly turned white (1-3 days after development of the respective leaves). Emergence of radicle (germination), hypocotyls and cotyledons and rosette leaves were considered in the NaCl assay together with development of green and white tissue.

Seeds were spread out on petri dishes (14 cm diameter) containing ½ x MS and different concentrations of NaCl. At least 180 seedlings of each line distributed in three biological replicates were analyzed (except for the double knockout line *idl6xidl7 1.9*, which only has been analyzed in 2 independent replicas, about 120 seedlings, and *idl6xidl7 2.20*, which only represent one biological replica of about 60 seeds).

The *salt overly sensitive* (*sos*) mutants *sos 1.1* and *sos 1.2* (Wu et al., 1996) were used as controls; however, this could only give indications of salt tolerance/intolerance in relation to germination because the *sos* mutants do not develop beyond the germination stage when grown on NaCl-containing medium. Figure 3.10 displays a graphical overview of average germination percentage of Wt, knockout lines, double knockout lines and over-expression lines. Only selected concentrations of NaCl are displayed (0 mM control, 100 mM and 130 mM) because the 120 mM NaCl treatment showed similar development patterns as the 130 mM treatment. 150 mM NaCl treatment was excluded because the NaCl concentration seemed to be too high to evaluate salt tolerance in growth and development assay on solid ½ x MS medium. Table A13, Appendix D, gives p-values from Student's t-test exploring the differences in germination percentage between Wt *A. thaliana* and the transgenic lines.

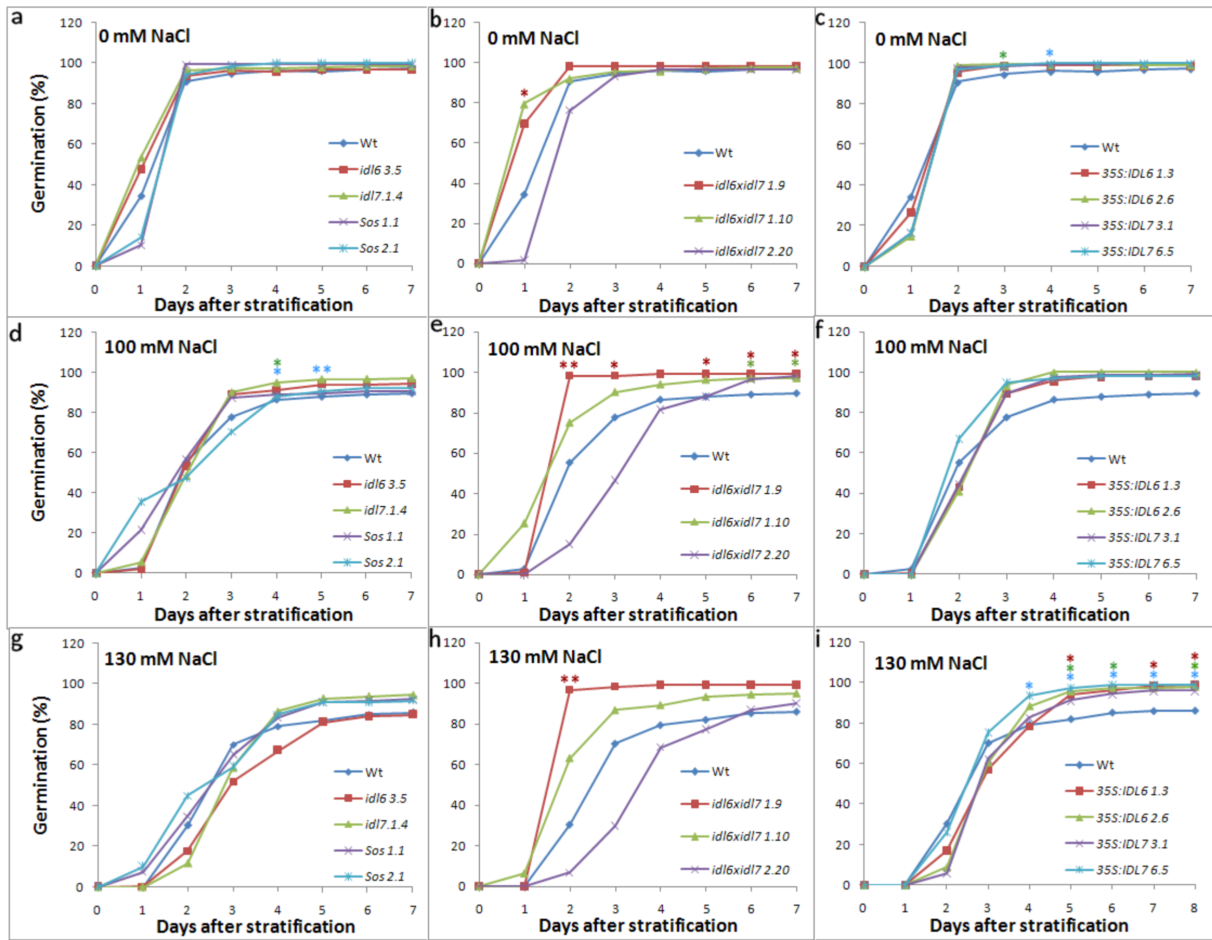

Figure 3.10 Effect of salt stress on germination in *A. thaliana* mutants: *idl6 3.5, idl7 1.4* and *sos1.1* and *sos2.1* (a, d, and g), double knockout lines *idl6xidl7 1.9, 1.10,* and *2.20* (b, e, and h), and over-expression lines *35S:IDL6 1.3* and *2.6* and *35S:IDL7 3.1* and *6.5* (c, f, and i), compared to wild type (Wt). Average germination percentage in three replicates (each containing at least 60 seeds from each transgenic line and Wt) of different concentrations of NaCl (0 mM; a-c, 100mM; d-f, 130 mM; g-i) in ½ x MS is displayed. Germination was scored every day for ten days after stratification. Asterisks indicate significant deviations between transgenic lines and Wt. One asterisk (*) indicates p-values ≤ 0.1, two asterisks (**) indicate p-values ≤ 0.05. Colors of the asterisks are consistent with the colors of the graphs.

Germination percentage on ½ x MS without NaCl (Figure 3.10 a-c) showed small or no significant deviations between Wt and transgenic lines. The significant deviations were observed early in the germination process and no significant deviation was observed after day 4. Similar observations were made in the plate-based analysis of phenotypic characterization (Figure 3.3). The 0 mM NaCl control is analogous to the plate-based phenotypic characterization.

In control plates, *idl6xidl7 1.9* germinated significantly earlier than Wt and *idl6xidl7 2.20* (Figure 3.10 b). *Idl6xidl7 1.10* showed the same trend as *idl6xidl7 1.9* although not significantly supported by the Student's t-test due to large standard deviations. However, all lines seemed to reach the same total percentage of germinated individuals when NaCl was not present (Figure 3.10 a-c). In 100 mM NaCl the same statistically significant observations were made for *idl6xidl7 1.9* in respect to germination rate, but in the presence of 100 mM NaCl, Wt seeds also seemed to reach a lower number of

germinating individuals (Figure 3.10 d-f). This was also the case for 130 mM NaCl (Figure 3.10 g-i) and statistically significantly true ($p \leq 0.1$) for differences between Wt and over-expression lines. Only about 80% of Wt seeds germinated in the presence of 100 mM or 130 mM NaCl, but almost 100% of seeds from the over-expression lines germinated at high NaCl concentrations (Figure 3.10 i). Other lines showed varying total germination percentages, but none were significantly different from Wt at e.g. day 7, or even at day 10 (data not shown).

There was no difference between the over-expression lines and Wt in the first days after stratification. Several of the over-expression lines even had an average lower germination percentage (not significantly) in the beginning, but at later stages more of the over-expression lines had a significantly higher germination percentage than Wt (p-values ≤ 0.1 level). At day 8, all of the over-expression lines had a significantly higher germination percentage than Wt in 130 mM NaCl except for *35S:IDL7 3.1*.

Emergence of hypocotyls and cotyledons (stage 0.7) was scored as number of individuals within a line where the hypocotyls and cotyledons had emerged from the seed coat at a given time point. Figure 3.11 display the results obtained from analyzing hypocotyls and cotyledon emergence. Significant deviations between Wt and any of the transgenic lines are displayed as asterisks and p-values from Student's t-test between Wt and transgenic lines are given in Table A14, Appendix D, together with average percentage of seedlings that developed hypocotyls and cotyledons at given time points.

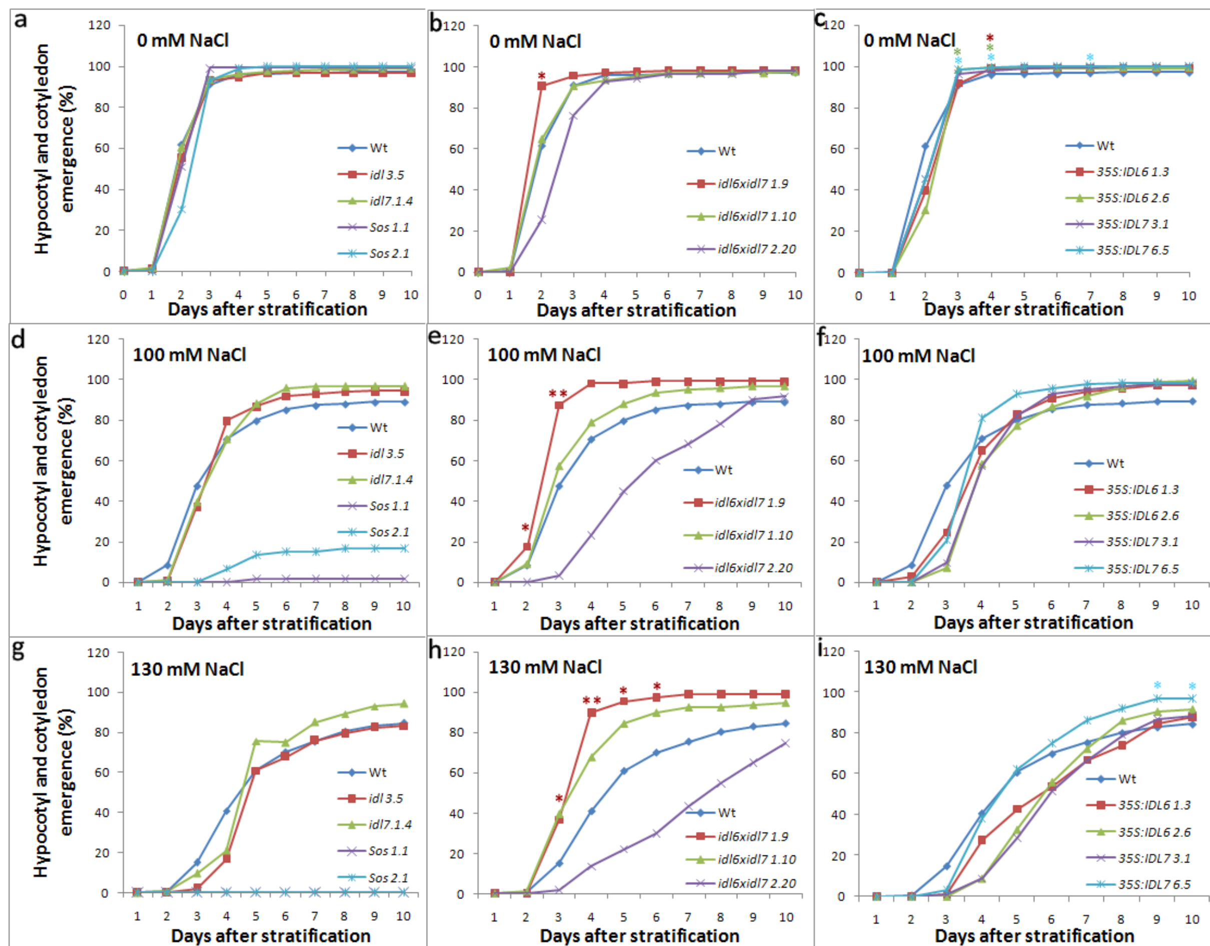

Figure 3.11 Effect of salt stress on development of hypocotyl and cotyledons in *A. thaliana* mutants: *idl6 3.5, idl7 1.4* and *sos1.1* and *sos2.1* (a, d, and g), double knockout lines *idl6xidl7 1.9, 1.10* and *2.20* (b, e, and h), and over-expression lines *35S:IDL6 1.3* and *2.6* and *35S:IDL7 3.1* and *6.5* (c, f, and i), compared to wild type (Wt). Average percentage of individuals with emerging hypocotyls and cotyledons in three replicates (each containing at least 60 seeds from each transgenic line and Wt) of different concentrations of NaCl (0 mM; a-c, 100mM; d-f, 130 mM; g-i) in ½ x MS is displayed. Hypocotyl and cotyledon emergence was scored every day for ten days after stratification. Asterisks indicate significant deviations between transgenic lines and Wt. One asterisk (*) indicates p-values ≤ 0.1, two asterisks (**) indicate p-values ≤ 0.05. Colors of the asterisks are consistent with the colors of the graphs.

Only minor deviations were observed in control plates without NaCl (Figure 3.11 a-c), and no differences were supported by p-values ≤ 0.05. At day 2, on average, about 60% of the seedlings of Wt and *idl6xidl7 1.10* line analyzed had developed hypocotyls and cotyledons. This was in contrast to about 90% in *idl6xidl7 1.9* (p ≤ 0.1). The *idl6xidl7 2.20* showed some tendency to develop slower in respect to hypocotyls and cotyledon emergence but as previously noted the *2.20* line is only represented by one replica and this trend was not confirmed by statistics.

T-test also establishes significant deviation between Wt and *idl6xidl7 1.9* on 100 mM and 130 mM NaCl. 50% of Wt seedlings and almost 90% of *idl6xidl7 1.9* seedlings had developing hypocotyls and cotyledons at day 3 on 100 mM NaCl (Figure 3.11 e). On 130 mM NaCl, 40% of Wt seedlings had

reached stage 0.7 at day 4, while 90% of *idl6xidl7 1.9* had reached this growth stage at day 4 (Figure 3.11 h).

For all other lines no significance was found in relations to speed of development, and the small significance that was observed (e.g. Figure 3.11 i) is related to total amount of seedlings that will develop through stage 0.7 and is further related to total germination percentage (recall that germination percentage showed that Wt significantly had lower total germination percentage than some of the over-expression lines at day 8, Figure 3.10). It is further notable that the tendency of the two double knockout lines *idl6xidl7 1.9* and *idl6xidl7 1.10* (not significant for the latter) showed a tendency to have a higher total percentage of individuals that developed hypocotyls and cotyledons at all concentrations of NaCl and that this was not the case on control plates with no supplementary NaCl.

It is obvious from Figure 3.10 and 3.11 that all lines eventually developed hypocotyl and cotyledons according to number of individuals that germinated, except for *sos*-mutants which do not develop past germination stage as previously noted.

Plants with cotyledons that are fully opened (stage 1.0) were scored as number of individuals with cotyledons that were opened with $\geq 180^o$ angle. Average numbers of individuals with fully opened cotyledons in each independent line and Wt are displayed in Figure 3.12. Average percentage, standard deviations and p-values from Student's t-test are given in Table A15, Appendix D.

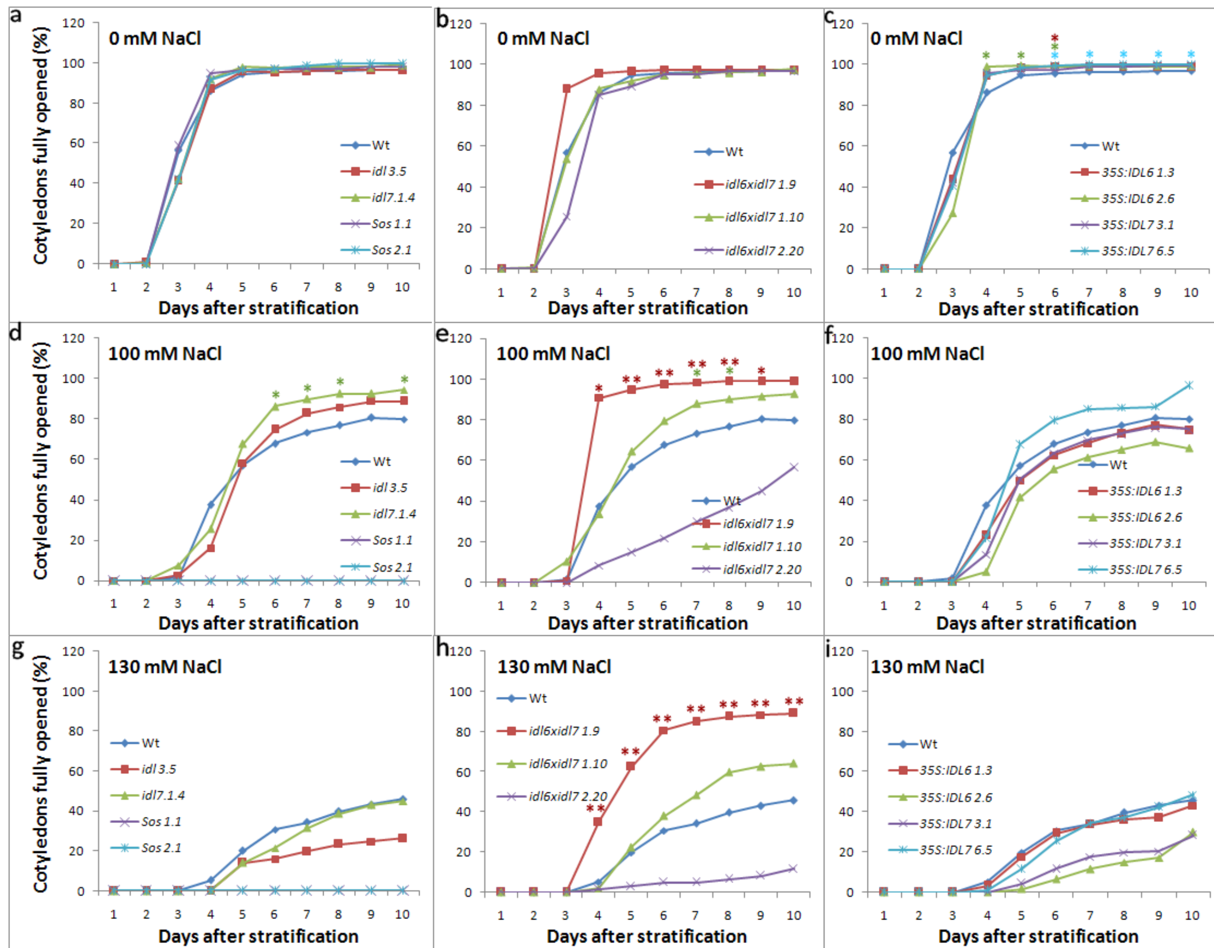

Figure 3.12 Effect of salt stress on development of fully opened cotyledons in *A. thaliana* mutants: *idl6 3.5, idl7 1.4* and *sos1.1* and *sos2.1* (a, d, and g), double knockout lines *idl6xidl7 1.9, 1.10* and *2.20* (b, e, and h), and over-expression lines *35S:IDL6 1.3* and *2.6* and *35S:IDL7 3.1* and *6.5* (c, f, and i), compared to wild type (Wt). Average percentage of individuals with fully opened cotyledons in three replicates (each containing at least 60 seeds from each transgenic line and Wt) of different concentrations of NaCl (0 mM; a-c, 100mM; d-f, 130 mM; g-i) in ½ x MS is displayed. Proportion of individuals with fully opened cotyledons was scored every day for ten days after stratification. Asterisks indicate significant deviations between transgenic lines and Wt. One asterisk (*) indicates p-values ≤ 0.1, two asterisks (**) indicate p-values ≤ 0.05. Colors of the asterisks are consistent with the colors of the graphs.

Development of fully opened cotyledons also followed total germination percentage, i.e. all seeds that germinate will further develop hypocotyls and cotyledons, and cotyledons will fully open under normal growth conditions. Therefore, the same deviations were observed for seedlings on ½ x MS in respect to percentage of individuals with cotyledons fully opened (Figure 3.12 a-c) that was observed for germination and development of hypocotyls and cotyledons.

This is not the case for plants grown in medium containing NaCl. A decreased total number of individuals that develop past stage 0.5 will be observed, and the effect is additive for later development stages. Therefore, large variations can occur between replicates, making it difficult to draw conclusions from the data collected.

Here also, analyses showed that *idl6xidl7 1.9* develops significantly earlier through stage 1.0 than Wt in both 100 mM and 130 mM NaCl and that *idl6xidl7 1.10* show a similar tendency although not significant (Figure 3.12 e and h). *Idl6xidl7 2.20* show a trend to behave differently than the other double knockout lines and Wt, but no statistical analyses were performed related to this line because of lack of replicas, as previously noted. Another small notable significant deviation was observed for line *idl7 1.4* which seemed to have a higher total number of individuals that reach developmental stage 1.0 than Wt (Figure 3.12 d).

No significant deviations were observed between Wt and over-expression lines concerning development through stage 1.0 on NaCl containing medium (Figure 3.12 f and i) as was the case for earlier developmental stages.

As previously described plants grown on medium containing NaCl will lose pigmentation in leaves and proportion of green tissue and white tissue was therefore registered for Wt and the different transgenic lines. Green tissue was scored as number of individuals having one or several green leaves. White tissue was scored as number of individuals with only white leaves. Development of green and white tissue is presented in Figures 3.13 and 3.14, respectively. P-values from Student's t-test of analyzing differences in green and white tissue between Wt *A. thaliana* and transgenic lines are provided in Tables A16 and A17 in Appendix D, together with average percentage and standard deviations of individuals that developed green and white tissue.

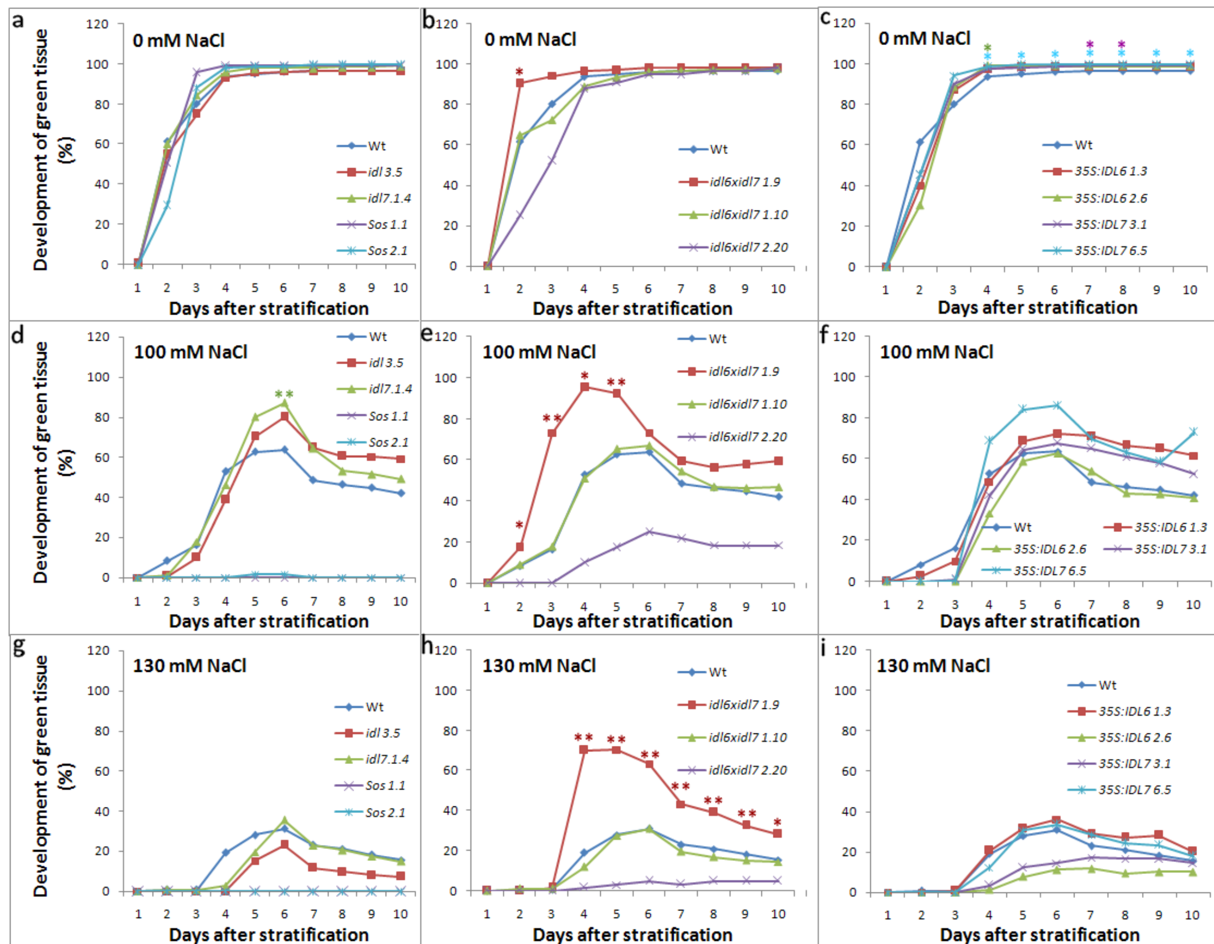

Figure 3.13 Effect of salt stress on development of green tissue in *A. thaliana* mutants: *idl6 3.5, idl7 1.4* and *sos1.1* and *sos2.1* (a, d, and g), double knockout lines *idl6xidl7 1.9, 1.10* and *2.20* (b, e, and h), and over-expression lines *35S:IDL6 1.3* and *2.6* and *35S:IDL7 3.1* and *6.5* (c, f, and i), compared to wild type (Wt). Average percentage of individuals with green tissue in three replicates (each containing at least 60 seeds from each transgenic line and Wt) of different concentrations of NaCl (0 mM; a-c, 100mM; d-f, 130 mM; g-i) in ½ x MS is displayed. Proportion of individuals with green tissue was scored every day for ten days after stratification. Asterisks indicate significant deviations between transgenic lines and Wt. One asterisk (*) indicates p-values ≤ 0.1, two asterisks (**) indicate p-values ≤ 0.05. Colors of the asterisks are consistent with the colors of the graphs.

Development of green tissue was reduced in plants grown in the presence of NaCl (Figure 3.13 d-i) compared to plants grown on regular ½ x MS (Figure 3.13 a-c). Student's t-test showed no significant deviations from Wt for the *idl6 3.5* line and the *idl7 1.4* line in any concentrations of NaCl. The *idl6xidl7 1.9* clearly showed a significant deviation from Wt when grown on medium containing NaCl (Figure 3.13 e and h). A higher total percentage of *idl6xidl7 1.9* seedlings developed green tissue than did Wt seedlings.

The other double knockout lines also seemed to have different developmental patterns in respect to development of green tissue in salty environment. While the *idl6xidl7 1.10* line behaves like Wt, the *idl6xidl7 2.20* line had poorer development of green tissue. However, these deviations were not significant compared to Wt by analysis with Student's t-test.

At later stages, the differences between *idl6xidl7 1.9* and Wt was less pronounced. On 100 mM NaCl (Figure 3.13 e) no significant deviations were observed at day 6 and onwards. At day 10 on 130 mM NaCl, *idl6xidl7 1.9* showed some deviations from Wt; these were only significant with 90% certainty.

Some small deviations were observed on medium containing no NaCl in respect to the over-expression lines compared to Wt. However, these were only significant with 90% certainty. No significant deviations were detected between over-expression lines and Wt in seedlings grown on medium containing NaCl.

The same trends were observed for development of white tissue, displayed in Figure 3.14. No significant deviations are observed between transgenic lines and Wt, except for the double knockout line *idl6xidl7 1.9* (p-value ≤ 0.05).

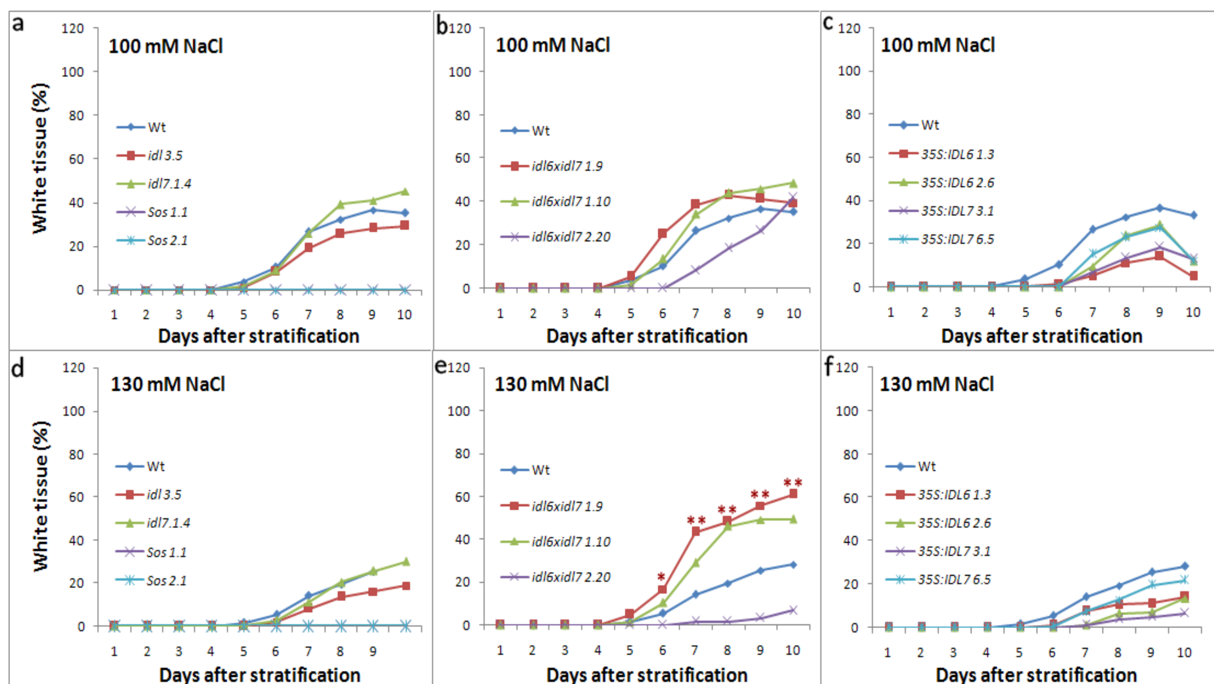

Figure 3.14 Effect of salt stress causing white leaves (due to loss of pigmentation) in *A. thaliana* mutants: *idl6 3.5, idl7 1.4* and *sos1.1* and *sos2.1* (a, d, and g), double knockout lines *idl6xidl7 1.9, 1.10* and *2.20* (b, e, and h), and over-expression lines *35S:IDL6 1.3* and *2.6* and *35S:IDL7 3.1* and *6.5* (c, f, and i), compared to wild type (Wt). Average percentage of individuals with only white tissue in three replicates (each containing at least 60 seeds from each transgenic line and Wt) of different concentrations of NaCl (0 mM; a-c, 100mM; d-f, 130 mM; g-i) in ½ x MS is displayed. Proportion of individuals with only white tissue was scored every day for ten days after stratification. Asterisks indicate significant deviations between transgenic lines and Wt. One asterisk (*) indicates p-values ≤ 0.1, two asterisks (**) indicate p-values ≤ 0.05. Colors of the asterisks are consistent with the colors of the graphs.

Idl6xidl7 1.9 had a higher number of individuals with only white tissue. This is related to the fact that *idl6xidl7 1.9* had a higher number of individuals that developed through the preceding growth and development stages.

3.4.2 Mannitol assay

Seeds were spread out on big petri dishes containing ½ x MS and different concentrations of mannitol to investigate the potential difference in tolerance of osmotic stress. At least 180 seedlings of each line distributed in three biological replicates were analyzed. Only selected concentrations of mannitol are displayed in the results presented here (control, 100 mM and 300 mM) because the 200 mM mannitol treatment showed similar development patterns as the 300 mM treatment. The 400 mM mannitol was excluded because it seemed to be a too high concentration to evaluate osmotic stress tolerance in growth and development assay on solid ½ x MS medium.

A. thaliana grown on medium containing mannitol showed decreased size and delayed development. The extent of the effect of mannitol on growth and development was dependent on the concentration of mannitol in the medium. Treatment with mannitol was not as detrimental as the NaCl treatment. Figure 3.15 displays a graphical overview of average germination percentage of Wt, knockout lines, double knockout lines and over-expression lines. Asterisks indicate significant differences between Wt and transgenic lines evaluated by Student's t-test. Average germination percentages, standard deviations and p-values from Student's t-test between Wt *A. thaliana* and transgenic lines are included in Table A18, Appendix D.

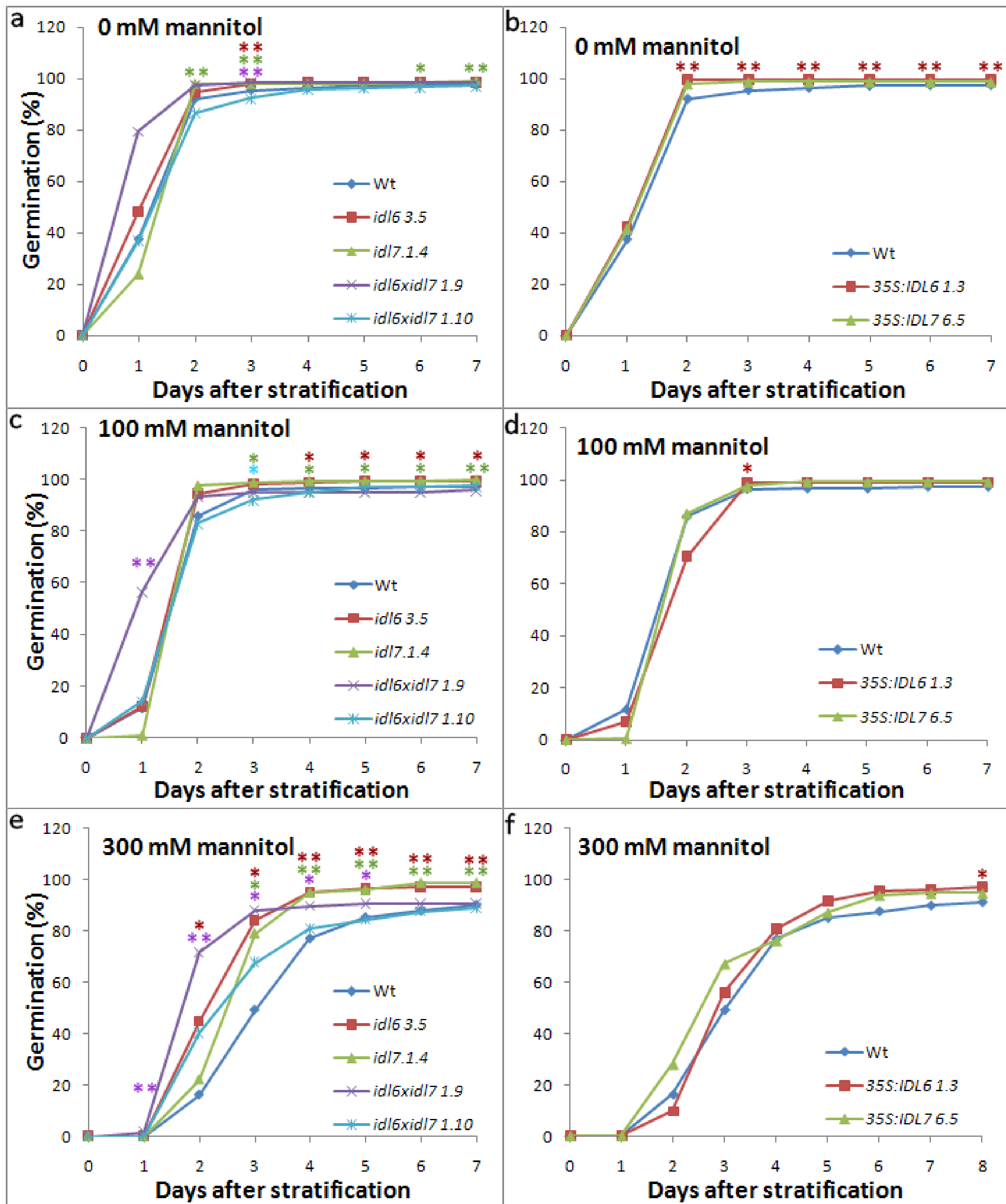

Figure 3.15 **Effect of osmotic stress on germination in *A.thaliana* mutants: *idl6 3.5, idl7 1.4, idl6xidl7 1.9* and *idl6xidl7 1.10* (a, c, and e), and over-expression lines *35S:IDL6 1.3* and *35S:IDL7 6.5* (b, d, and f), compared to wild type (Wt). Average germination percentage in three replicates (each containing at least 60 seeds from each transgenic line and Wt) of different concentrations of mannitol (0 mM; a and b, 100mM; c and d, 300 mM; e and f) in ½ x MS is displayed. Germination was scored every day for ten days after stratification. Asterisks indicate significant deviations between transgenic lines and Wt. One asterisk (*) indicates p-values ≤ 0.1, two asterisks (**) indicate p-values ≤ 0.05. Colors of the asterisks are consistent with the colors of the graphs.**

On medium without mannitol, results showed some significant deviations in total germination frequency between Wt and some of the transgenic lines (Figure 3.15 a and b). At day 7, *idl7 1.4* and *35S:IDL6 1.3* were found to have a higher total germination percentage than Wt, whereas all other lines did not deviate from Wt on ½ x MS according to the Student's t-test. No significant differences were found in germination speed on medium without mannitol, although the *idl6xidl7 1.9* line showed a trend to germinate faster than Wt (Figure 3.15 a).

The tendency of *idl6xidl7 1.9* to germinate faster was also clear on medium containing mannitol. At day 1 after stratification on 100 mM mannitol, about 57% of *idl6xidl7 1.9* seeds had germinated, while only about 12% of Wt seeds had germinated at the same time (Figure 3.15 c). On 130 mM mannitol, about 70% of *idl6xidl7 1.9* seeds had germinated at day 2 (Figure 3.15 e) but only 16% of Wt seeds had germinated 2 days after stratification. The differences between Wt and *idl6xidl7 1.9* in respect to germination rate on 100 mM and 300 mM mannitol holds with at least 95% certainty. In addition, *idl6 3.5* showed a tendency to germinate faster than Wt on 300 mM mannitol at day 2 ($p \leq 0.1$).

At day 4 and 6 after stratification on 100 mM and 300 mM mannitol, respectively, total germination percentage seemed to have been reached. For both concentrations significant differences were found, indicating that Wt had a lower germination percentage than *idl6 3.5* and *idl7 1.4* (Figure 3.15 c and e). *35S:IDL6 1.3* also seemed to have a higher total germination percentage than Wt (only at day 8) (Figure 3.15 f).

Emergence of hypocotyls and cotyledons (stage 0.7) was scored as number of individuals within a line where the hypocotyls and cotyledons had emerged from the seed coat at a given time point. Figure 3.16 display the results obtained from analyzing hypocotyls and cotyledon emergence. Significant deviations between Wt and any of the transgenic lines are displayed as asterisks and p-values from Student's t-test are included in Table A19, Appendix D, together with average percentage of individuals that reached growth stage 0.7 and standard deviations.

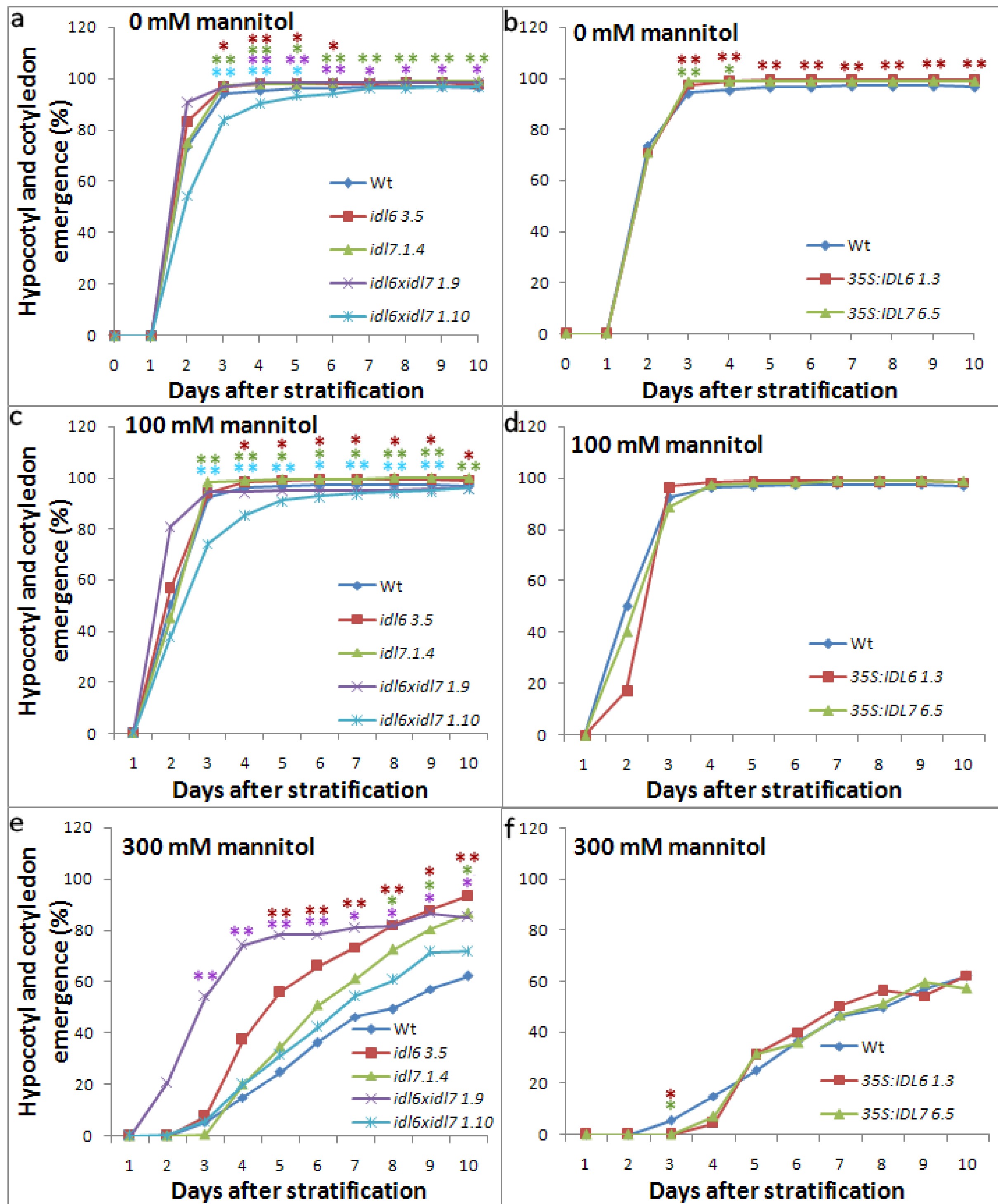

Figure 3.16 Effect of osmotic stress on development of hypocotyl and cotyledons in *A.thaliana* mutants: *idl6 3.5, idl7 1.4, idl6xidl7 1.9* and *idl6xidl7 1.10* (a, c, and e), and over-expression lines *35S:IDL6 1.3* and *35S:IDL7 6.5* (b, d, and f), compared to wild type (Wt). Average percentage of individuals with developing hypocotyls and cotyledons in three replicates (each containing at least 60 seeds from each transgenic line and Wt) of different concentrations of mannitol (0 mM; a and b, 100mM; c and d, 300 mM; e and f) in ½ x MS is displayed. Hypocotyl and cotyledon emergence was scored every day for ten days after stratification. Asterisks indicate significant deviations between transgenic lines and Wt. One asterisk (*) indicates p-values ≤ 0.1, two asterisks (**) indicate p-values ≤ 0.05. Colors of the asterisks are consistent with the colors of the graphs.

Some deviations in development of hypocotyls and cotyledons were observed between lines grown on ½ x MS without mannitol (Figure 3.16 a and b), but only between *idl6xidl7 1.10* compared to Wt and other transgenic lines in relation to speed of development. At day 3, on average 84% of individuals of *idl6xidl7 1.10* had developing hypocotyls and cotyledons, whereas in all other transgenic lines and Wt, between 94-98% of seedlings had emerging hypocotyls and cotyledons.

Variation in total percentage of seedlings that will develop through stage 0.7 was observed for seedlings grown on ½ x MS. At day 7, a significantly higher percentage of *idl7 3.5* (99%) and *35S:IDL6 1.3* (100%) seedlings reached this stage compared to Wt (97%).

On 100 mM mannitol, the same observations were made as on control plates. *idl6xidl7 1.10* grew significantly slower than Wt and significantly slower than some of the other lines (*idl7 1.4, idl6xidl7 1.9* and *35S:IDL6 1.3*) (Figure 3.16 c and d). This was clearly visual at day three, where only about 74% of *idl6xidl7 1.10* seedlings had developing hypocotyls and cotyledons. Total number of individuals that developed hypocotyl and cotyledons was reached later for plants grown on mannitol containing medium than on control plates. That is, at day 3 on control plates, approximately all individuals had developed hypocotyls and cotyledons. Whereas on mannitol containing medium; this was not observed until day 4 (on 100 mM) or beyond day 10 (in 300 mM). At day 10, only *idl7 1.4* had a significantly higher percentage than Wt of individuals that had reached this stage with $p \leq 0.05$, *idl6 3.5* had higher total percentage than Wt with a p-value of 0.064 (Figure 3.16 c).

On 300 mM mannitol, the speed at which hypocotyls and cotyledons developed varied largely between all transgenic lines and Wt (Figure 3.16 e and f). Hypocotyl and cotyledons emerged faster in *idl6xidl7 1.9* than in all other transgenic lines and Wt ($p \leq 0.05$). *Idl6 3.5* also developed hypocotyls and cotyledons faster than Wt ($p \leq 0.05$). Total number of individuals that developed through stage 0.7 was not reached at day 10 for plants grown in 300 mM mannitol, and was therefore not considered during these analysis. At day 8 and later a small significant difference between *idl7 1.4* and Wt was also observed.

An other observation made in respect to development through stage 0.7 in 300 mM was that Wt *A. thaliana* developed significantly faster than both the over-expression lines (*35S:IDL6 1.3* and *35S:IDL7 6.5*) at day 3 (Figure 3.16 f), but this early difference was not visible at later time points, and average emergence of hypocotyls and cotyledons was even lower than in over-expression lines after day 4, although this difference was not supported by t-tests.

Plants with cotyledons that were fully opened (stage 1.0) were scored as number of individuals with cotyledons that were opened with $\geq 180^{o}$ angle. Average number of individuals with fully opened cotyledons in each independent line and Wt are displayed in Figure 3.17. Asterisks indicate significant deviations and all p-values from Student's t-test are displayed in Table A20, Appendix D.

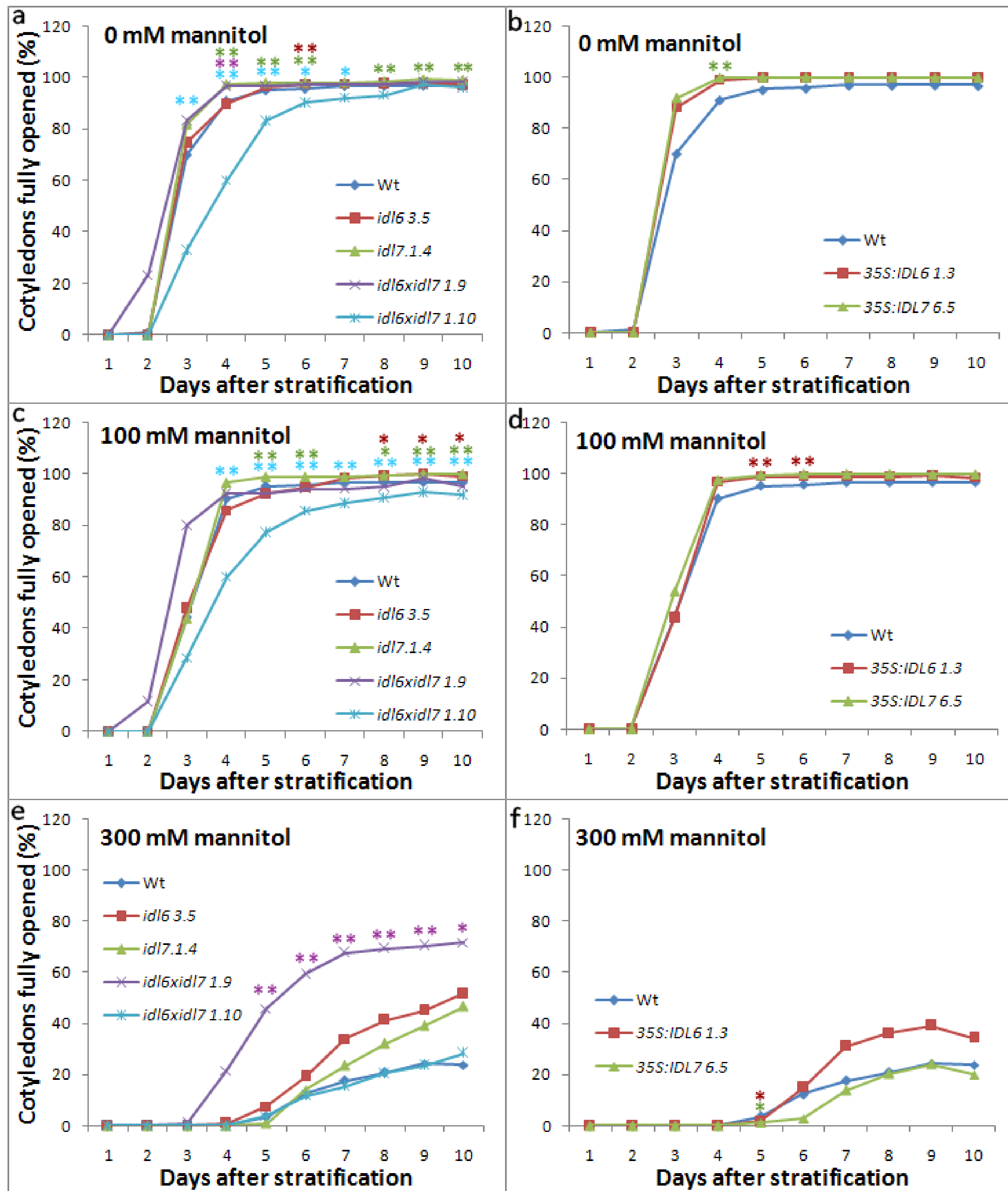

Figure 3.17 Effect of osmotic stress on development of fully opened cotyledons in *A.thaliana* mutants: *idl6 3.5, idl7 1.4, idl6xidl7 1.9* and *idl6xidl7 1.10* (a, c, and e), and over-expression lines *35S:IDL6 1.3* and *35S:IDL7 6.5* (b, d, and f), compared to wild type (Wt). Average percentage of individuals with fully opened cotyledons in three replicates (each containing at least 60 seeds from each transgenic line and Wt) of different concentrations of mannitol (0 mM; a and b, 100mM; c and d, 300 mM; e and f) in ½ x MS is displayed. Proportion of individuals with fully opened cotyledons was scored every day for ten days after stratification. Asterisks indicate significant deviations between transgenic lines and Wt. One asterisk (*) indicates p-values ≤ 0.1, two asterisks (**) indicate p-values ≤ 0.05. Colors of the asterisks are consistent with the colors of the graphs.

In general, percentage of individuals with fully opened cotyledons (Figure 3.17) showed the same significant deviations as development through stage 0.7 (Figure 3.16). However, fewer lines significantly deviated from Wt and major differences with p-values ≤ 0.05 were only observed for *idl6xidl7 1.9* and *idl6xidl7 1.10* in relation to developmental rate.

Most notably were the differences concerning total percentage of individuals that developed through stage 1.0 on medium containing 100 mM mannitol. *Idl6 3.5*, i*dl7 1.4* was observed to have an average higher number of individuals with fully opened cotyledons at day 10, whereas *idl6xidl7 1.10* was observed to have a significantly lower total average number of individuals with fully opened cotyledons (Figure 3.17 c). On 300 mM mannitol, *idl6xidl7 1.10* showed the same growth patterns as Wt (Figure 3.18 e).

Results showed that the *idl6xidl7 1.9* line grew significantly better in relations to developing fully opened cotyledons on 300 mM mannitol (Figure 3.17 e). The same trend was visible for development of green tissue, which is displayed in Figure 3.18 below. However, these results showed that this difference is less pronounced at day 8 and 9 and was not statistically supported at day 10 (Figure 3.18 e) (p-values are displayed in Table A21, Appendix D). The *idl6xidl7 1.9* line also reached a higher total number of individuals that developed green tissue compared to Wt; this is a direct consequence of higher total percentage that developed hypocotyls and cotyledons, as previously observed (Figure 3.16).

The *idl6xidl7 1.10* line also showed slower developmental rate of green tissue than Wt *A. thaliana* when grown on both regular ½ x MS (Figure 3.18 a) and on medium containing 100 mM mannitol (Figure 3.18 c). On 300 mM, *idl6xidl7 1.10* showed the same developmental trend as Wt in relation to developing green tissue (Figure 3.18 e), as was also evident at earlier developmental stages. All other lines showed similar trends as in the preceding growth stage.

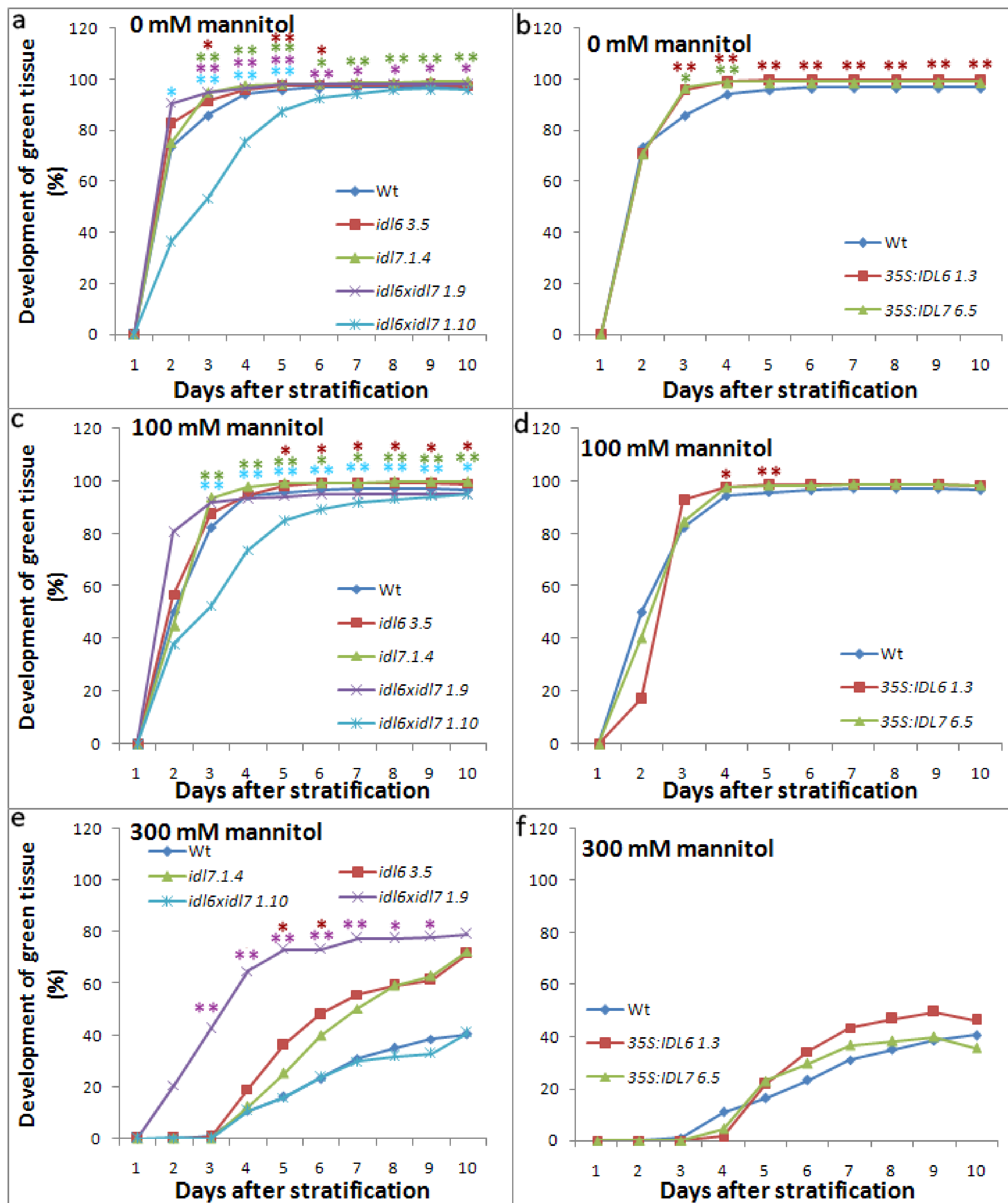

Figure 3.18 Effect of osmotic stress on development of green tissue in *A.thaliana* mutants: *idl6 3.5, idl7 1.4, idl6xidl7 1.9* and *idl6xidl7 1.10* (a, c, and e), and over-expression lines *35S:IDL6 1.3* and *35S:IDL7 6.5* (b, d, and f), compared to wild type (Wt). Average percentage of individuals with green tissue in three replicates (each containing at least 60 seeds from each transgenic line and Wt) of different concentrations of mannitol (0 mM; a and b, 100mM; c and d, 300 mM; e and f) in ½ x MS is displayed. Proportion of individuals with green tissue was scored every day for ten days after stratification. Asterisks indicate significant deviations between transgenic lines and Wt. One asterisk (*) indicates p-values ≤ 0.1, two asterisks (**) indicate p-values ≤ 0.05. Colors of the asterisks are consistent with the colors of the graphs.

3.5 UV treatment

UV cause severe oxidative stress in plant tissue (Jansen et al., 1998; Roldan-Arjona and Ariza, 2009), therefore, *A. thaliana* transgenic lines were subjected to UV light to analyze differences in oxidative stress by DAB assay. *In silico* data also shows *IDL6* and *IDL7* to be up-regulated upon UV treatment UV treated tissue from Wt *A. thaliana* was also subjected to expression analysis of *IDL6* and *IDL7* by qPCR.

3.5.1 Detection of ROS

Oxidative stress can be identified in plant tissue by detecting H_2O_2 through the DAB assay, as described in chapter 2.4. A reddish-brown color in the tissue indicates presence of H_2O_2. 15 days old plant grown on ½ x MS in glass containers irradiated with different doses of UV light showed to have H_2O_2 related enzyme activity in roots. Little or no staining was detected in rosette leaves. Root tissue from Wt, *idl6 3.5*, *idl7 1.4* and *idl6xidl7 1.10* after DAB assay is displayed in Figure 3.19.

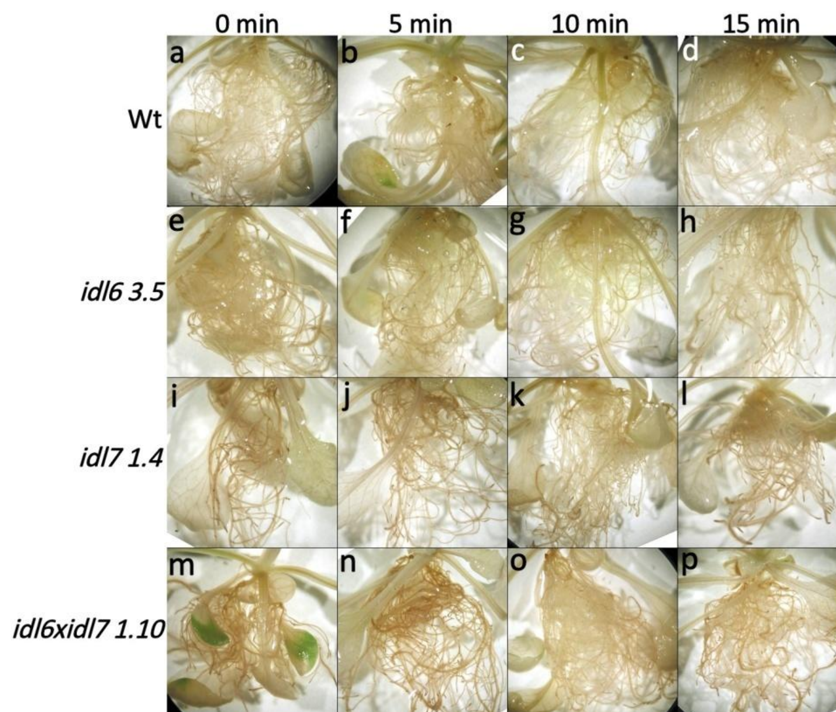

Figure 3.19 DAB assay showed small differences in ROS production in roots from plants treated with UV (365nm) for 5 minutes (b, f, j and n), 10 minutes (c, g, k and o) or 15 minutes (d, h, l and p), compared to untreated tissue (a, e, i and m). A darker reddish-brown color indicates the presence of H_2O_2. Wild type (Wt) *A. thaliana* (a-d) was used as a reference compared to ROS production in mutants; *idl6 3.5* (e-h), *idl7 1.4* (i-l) and *idl6xidl7 1.10* (m-p).

Wt *A. thaliana* (Figure 3.19 a-d) showed no major generation of H_2O_2 in any of treatments. This holds also for the *idl6 3.5* line (Figure 3.19 e-h). The *idl7 1.4* line showed higher H_2O_2 levels in roots (Figure 3.19 i-l) than other lines and Wt. This is visible as darker coloring of the roots in this line. However, the tissue in control treatments of *idl7 1.4* that was not irradiated by UV (i) also showed increased H_2O_2 levels in roots compared to Wt and *idl6 3.5*. H_2O_2 levels in *idl6xidl7 1.10* (Figure 3.19 m-p)

appeared to be even higher than in *idl7 1.4*. This was especially evident after 5 minutes dose of UV (n), but the control treatment (m) did also show increased H_2O_2 levels in this case.

No significant differences were observed between 10 min and 15 min UV dose, and the experiment was repeated with only 0, 5 and 15 min UV doses. Results are presented in Figure 3.20.

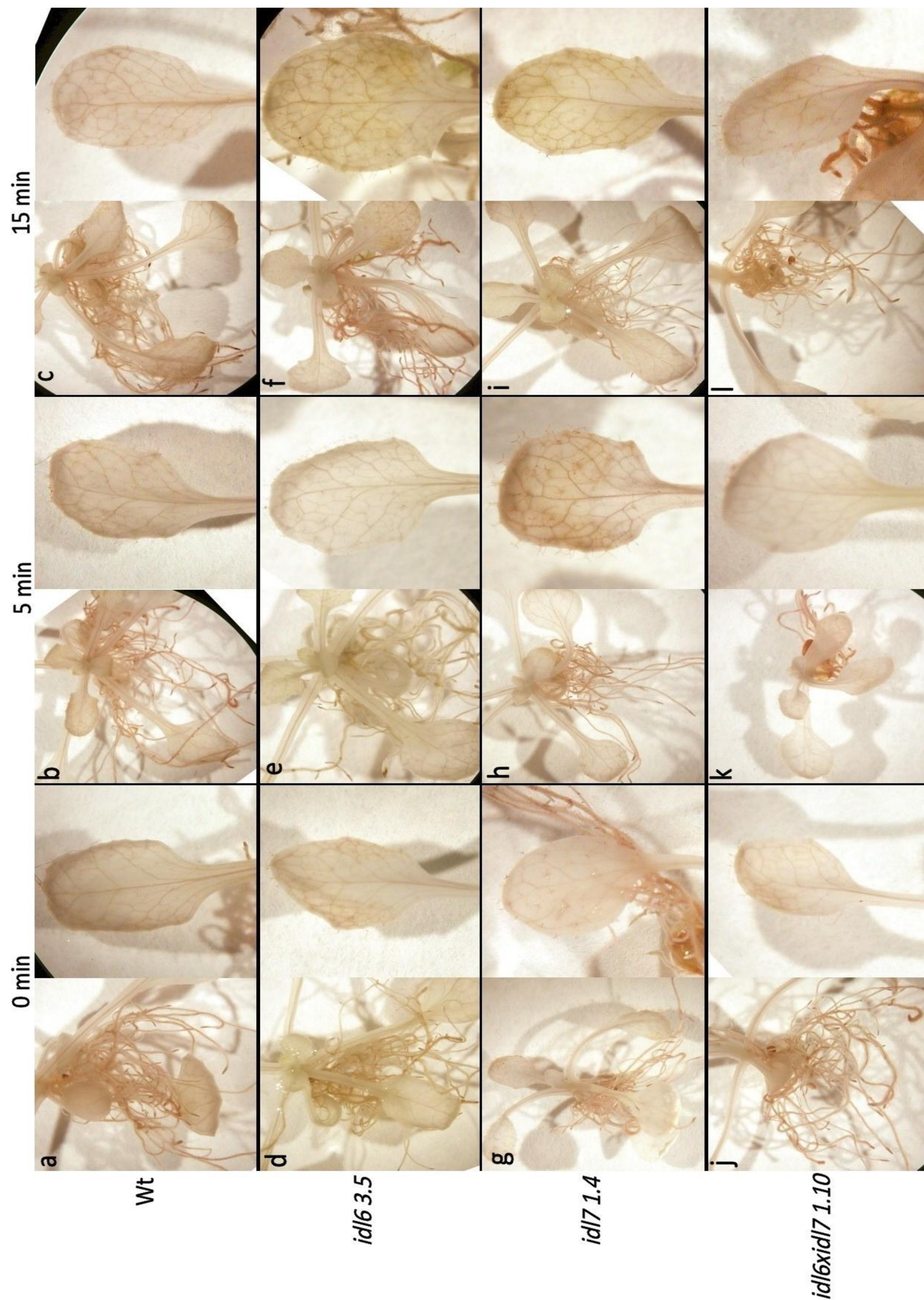

Figure 3.20 DAB assay of UV treated tissue from Wt *A. thaliana* (a-c), *idl6 3.5* (d-f), *idl7 1.4* (g-i) and *idl6xidl7 1.10* (j-l). Dark reddish-brown color indicates the presence of H_2O_2. Plants were grown on ½ x MS in glass containers and 15 days old plants were treated with UV (365 nm) for 0 min (control) (a, d, g and j), for 5 min (b, e, h and k) or for 15 min (c, f, I and l).

Results from DAB assay after UV-treatment did not give any clear indications of altered H_2O_2 levels between Wt *A. thaliana* and *idl6 3.5, idl7 1.4* or *idl6xidl7 1.10* (Figure 3.20). Only roots showed the strong red/brownish color in the tissue, which indicates the presence of H_2O_2. No obvious differences were detected between Wt and transgenic lines or differences within the lines related to UV-dose.

3.5.2 Expression analysis of UV radiated plants

15 days old Wt *A. thaliana* grown on soil was irradiated with 15 min dose of 365 nm UV light. Above ground tissue was harvested at different time points after treatment (0.5 h, 1 h, 3 h, 6 hand 24 h). RNA was isolated from the harvested tissue and qPCR was performed on cDNA with primers for amplifying *IDL6* and *IDL7*. *PHYTOALEXIN DEFICIENT 4 (PAD4)* and *ELONGATED HYPOCOTYL 5 (HY5)* were used as control genes. *PAD4* is required for R-gene action and has previously been shown to be involved in regulation of H_2O_2 metabolism (Rusterucci et al., 2001). *in silico* data also show *PAD4* to be up-regulated upon UV-B treatment (BAR, 2007). *HY5* is a transcription factor that has been shown to regulate several UV-B induced genes and has previously been shown to be up-regulated upon UV-B treatment by both qPCR (Brown et al., 2005) and in *in siilico* data (BAR, 2007). *TIP41*-like and *PP2A* were used as reference genes. Figure 3.21 displays the log2 ratios of relative expression of *IDL6* and *IDL7*. Output data from analyses in LinReg and REST2009 are provided in Table A23, Appendix D.

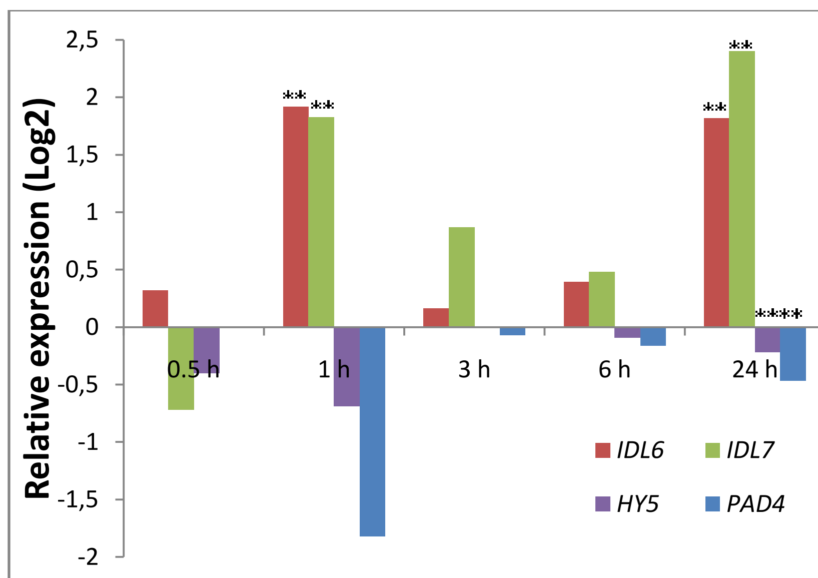

Figure 3.21 Relative expression (Log2) of *IDL6*, *IDL7*, *HY5* and *PAD4* in 15 days old Wt *A. thaliana* grown on soil, tissue harvested 0.5 h- 24 h after UV treatment (15 min 365 nm) compared to untreated tissue harvested at the different time points. Asterisks (**) indicate significant difference from untreated tissue with p-values ≤ 0.05.

Significant regulation of *IDL6* and *IDL7* was only observed in tissue harvested 1 h and 24 h after treatment. REST analyses showed that the control genes *PAD4* and *HY5* were significantly down-

regulated 24 h after treatment compared to non-treated control groups. No significant regulation of the *PAD4* and *HY5* was observed at earlier time points.

3.6 Promoter activity of *IDL6* and *IDL7*

Several different experiments was conducted with *promoter:GUS* lines to investigate promoter activity of *IDL6* and *IDL7*. Promoter studies can give results indicating the extent of expression of a gene of interest. They may also give indications of possible localization of the gene product. Stress treatments that were analyzed in this thesis were chosen using the *Arabidopsis* eFP Browser, as previously described, other treatments were chosen based on previously published literature and are referred to in the presentation of the results from the respective treatments. *Promoter:GUS* lines were chosen based on segregation analyses previously presented (chapter 3.1). Only lines which showed 3:1 distribution in T_1 generation or 1:0 distribution in T_2 generation were used in experiments described here.

3.6.1 Infestation with aphids

Previous research has shown *IDL6* and *IDL7* to be up-regulated on microarray upon infestation with aphids (Kusnierczyk et al., 2008). *promoter:GUS* lines were therefore used to investigate the potential expression patterns of *IDL6* and *IDL7* upon aphid infestation.

23 days old soil-grown plants of *pIDL6:GUS 18.4* and *pIDL6:GUS 25.4* were infested with aphids (*Myzus persicae*) in a pilot study. 6 adult aphids were transferred to each plant individual and kept in plexi glass cylinders for 72h before GUS staining. Results are presented in Figure 3.22. Plants grown under same conditions without aphids were used as controls.

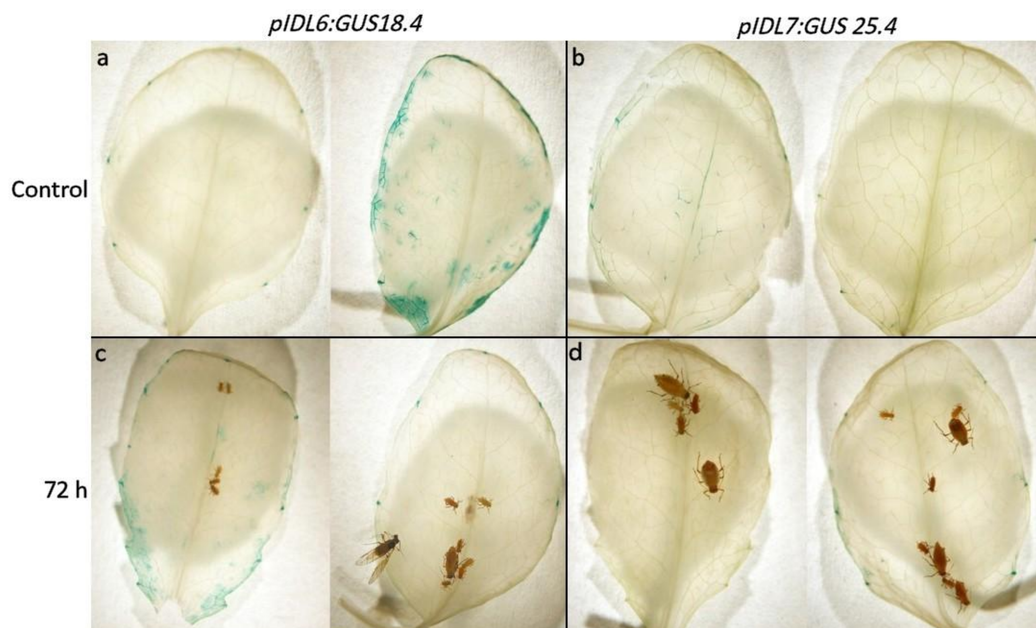

Figure 3.22 23 days old *pIDL6:GUS 18.4* (a and c) and *pIDL6:GUS 25.4* (b and d) plants were infested with aphids for 72 hours (c and d) upon harvesting for GUS assay. Tissue from plants not infested with aphids was harvested as negative controls (a and b).

Patterns of GUS staining were not observed to be consistent with aphid infestation and replicates are too few to give any conclusions about promoter activity of *IDL6* in this pilot study.

A new experiment was performed with both *pIDL6:GUS* and *pIDL7:GUS* lines. 4 adult aphids were transferred to each rosette leaf of 21 days old plants (6-8 rosette leaves). 7 plant individuals each of *pIDL6:GUS 4.1, pIDL6:GUS 18.4* and *pIDL7:GUS 3.5* were infested with aphids, equal amounts of control individuals were grown under the same conditions. *pCYP79B2:GUS* and *pCYP79B3:GUS* were used as additional controls. *CYP79B2* and CYP79B3 are two cytochrome P450 enzymes which previously have been shown to be up-regulated upon aphid infestation in both microarray and by GUS assay (Kusnierczyk et al., 2007). 2 leaves from each plant infested with aphids and controls were harvested for GUS assay. Figure 3.23 below shows GUS staining in the harvested leaves.

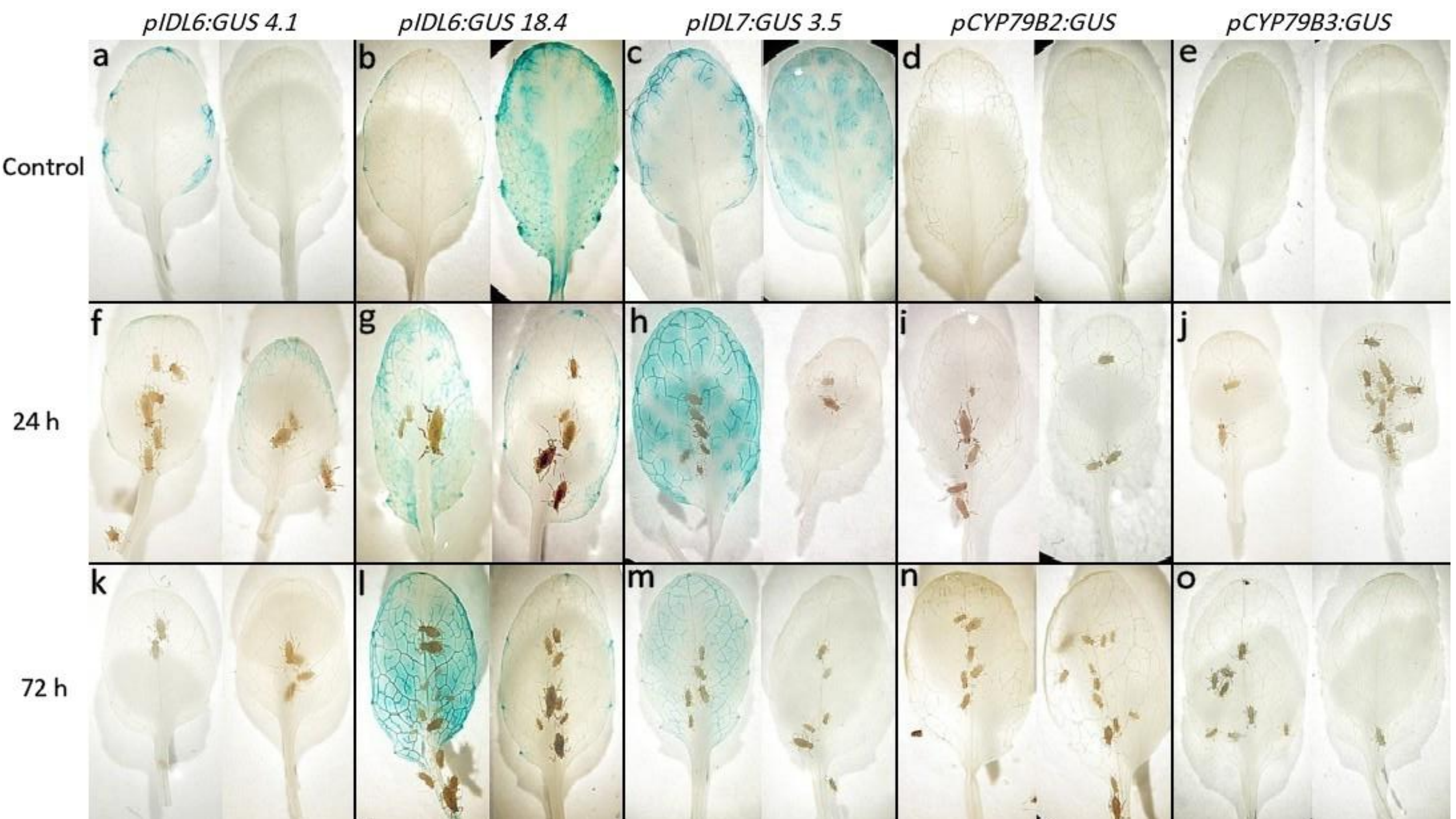

Figure 3.23 21 days old *A. thaliana* GUS lines grown on soil were infested with aphids for 24 h (f-j) and 72 h (k-o). GUS assay was also performed on plants not infested with aphids (a-e). Promoter activity of *IDL6* was analyzed in *pIDL6:GUS 4.1* (a,f and k) and *pIDL6:18.4* (b, g and l). Promoter activity of *IDL7* was analyzed in *pIDL7:GUS 3.5* (c, h and m). Promoter activity in *pcyp79B2:GUS* (d, I and n) and *pcyp79B3:GUS* (e, j and o) were analyzed as controls.

pCYP79B2:GUS and *pCYP79B3:GUS* showed expression only in vascular tissue, whereas the GUS expression of *pIDL6:GUS* and *pIDL7:GUS* was mainly located to other leaf tissue than vascular tissue.

Control plants of *pIDL6:GUS 4.1, pIDL6:GUS 18.4* and *pIDL7:GUS 3.5* all showed GUS staining. This makes the GUS staining in tissue infested with aphids complicated to interpret. GUS staining in treated tissue is very variable, both in relations to infestations time (24 hours or 72 hours) and in relation to variation within each of the *promoter:GUS* lines investigated. Although effort was made to collect leaves which definitely had been fed on by the aphids by only harvesting rosette leaves for GUS assay that had several aphids on, no control exists for the actual feeding patterns of aphid since they are mobile on the plant.

3.6.2 Stress assay of plants growing in liquid ½ x MS

5 days old seedlings of *A. thaliana* GUS lines (*pIDL6:GUS* and *pIDL7:GUS*) grown on solid ½ x MS were transferred to liquid ½ x MS for adaptation for three days. 8 days old seedlings were treated with NaCl, mannitol, H_2O_2, Paraquat and Flg22. GUS assay after treatments of 1 and 2 hours revealed promoter activity of *IDL6* and *IDL7* but no consistent differences between lines or treatment were detected. Figure 3.24 shows GUS expression upon the different treatments.

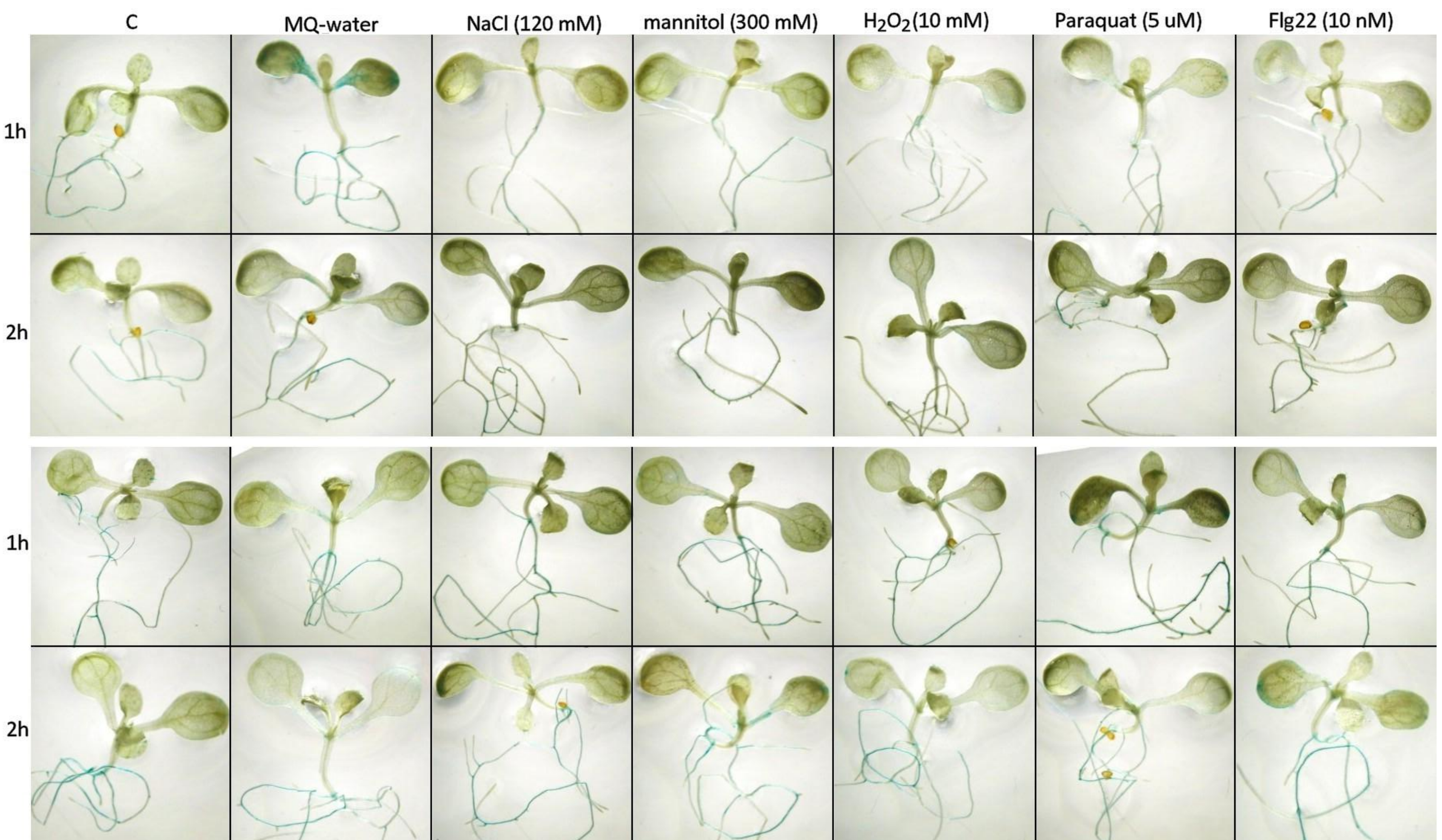

Figure 3.24 GUS assay of *A.thaliana* GUS lines. 8 days old seedling of *pIDL6:GUS 18.4* (upper two rows) and *pIDL7:GUS 6.5* (lower two rows) were treated for 1 or 2 hours with different solvents in liquid ½ MS. Untreated plants not treated in liquid ½ x MS was included as negative controls (C), MQ-water was used as mock treatment.

GUS assay shows promoter activity of *IDL6* and *IDL7* in roots after all treatments, but also in controls that were treated with MQ-water and in untreated seedlings which was grown on solid ½ x MS and harvested directly for GUS assay. The untreated plants were included to investigate if growing in liquid ½ x MS would influence the promoter activity and thus expression of *IDL6* and *IDL7*. As Figure 3.24 shows, GUS activity was detected in roots of seedlings treated with MQ-water and in seedlings only grown in liquid ½ x MS. *pIDL7:GUS 6.5* showed more GUS activity in roots than *pIDL6:GUS 18.4* for all treatments. In addition, *pIDL6:GUS 18.4* seedlings treated with MQ-water strangely showed more GUS activity in cotyledons after 1 h, than in seedlings treated with any of the other compounds.

3.6.4 Infiltration of H_2O_2 or *Pseudomonas syringae* pv. *tomato*

5 weeks old GUS lines of *A. thaliana* were infiltrated with H_2O_2 and Pst to investigate promoter activity and localization upon stress treatment of ROS and bacteria. Only one half of each leaf was treated. Different solutions were forced into the intracellular space through stomatal openings on one half of the underside of each leaf. Two leaves on each plant were treated. Tissue was harvested 0.5 h, 1 h and 24 h after infiltration. Figure 3.25, 3.26 and 3.27 show promoter activity of *pIDL7:GUS 3.5* after 0.5 h, 1 h and 24 h respectively. The leaves are representative of the GUS staining pattern throughout the plants that were analyzed and effort was made to include leaves representing leaves with the most and the least GUS staining if variation was observed within a certain treatment. When harvesting tissue after 24 h, additional neighboring leaves of infiltrated leaves were harvested. Pst was solved in 10 mM $MgCl_2$ and H_2O_2 was solved in MQ-water. The solvents were used as mock treatment to represent a negative control for effects caused by treatment or solvent.

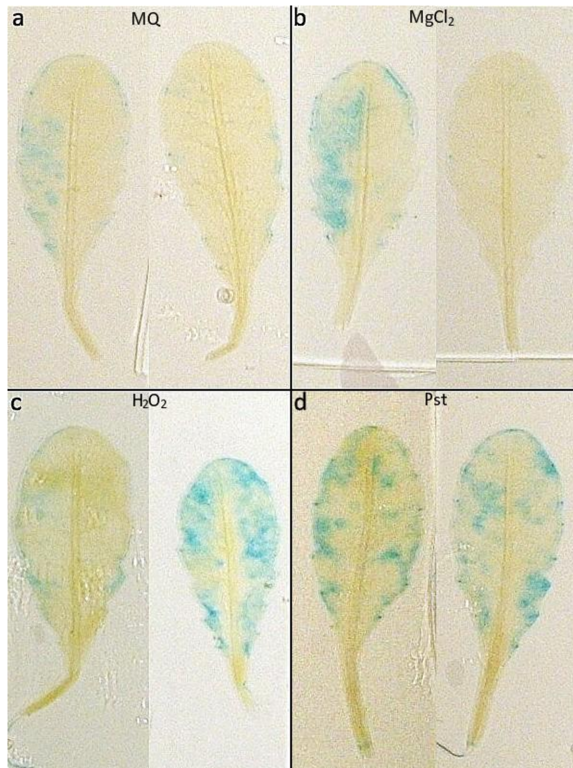

Figure 3.25 Effect of treatment with MQ-water (a) and 10mM $MgCl_2$ (b) as controls for treatment with 10 mM H_2O_2 (c) and *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (10^4 CFU/mL) (d), respectively, reveals activity of *IDL7* promoter in *pIDL7:GUS 3.5* 0.5 h after infiltration. All leaves are shown with the side of infiltration to the left.

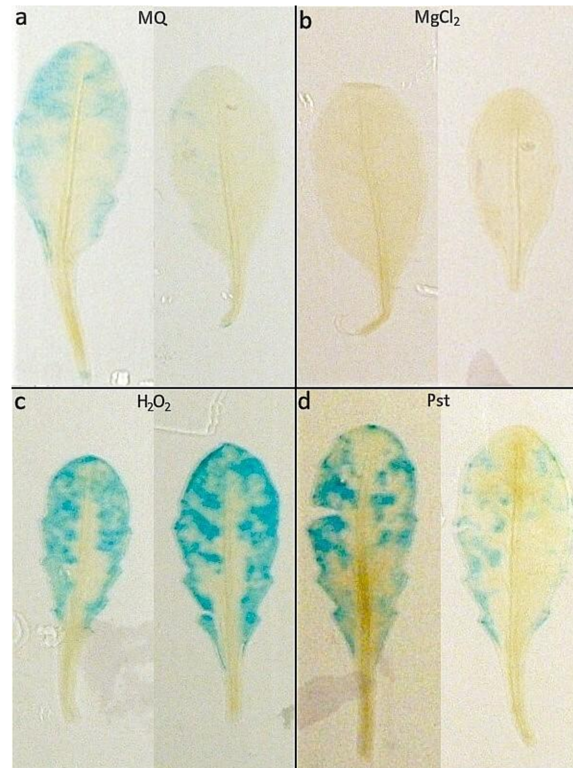

Figure 3.26 Effect of treatment with MQ-water (a) and 10 mM $MgCl_2$ (b) as controls for treatment with 10 mM H_2O_2 (c) and *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (10^4 CFU/mL) (d), respectively, reveals activity of *IDL7* promoter in *pIDL7:GUS 3.5* 1 h after infiltration. All leaves are shown with the side of infiltration to the left.

More GUS activity was observed in tissue harvested 0.5 h after treatment of H_2O_2 (Figure 3.25 c) and Pst (Figure 3.25 d) than in tissue treated with MQ-water (Figure 3.25 a) or $MgCl_2$ (Figure 3.25 b). Some observable effects of infiltration by the syringe were also visible in the control leaves by the patterns of *GUS* expression. Only one side of the leaf showed *GUS* expression, which is consistent with the infiltration pattern. However, this was not the case for all control leaves, as some of them did not show a specific pattern potentially caused by the infiltration. All leaves treated with either H_2O_2 or Pst showed GUS expression 0.5 h after treatment, but no obvious patterns consistent with infiltration position was observed.

GUS activity in leaves harvested 1 h after treatment (Figure 3.26) showed similar patterns as the 0.5 h treatment except for control leaves, which showed less GUS activity (Figure 3.26 a and b). The leaves treated with H_2O_2 or Pst (Figure 3.26 c and d) also showed a tendency to have more expression 1 h after treatment than 0.5 h after treatment.

In tissue harvested after 24 hours, leaves from control treatments with MQ-water or $MgCl_2$ showed some GUS expression (Figure 3.27 a and b). One of the leaves from control leaves harvested showed

severe GUS expression. However, most of the leaves did not show any GUS expression. The rest of the leaves showed no or very little GUS expression after control treatments. The little GUS expression observed in control leaves after 24 h was located to the edges of the rosette leaves and some also at the cutting site in the petioles.

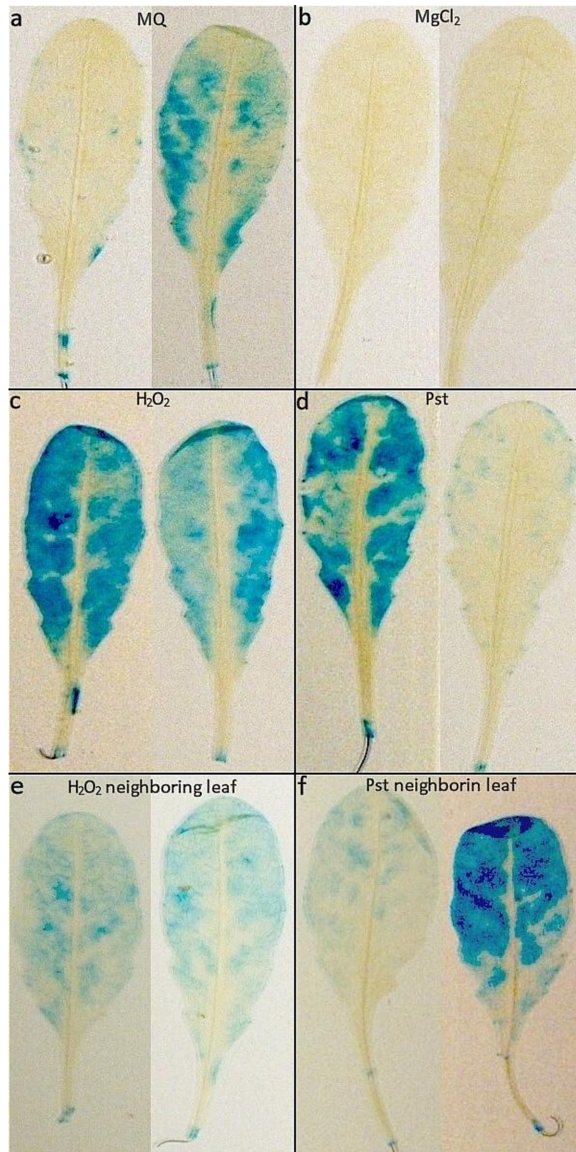

Figure 3.27 Effect of treatment with MQ-water (a) and 10 mM $MgCl_2$ (b) as controls for treatment with 10 mM H_2O_2 (c) and *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (10^4 CFU/mL) (d), respectively, reveals activity of *IDL7* promoter in *pIDL7:GUS 3.5* 24 h after infiltration. Neighboring leaves were harvested for GUS assay to investigate systemic response (e and f, for H_2O_2 treated and Pst treated plants respectively). All infiltrated leaves are shown with the side of infiltration to the left.

Leaves infiltrated with H_2O_2 or Pst (Figure 3.27 c and d) all showed GUS expression 24 h after infiltration, and the GUS staining was even stronger than in leaves harvested 0.5 h and 1 h after infiltration.

In plants infiltrated with either H_2O_2 or Pst, neighboring leaves of the leaves infiltrated were also harvested for GUS assay. This revealed extensive GUS expression in neighboring leaves and the same pattern of GUS expression was observed, although, it seemed to be weaker than in leaves directly infiltrated by H_2O_2 or Pst (Figure 3.27 e and f).

A separate experiment was conducted on 5 weeks old plants including several different control lines for possibly detecting similarities of known stress induced expression patterns and as a control to verify that the GUS staining is actually caused by Pst and H_2O_2 treatment. Further the controls may possibly indicate a connection of *IDL6* and *IDL7* to salicylic acid or jasmonic acid in stress response.

pPDF1.2:GUS is a GUS reporter line for the promoter of *PDF 1.2* which previously has been shown to be responsive to microbial attacks. The gene encodes a plant defensin, a small peptide involved in defense response, which is responsive to ethylene, methyl jasmonate and ROS. *pdf 1.2* has been widely investigated, and expression pattern and GUS reporter lines has been studied upon different stress treatments and treatments with chemical compounds (Manners et al., 1998). GUS staining in rosette leaves from *pPDF1.2:GUS* 1 h after treatment with H_2O_2 or Pst as previously described is shown in Figure 3.28. Both mock treatments and treatments with H_2O_2 or Pst showed extensive GUS expression 1 h after treatment.

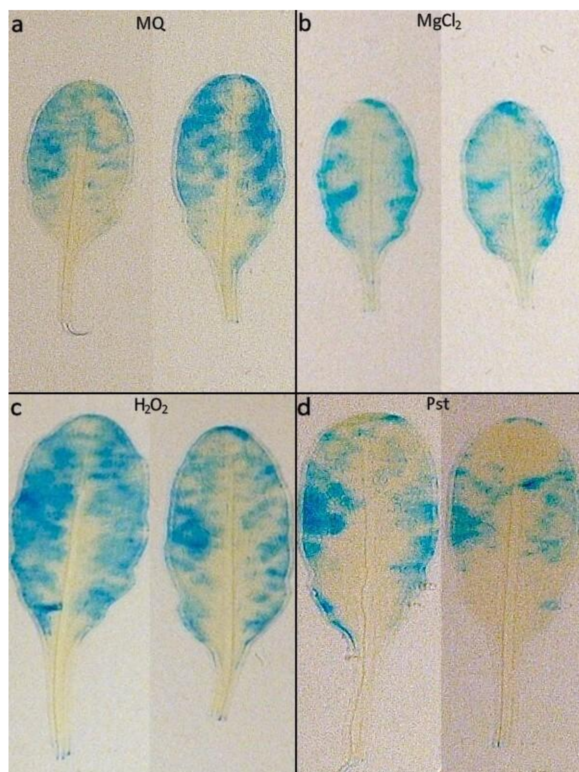

Figure 3.28 Effect of treatment with MQ-water (a) and 10 mM $MgCl_2$ (b) as controls for treatment with 10 mM H_2O_2 (c) and *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (10^4 CFU/mL) (d), respectively, reveals activity of *PDF* promoter in *pPDF 1.2:GUS* 1 h after infiltration. All leaves are shown with the side of infiltration to the left.

pPR1:GUS was also used to compare GUS expression of *IDL6* and *IDL7 promoter:GUS* transgenic lines upon treatment with H_2O_2 or Pst. *PATHOGENESIS RELATED 1 (PR1)* gene has previously been shown to be up-regulated following hypersensitive response and systemic acquired resistance (Van Loon and Van Strien, 1999) and therefore should display promoter activity upon H_2O_2 or Pst treatment. The expression patterns of *pPR1:GUS* are displayed in Figure 3.29.

A second *pPR1:GUS* line was also subjected to H_2O_2 or Pst treatment to investigate promoter activity of *PR1*. However, the promoter of *PR1* was in this case investigated in the *nonrace-specific disease resistance 1(ndr1)* mutant background (Zhang and Shapiro, 2002). Mutations in *ndr1* will suppress the resistance response of several different resistance proteins in addition to blocking ROS dependant induction of SA biosynthesis. In other words, the *ndr1-1* mutant has a defect HR and SAR and is not able to fight of pathogens. Expression of *PR1* is induced by HR and SAR, and will thus be reduced in *ndr1-1* and it is expected that the promoter will be rendered inactive and will not lead to expression of *GUS* during treatment of H_2O_2 or Pst. Results after 1 h treatment with H_2O_2 or Pst are displayed in Figure 3.30.

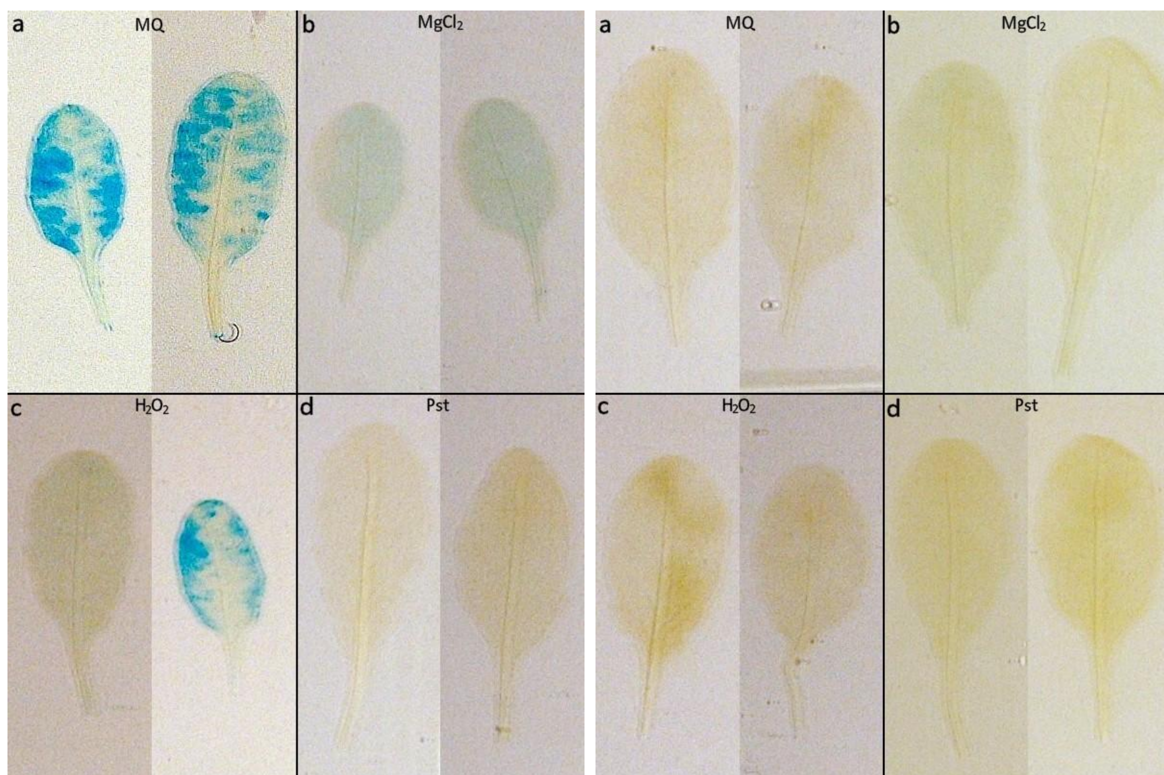

Figure 3.29 Effect of treatment with MQ-water (a) and 10 mM $MgCl_2$ (b) as controls for treatment with 10 mM H_2O_2 (c) and *Pseudomonas syringae* pv. tomato (Pst) DC3000 (10^4 CFU/mL) (d), respectively, reveals activity of *PR1* promoter in *pPR1:GUS* 1 h after infiltration. All leaves are shown with the side of infiltration to the left.

Figure 3.30 Effect of treatment with MQ-water (a) and 10 mM $MgCl_2$ (b) as controls for treatment with 10 mM H_2O_2 (c) and *Pseudomonas syringae* pv. (Pst) DC3000 (10^4 CFU/mL) (d), respectively, reveals activity of *PR1* promoter in *pPR1:GUS* in *ndr1-1* mutant background 1 h after infiltration. All leaves are shown with the side of infiltration to the left.

Results showed that in tissue harvested 1 h after mock treatment or H_2O_2 or Pst treatment GUS staining was very variable. Some GUS staining was observed for mock treatment with MQ-water and treatment with H_2O_2 in the *pPR1:GUS* line after 1 h (Figure 3.29 a and c), however, no staining of tissue treated with $MgCl_2$ or Pst was observed in this experiment (Figure 3.29 b and d). More staining in mock treated tissue was also observed in comparison to H_2O_2 treated tissue. No observation of promoter activity was observed in the *pPR1:GUS/ndr1-1* line (Figure 3.30 a-d).

Results from treatments of *pIDL6:GUS 4.4* and *pIDL7:GUS 6.5* are presented in Figure 3.31 and 3.32, respectively. Treatment with MQ-water or H_2O_2 did not lead to any GUS staining and thus promoter activity of *IDL6* in the harvested tissue (Figure 3.31 a and c). Treatment with Pst gave indications of promoter activity of *IDL7* in tissue harvested 1 h after treatment (Figure 3.32 d), this was also visible in control treatment with $MgCl_2$ (Figure 3.32 b).

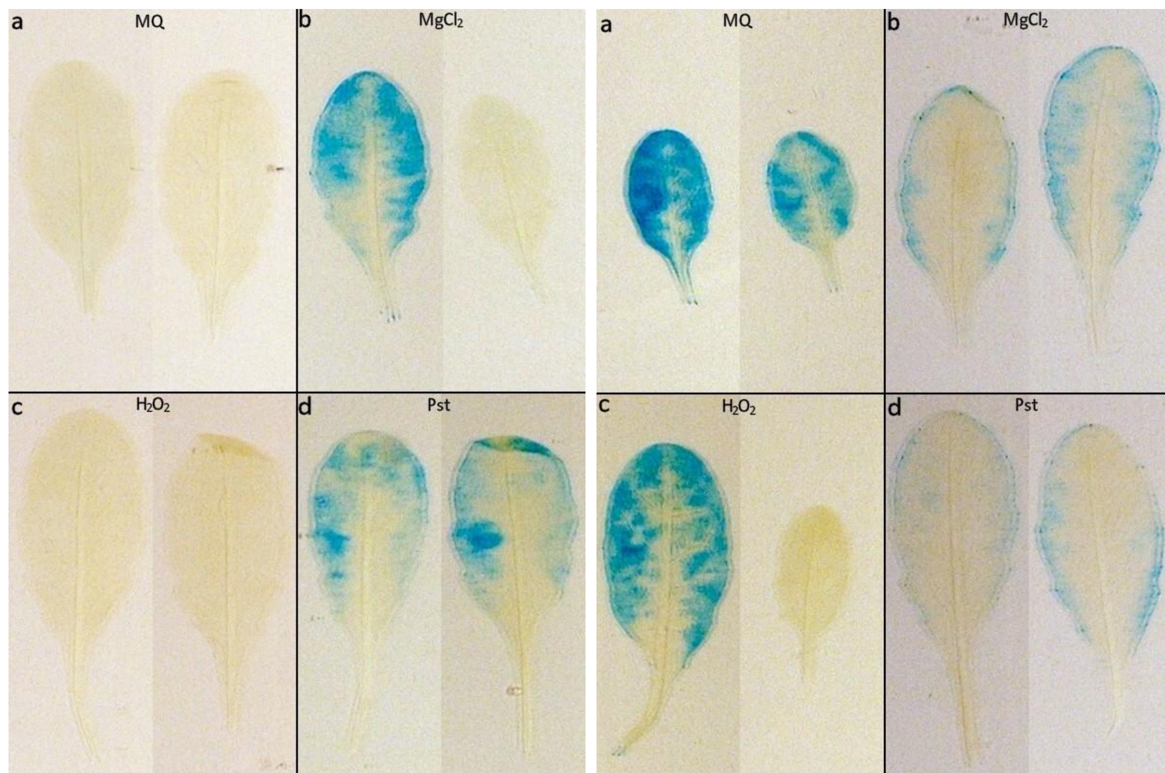

Figure 3.31 Effect of treatment with MQ-water (a) and 10 mM $MgCl_2$ (b) as controls for treatment with 10 mM H_2O_2 (c) and *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (10^4 CFU/mL) (d), respectively, reveals activity of *IDL6* promoter in *pIDL6:GUS 4.4* 1 h after infiltration. All leaves are shown with the side of infiltration to the left.

Figure 3.32 Effect of treatment with MQ-water (a) and 10 mM $MgCl_2$ (b) as controls for treatment with 10 mM H_2O_2 (c) and *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (10^4 CFU/mL) (d), respectively, reveals activity of *IDL7* promoter in *pIDL7:GUS 6.5* 1 h after infiltration. All leaves are shown with the side of infiltration to the left.

The *IDL7* promoter showed extensive GUS staining in both mock treatment and treatments with H_2O_2 and Pst (Figure 3.32). The results of *pIDL7:GUS 6.5* presented here showed that mock treatments with MQ-water and $MgCl_2$ caused equal amounts or even more GUS staining than treatments with H_2O_2 or Pst.

3.7 Screening of GUS lines

A thorough large scale screen was performed on several *promoter:GUS* lines of *IDL6* and *IDL7* because of varying results in *promoter:GUS* experiments, as described above. New segregation analyses were conducted of T_2 generations. Screens of *promoter:GUS* lines at several different growth stages were performed to define the level of background *GUS* expression in plants grown under normal conditions. Tissue was also harvested from different growth stages for expression analyses of *IDL6* and *IDL7* amplicons in comparison to *GUS* expression (*UidA* amplicon) under the control of the *IDL6* or *IDL7* promoter.

3.7.1 Reanalysis of single T-DNA insertion GUS lines

For analyses of promoter activity of *IDL6* and *IDL7*, segregation analyses of T_2 *pIDL6:GUS* lines and *pIDL7:GUS* lines were performed. Table A6 and A7 in Appendix D shows the segregation analyses of seedlings grown on ½ x MS with hygromycin for selection of lines containing T-DNA. A χ^2-test (Appendix C) was used to evaluate the null hypothesis "1:0 distribution" of homozygous resistant individuals.

3.7.2 Promoter activity screen of GUS lines

pIDL6:GUS lines and *pIDL7:GUS* lines were grown on ½ x MS to investigate *GUS* expression and promoter activity of *IDL6* and *IDL7* under normal growth conditions. 10 days old seedlings were harvested for GUS assay. Figure 3.33 shows a selection of the lines that were analyzed. Selections were made to include both lines with the least and the most amount of GUS staining.

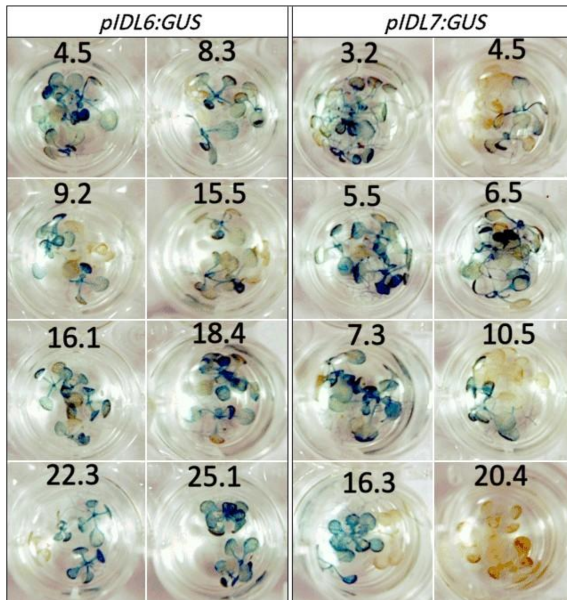

Figure 3.33 GUS assay of 10 days old *A. thaliana* seedlings of different *promoter:GUS* lines of both *IDL6* and *IDL7* promoters reveals promoter activity in plants grown under normal conditions.

All lines of *pIDL6:GUS* and *pIDL7:GUS* (except *pIDL7:GUS 20.4*) show extensive GUS staining during normal conditions in 10 days old seedlings. Some of the lines were chosen for investigation of GUS activity at later growth stages. Figures 3.34 and 3.35 show results of GUS staining in *promoter:GUS* lines. Rosette leaves were harvested from 23 days and 35 days old plants grown under normal conditions. Inflorescence was collected from 35 days old plants with 12- 15 open flowers; the inflorescence was collected to include flowers in position 1-6.

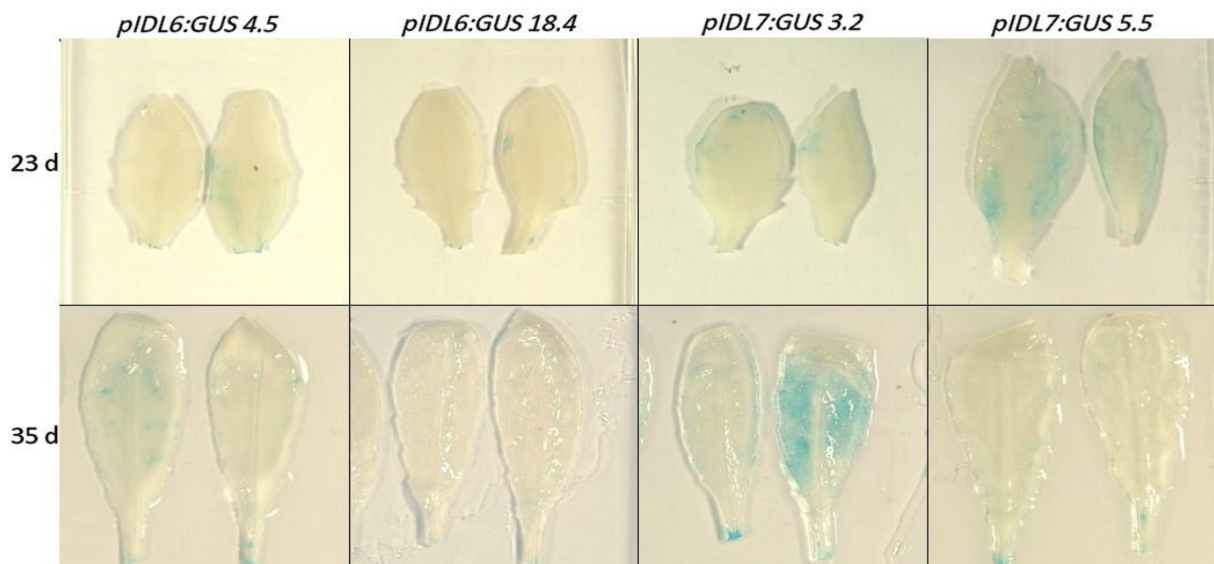

Figure 3.34 Rosette leaves were harvested from 23 and 35 days old plants of *promoter:GUS* lines grown under normal conditions: *pIDL6:GUS 4.5*, *pIDL6:GUS 18.4*, *pIDL7:GUS 3.2* and *pIDL7:GUS 5.5*. Little or no GUS activity was detected in rosette leaves in both lines at both stages.

Little or no GUS activity was detected by GUS assay in rosette leaves in 23 and 35 days old *promoter:GUS* lines. Some GUS activity was also detected in sites at the petiole where leaves were cut from the plant. On the contrary, inflorescence of 35 days old plants shows more GUS activity.

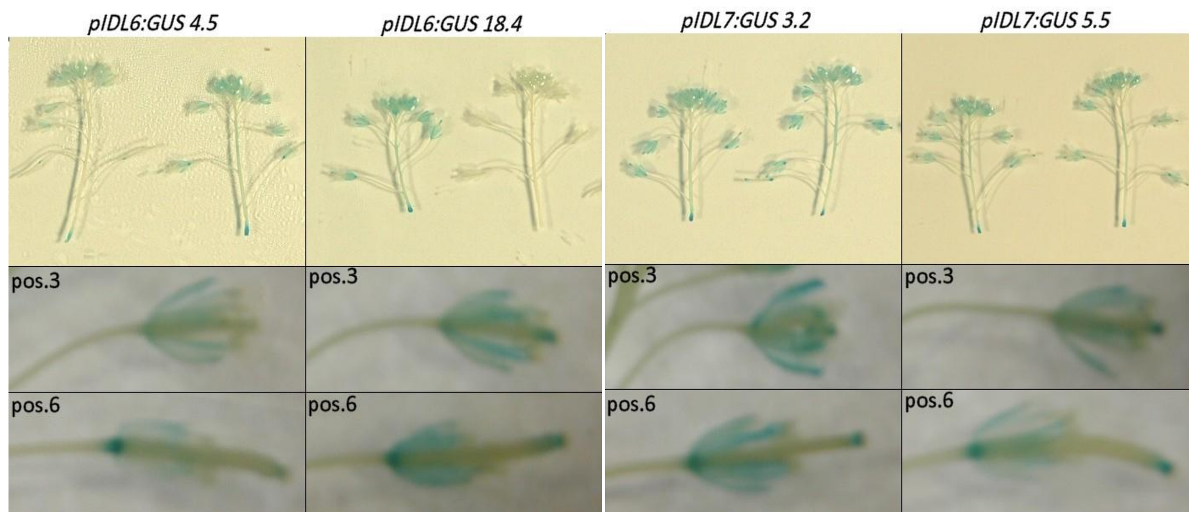

Figure 3.35 Inflorescence was harvested from 35 days old plants of *promoter:GUS* lines grown under normal conditions: *pIDL6:GUS 4.5*, *pIDL6:GUS 18.4*, *pIDL7:GUS 3.2* and *pIDL7:GUS 5.5*. GUS activity was detected in inflorescence in both lines. Inflorescence including flowers in position 1-6 from plants with 12-15 open flowers shows a consistent amount of GUS activity. Close up of flowers in positions 3 and 6 (pos.3 and pos.6) imaged in light microscopy are shown in the lower panels.

GUS activity was detected in the inflorescence of all *promoter:GUS* lines tested of the *IDL*-promoters. *GUS* expression was most pronounced in flower organs, as the close ups of flowers in position 3 and 6 in Figure 3.35 shows. GUS activity was restricted to petals and the tip of the developing siliques. In flowers of position 6, a strong GUS staining was also detected at the base of the flower and silique. GUS staining was also detected in the stem where the inflorescence had been cut during harvesting.

3.7.3 Expression analyses of GUS lines

Data in the *Arabidopsis* eFP Browser (BAR, 2007) shows *IDL6* and *IDL7* to have low expression during normal conditions, but analyses of promoter activity done here shows that the genes are highly active during normal conditions. It would therefore be interesting to analyze the mRNA content of *IDL6* and *IDL7* compared to *UidA* (the gene encoding GUS) controlled by the *IDL6* and *IDL7* promoters.

10 days old seedlings of *pIDL6:GUS* and *pIDL7:GUS* lines were harvested for qPCR analysis to investigate expression of *UidA* controlled by promoters of *IDL6* or *IDL7* compared to expression of *IDL6* and *IDL7* in the same transgenic lines. Ct-values of *IDL6, IDL7* and *UidA* in four different lines of each *pIDL6:GUS* and *pIDL7:GUS* is presented in Table 3.3 together with an estimated fold change in expression obtained from REST2009. Reaction efficiencies from experiments are provided in Table A24, Appendix D.

Table 3.3 Mean Ct-values obtained from LinReg and standard deviations (SD) from qPCR analyses of tissue harvested from 10 days old seedlings of different *promoter:GUS* lines and Wt grown on ½ x MS under normal conditions. An estimate of fold change expression was obtained from REST2009 analyses.

	Mean Ct *IDL6* ± SD	**Mean Ct *UidA* ± SD**	**Estimated fold change in expression**
Wt	31.36 ± 5.11	40.04 ± 3.18	
pIDL6:GUS 4.5	28.14 ± 0.83	21.92 ± 0.39	1000 fold
pIDL6:GUS 15.8	27.87 ± 0.12	25.70 ± 0.16	100 fold
pIDL6:GUS 18.4	27.09 ± 0.43	22.63 ± 0.38	1000 fold
pIDL6:GUS 25.1	28.25 ± 1.11	22.94 ± 1.11	1000 fold
	Mean Ct *IDL7* ± SD	**Mean Ct *UidA* ± SD**	
Wt	25.98 ± 0.20	37.37 ± 1.21	
pIDL7:GUS 3.2	26.38 ± 0.36	19.78 ± 0.23	100 fold
pIDL7:GUS 5.5	24.85 ± 0.24	20.11 ± 0.20	10 fold
pIDL7:GUS 16.3	26.51 ± 0.41	20.71 ± 0.94	100 fold
pIDL7:GUS 20.4	26.47 ± 0.31	22.87 ± 0.01	10 fold

Ct-values showed large deviations between *IDL6* and *IDL7* amplicon and the *UidA* amplicon. *UidA* had much lower Ct-values than either *IDL6* or *IDL7.* The *UidA* amplicon showed up several cycles before the *IDL6* and *IDL7* amplicons for all *promoter:GUS* lines investigated. No dramtic change was observed comparing *IDL6* and *IDL7* amplicons in Wt and *promoter:GUS* lines indicating that the *promoter:GUS* lines behave like Wt in respect mRNA levels of *IDL6* and *IDL7*.

As expected, great difference was observed comparing Ct-values of *UidA* in Wt and *promoter:GUS* lines since Wt *A. thaliana* normally does not contain the bacterial gene *UidA*.

Rosette leaves of 23 days old plants grown on soil under normal conditions were harvested from the same *promoter:GUS* lines. Ct-values of *IDL6, IDL7* and *UidA* in four different lines of each *pIDL6:GUS* and *pIDL7:GUS* is presented in Table 3.4 together with an estimated fold change in expression obtained from REST2009. Reaction efficiencies from experiments are provided in Table A25, Appendix D.

Table 3.4 Mean Ct-values obtained from LinReg and standard deviations (SD) from qPCR analyses of tissue harvested from 23 days old plants of different *promoter:GUS* lines and Wt grown on soil under normal conditions. An estimate of fold change expression was obtained from REST2009 analyses.

	Mean Ct *IDL6* ± SD	Mean Ct *UidA* ± SD	Estimated fold change in expression
Wt	31.36 ± 5.11	40.04 ± 3.18	
pIDL6:GUS 4.5	28.14 ± 0.83	21.92 ± 0.39	10 fold
pIDL6:GUS 15.8	27.87 ± 0.12	25.70 ± 0.16	10 fold
pIDL6:GUS 18.4	27.09 ± 0.43	22.63 ± 0.38	10 fold
pIDL6:GUS 25.1	28.25 ± 1.11	22.94 ± 1.11	10 fold
	Mean Ct *IDL7* ± SD	**Mean Ct *UidA* ± SD**	
Wt	25.98 ± 0.20	37.37 ± 1.21	
pIDL7:GUS 3.2	26.38 ± 0.36	19.78 ± 0.23	10 fold
pIDL7:GUS 5.5	24.85 ± 0.24	20.11 ± 0.20	10 fold
pIDL7:GUS 16.3	26.51 ± 0.41	20.71 ± 0.94	10 fold
pIDL7:GUS 20.4	26.47 ± 0.31	22.87 ± 0.01	10 fold

Difference in Ct-values between *IDL6* and *UidA* and *IDL7* and *UidA* was observed to follow the same pattern as in 10 days old seedlings with lower Ct-values for the *UidA* amplicon than *IDL6* or *IDL7*..

Table 3.5 show Ct-values analyses together with an estimated fold change in expression obtained from REST2009 of rosette leaves of 35 days old plants of the same *promoter:GUS* lines grown on soil under normal conditions. Reaction efficiencies from experiments are provided in Table A26, Appendix D.

Table 3.5 Mean Ct-values obtained from LinReg and standard deviations (SD) from qPCR analyses of tissue harvested from 35 days old plants of different *promoter:GUS* lines and Wt grown on soil under normal conditions. An estimate of fold change expression was obtained from REST2009 analyses.

	Mean Ct *IDL6* ± SD	Mean Ct *UidA* ± SD	Estimated fold change in expression
Wt	28.48 ± 3.16	34.39 ± 0.05	
pIDL6:GUS 4.5	26.59 ± 1.23	21.97 ± 0.61	10 fold
pIDL6:GUS 15.8	27.92 ± 0.20	26.48 ± 0.22	10 fold
pIDL6:GUS 18.4	26.10 ± 0.47	22.65 ± 0.51	10 fold
pIDL6:GUS 25.1	27.87 ± 0.26	21.82 ± 0.36	10 fold
	Mean Ct *IDL7* ± SD	**Mean Ct *UidA* ± SD**	
Wt	24.72 ± 0.61	33.57 ± 0.30	
pIDL7:GUS 3.2	25.85 ± 0.39	20.14 ± 0.38	10 fold
pIDL7:GUS 5.5	24.57 ± 0.38	20.55 ± 0.19	10 fold
pIDL7:GUS 16.3	25.15 ± 0.31	21.09 ± 0.09	10 fold
pIDL7:GUS 20.4	25.50 ± 0.79	22.17 ± 0.43	10 fold

These results also show that the *UidA* amplicon under the control of either the promoter of *IDL6* or *IDL7* has much lower Ct-values than the *IDL6* and *IDL7* amplicons in 35 days old plants.

3.8 Cycloheximide treatment

Cycloheximide (CHX) is a compound that inhibits the protein synthesis machinery in eukaryotic organisms (Ohh and Takei, 1995). *In silico* data shows that *IDL6* and *IDL7* expression is highly up-regulated upon treatment with CHX (Table 3.2). CHX is also a widely used agent to stabilize labile mRNA molecules.

To investigate if the observed differences in mRNA levels of *IDL6* and *IDL7* compared to *UidA* mRNA levels (Tables 3.3, 3.4 and 3.5) is an effect of rapid degradation of *IDL6* and *IDL7* mRNAs, seedlings were treated with CHX. By treating *promoter:GUS* lines of *IDL6* or *IDL7* with CHX, it was expected that mRNA levels in the plant tissue would increase and be visible in qPCR analysis if the difference previously detected were caused by rapid mRNA degradation.

10 days old seedlings were treated with 10 µM CHX solved in DMSO in liquid ½ x MS for three hours, MQ-water was used as a negative control and DMSO was used as a mock control. Figure 3.36 shows the results after GUS staining of treated seedlings of *pIDL6:GUS 4.5, pIDL6:GUS 18.4, pIDL7:GUS 3.2* and *pIDL7:GUS 20.4*.

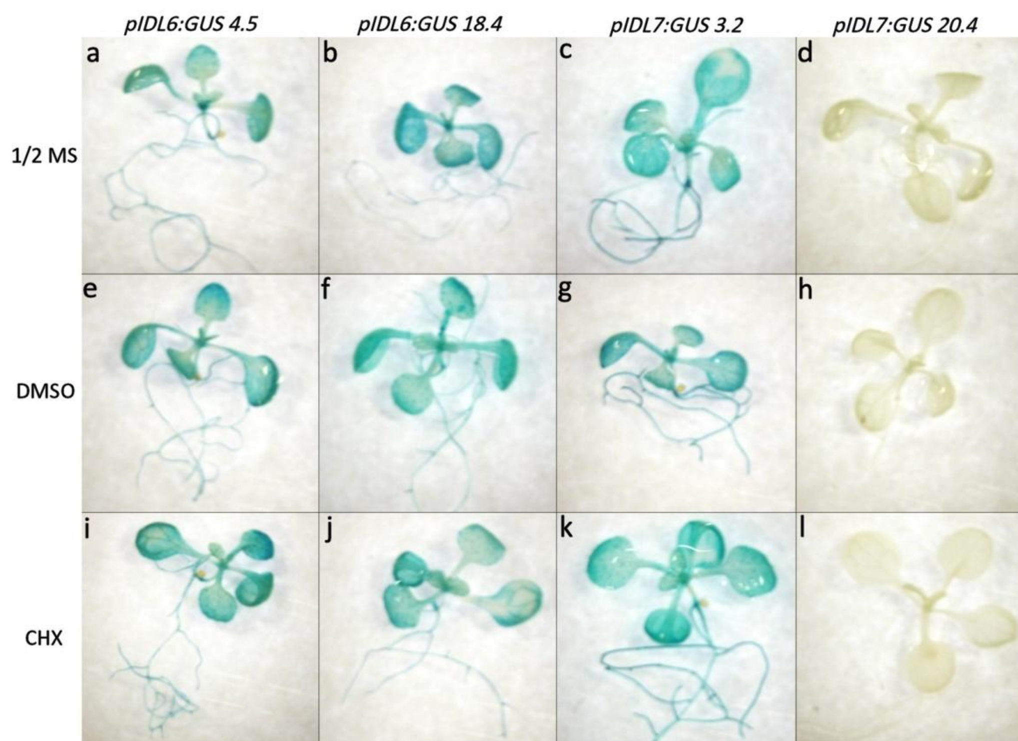

Figure 3.36 GUS staining from cycloheximide (CHX) treatment of 10 days old plants of *promoter:GUS* lines: *pIDL6:GUS 4.5, pIDL6:GUS 18.4, pIDL7:GUS 3.2* and *pIDL7:GUS 20.4* were treated with 10 µM CHX for three hours in liquid ½ x MS to investigate *GUS* expression (i-l). Plants only imbedded in ½ x MS was included as a negative control (a-d). CHX was solved in DMSO and DMSO was therefore included as a mock treatment (e-h).

Extensive GUS staining was detected throughout the plant tissue in all *promoter:GUS* lines of all treatments, except for *pIDL7:GUS 20.4* which did not show any GUS staining. This was also noted for the same line in the screening experiment (Figure 3.33). Tissue was harvested from the same treatment for qPCR analysis of expression patterns of *UidA* compared to *IDL6* and *IDL7* upon treatment with CHX. Ct-values obtained from LinReg are given in Table 3.6 below and reaction efficiencies are given in Table A27, Appendix D.

Table 3.6 Mean Ct-values and standard deviations (SD) from qPCR of *pIDL6:GUS* and *pIDL7:GUS* lines treated with 10 µM cycloheximide (CHX) in liquid ½ x MS for three hours were performed on 10 days old plants of *A. thaliana* to investigate the relative expression of *IDL6, IDL7* and *UidA*. Seedlings only embedded in liquid ½ x MS, and seedlings treated with DMSO were used as negative controls. Ct-values were calculated by linear regression by the LinReg.

	1/2 x MS	DMSO MOCK	CHX
		Mean Ct *IDL6* ± SD	
Wt	30.54 ± 0.09	31.35 ± 0.95	23.30 ± 1.73
pIDL6:GUS 4.5	30.85 ± 0.94	32.53 ± 0.67	23.38 ± 1.09
pIDL6:GUS 18.4	32.71 ± 0.57	31.38 ± 1.58	23.71 ± 0.46
		Mean Ct *IDL7* ± SD	
Wt	28.48 ± 0.37	27.88 ± 0.59	20.46 ± 0.54
pIDL7:GUS 3.2	29.24 ± 0.28	29.26 ± 0.08	20.29 ± 0.35
pIDL7:GUS 20.4	29.68 ± 0.34	29.26 ± 0.54	20.91 ± 0.87
		Mean Ct *UidA* ± SD	
Wt	36.09 ± 1.69	30.85 ± 4.92	34.68 ± 1.00
pIDL6:GUS 4.5	20.79 ± 0.22	20.37 ± 0.20	19.56 ± 0.25
pIDL6:GUS 18.4	13.92 ± 12.06	20.16 ± 0.09	18.27 ± 0.44
pIDL7:GUS 3.2	20.81 ± 0.13	20.33 ± 0.12	18.08 ± 0.29
pIDL7:GUS 20.4	23.07 ± 1.12	23.10 ± 0.12	23.77 ± 0.62

Ct-values showed to be highly affected by CHX treatment in relations to the *IDL6* and *IDL7* amplicon. In Wt and all *promoter:GUS* lines, the Ct-values were decreased with 8-10 cycles for the *IDL6* and *IDL7* amplicon in CHX-treated tissue compared to control treatments (½ x MS and DMSO). Ct-values of the *UidA* amplicon only decreased by one cycle in CHX-treated tissue of the same lines.

Figure 3.37 displays the relative expression (Log2 ratios) of *UidA, IDL6* and *IDL7* in CHX-treated tissue obtained from analysis of Ct-values in REST2009 compared to DMSO-treated tissue. Relative expression and p-values from analyses in REST2009 are given in Table A28, Appendix D.

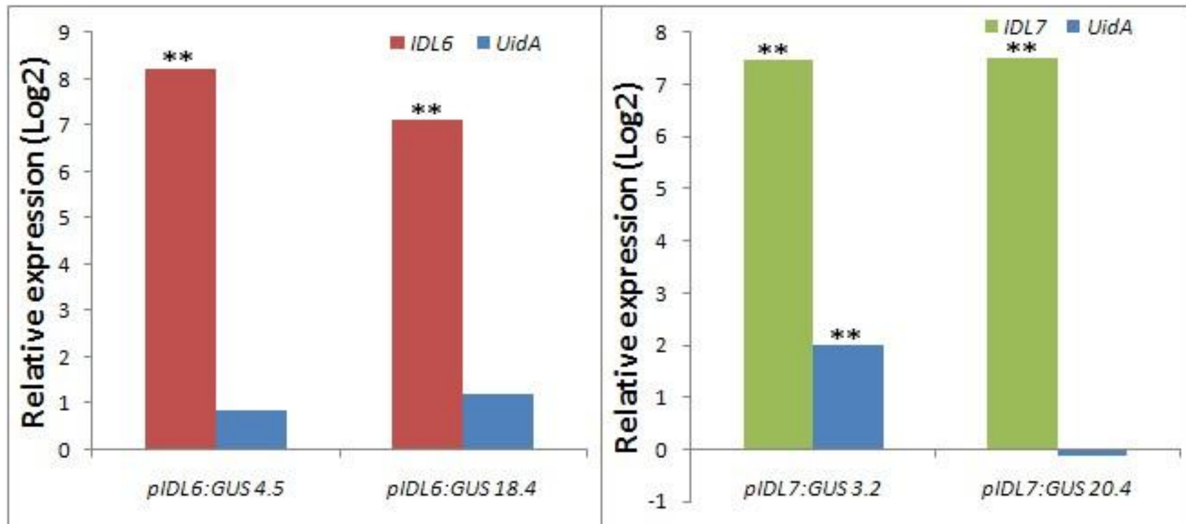

Figure 3.37 Relative expression (Log2 ratios) of *UidA, IDL6* and *IDL7* in 10 days old plants of *promoter:GUS* lines: *pIDL6:GUS 4.5, pIDL6:GUS 18.4, pIDL7:GUS 3.2* and *pIDL7:GUD 20.4* treated with 10 µM cycloheximide (CHX) for three hours in liquid ½ x MS. CHX was solved in DMSO; DMSO was therefore included as a mock treatment and was used as a control group in statistical analysis. Two asterisks (**) indicate significant difference from DMSO treated tissue with p-values ≤ 0.05.

Significant difference was established by REST2009 when comparing *IDL6* or *IDL7* expression in *promoter:GUS* lines to control group (DMSO treated). *IDL6* and *IDL7* were up-regulated hundred folds in all lines treated with CHX compared to treatment with DMSO.

Expression levels of *UidA* showed to be up-regulated in one of the lines (*pIDL7:GUS 3.2*), but no regulation was detected upon treatment with CHX in the other *promoter:GUS* lines.

The same expression patterns of *UidA* was observed when compared to Wt as a control group (data not shown) as in the initial expression screening of 10 days old *promoter:GUS* lines (Figure 3.36).

3.9 Work on *IDL8* transgenic lines

Basic groundwork and analyses was done to investigate single mutant lines of *idl8* and *pIDL8:GUS* lines. Over-expression lines of *IDL8* were constructed, and together this will potentially provide a basis for further work.

3.9.1 Identification of single insertion lines and genotyping

Two knockout lines of *idl8* were analyzed to identify homozygous individuals by segregation analyses of plants grown on ½ x MS and kanamycin. Table 3.7 displays the χ^2-values of the two lines tested. *Idl8 1.2* was heterozygous (shown in bold values), whereas *idl8 1.1* was homozygous (underlined values). χ^2-values for testing 1:0 distribution of homozygous individuals are not shown here for simplicity of the table.

Table 3.7 Segregation analyses of T-DNA knockout lines of *idl8* grown on ½ x MS containing kanamycin (Km) as a selection marker. Expected number of resistant (r) and sensitive (s) individuals was calculated according to the 3:0 distribution of homozygous resistant individuals based on total number of seeds analyzed. A χ^2-test was used to evaluate the hypothesis; H_0 (3:1 distribution) and H_1 (not 3:1 distribution). Heterozygous lines are shown in bold, homozygous lines are indicated with underlined values.

KO line	Observed Km^r	Observed Km^s	Expected Km^r	Expected Km^s	χ^2
<u>*idl8 1.1*</u>	<u>92</u>	<u>0</u>	<u>69</u>	<u>23</u>	<u>30.667</u>
idl8 1.2	**60**	**30**	**69**	**23**	**3.304**

Genotyping using the Extract-N-Amp™ Plant PCR Kit was performed on several individuals of the mutant lines to verify that the lines were homozygous or heterozygous. PCR products were separated by gel electrophoresis and the results obtained are displayed in Figure 3.38.

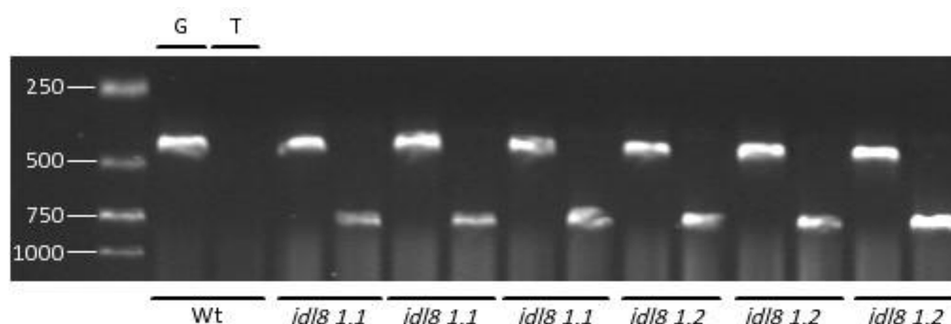

Figure 3.38 Genotyping of the *idl8 1.1* and *idl8 1.2* knockout lines, Wt *A. thaliana* was included as a control. Primers for detecting genomic *IDL8* (bands around 450 bp) (G) and primers for detecting T-DNA insert (bands around 750 bp) (T) were applied. Fermentas Gene Ruler™ 1 kb Plus DNA ladder was used.

Wt was used as negative control for T-DNA detection. It is expected that Wt will not give any PCR product when employing primers for detecting T-DNA in the reaction. Figure 3.38 shows that Wt contained only genomic DNA. As expected, both knockout lines showed PCR products from reaction with T-DNA primers (PCR products around 750 bp). PCR products from reaction with *IDL8* primers were expected only in individuals carrying one or more intact copies of the *IDL8* gene. As expected, Wt and the heterozygous *idl8 1.2* showed an intact *IDL8* gene (band of 422 bp). However, the predicted homozygous *idl8 1.1* line also carried an intact *IDL8* gene with PCR products of around 450 bp.

The postulated homozygous line, *idl8 1.1,* from segregation analyses can therefore not be identified as a complete knockout line of the *IDL8* gene. The reason for its complete kanamycin resistance could be caused by insertion of multiple T-DNA inserts in positions outside the *IDL8* gene in the plant genome.

To investigate if the T-DNA was inserted into regulatory elements outside the *IDL8* sequence, such as promoter region, the exact position of the T-DNA in *idl8 1.1* was examined. The sequence was

isolated from rosette leaves by PCR and cloned into a TOPO vector for sequence analysis. The TOPO vector was transformed into *E. coli* for blue-white screening and further amplifying. After successful blue-white screen, the plasmid was isolated, and the quality and concentration of the DNA was checked by Nanodrop.

The plasmid was further analyzed by digestion with restriction enzyme EcoRI. This reaction will yield different fragments of the plasmid when analyzed in agarose gel. An empty TOPO vector (without insert) will give one large visible band in the gel (linear plasmid). A TOPO vector with an insert will give two defined bands in the gel: 1) linear vector, same size as the empty vector (3900 bp) and 2) a smaller fragment with the T-DNA insert with additional flanking nucleotides. Figure 3.39 shows the results from gel electrophoresis after digestion with EcoRI of plasmids originating from four different *E. coli* colonies in the blue- white screen.

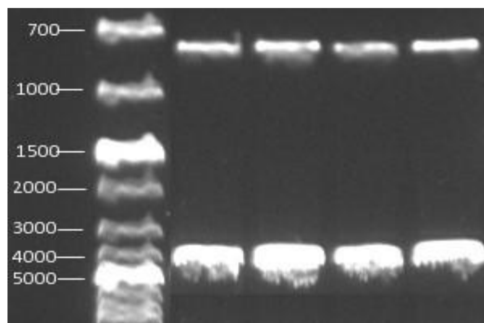

Figure 3.39 Restriction fragments from digestion of TOPO plasmid with T-DNA insert in *idl8* with the restriction enzyme EcoRI separated in agarose gel. Four different restriction enzyme reactions were conducted. Fermentas Gene Ruler™ 1 kb Plus DNA ladder was used.

Restriction cutting yielded desired fragments, one large (around 4000 bp in Figure 3.39) representing linearized vector, and one small (around 750 bp in Figure 3.39) representing the T-DNA insert and additional flanking nucleotides.

DNA of good quality was sent to "Universitetssykehuset i Nord-Norge",Tromsø, for Big Dye v3.1 sequencing to verify the exact position of the T-DNA. Analysis of chromatograms obtained from Tromsø showed that the postulated mutated *idl8* gene perfectly aligned with *IDL8* coding sequence by alignments analyses in MACAW (Alignments are provided in Figure A4, appendix E). However, the T-DNA was inserted in the promoter region about 150 bp upstream of the coding sequence in the *idl8 1.1* mutant line.

3.9.2 Construction of *35S:IDL8*

First step in construction of an over-expression line of any gene is isolating the gene from genomic DNA obtained from *A. thaliana* (rosette leaves). This was performed by PCR with *IDL8*-specific primers (Table 2.1), and the PCR products were separated by gel electrophoresis to verify that desired sequence had been amplified. Figure 3.40 shows the results from gel electrophoresis.

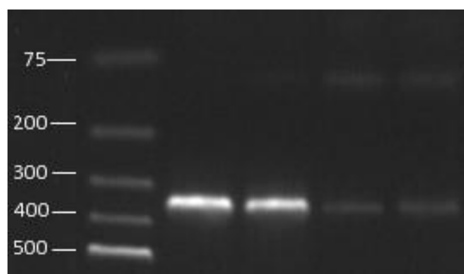

Figure 3.40 PCR fragments separated in gel shows desired PCR products of genomic *IDL8,* 325 bp for all reaction products. Fermentas Gene Ruler^{TM} 1 kb DNA ladder was used.

Gel electrophoresis showed desired bands of the *IDL8* gene (325 bp). PCR product was further mixed with pDONR vector solution in BP reaction and subsequently transformed into *E. coli* by heat shock transformation. Colonies of *E. coli* grown on LA with Zeocin over night was picked for bacterial PCR and for further culturing in LB. Bacterial PCR was performed using primers for detection of intact *IDL8* gene (Table 2.1) and the PCR product was investigated by gel electrophoresis to investigate the presence of *IDL8* in *E. coli.* Figure 3.41 show the results from PCR fragments separated in agarose gel.

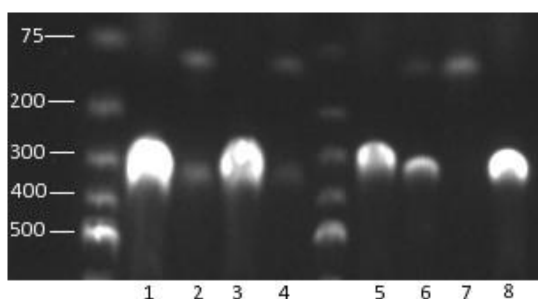

Figure 3.41 PCR fragments from bacterial PCR separated in gel shows desired PCR products of *IDL8* (325 bp). 8 different colonies of *E. coli* from LA with Zeocin were investigated. Fermentas Gene Ruler^{TM} 1 kb DNA ladder was used.

All colonies checked harbored the PCR fragment from *IDL8* (325 bp). QIAgen Miniprep was used to isolate amplified plasmids from *E. coli* cultured in LB. The quality of the isolated DNA was investigated by Nanodrop.

DNA of good quality was sent to “Universitetssykehuset i Nord-Norge”,Tromsø, for Big Dye v3.1 sequencing to verify that the pDONR vector encompassed the sequence of *IDL8* without any mutations or shifts that might have occurred during cloning work.

Analysis of chromatograms (performed in Chromas Lite) of sequences obtained from sequencing and subsequent alignment analysis (performed in MACAW) showed that two of the plasmid clones encompassed the *IDL8* gene without shifts or mutations. Sequence alignment of one of them, “U-05”, and *IDL8* sequence is provided in Figure A5, Appendix E.

Two separate LR reactions were performed with the two pDONR vector clones containing *IDL8* to transfer the gene into the destination vector pEG100. pEG100-*IDL8* was then heat shock transformed into *E. coli* for amplification (in LB and LA containing kanamycin which selects for *E. coli* carrying pEG100). Plasmids were isolated by QIAgen Miniprep and purity of the DNA was measured by Nanodrop.

One plasmid clone was chosen to be further transformed into *A. tumefaciens*. After transformation of *A. tumefaciens* C58 by electroporation, 8 colonies grown on LA with carbenicillin, rifampicin and kanamycin were picked for bacterial PCR (with *IDL8* specific primers (Table 2.1)) and further culturing in LB. PCR product were analyzed by gel electrophoresis, results are displayed in Figure 3.42.

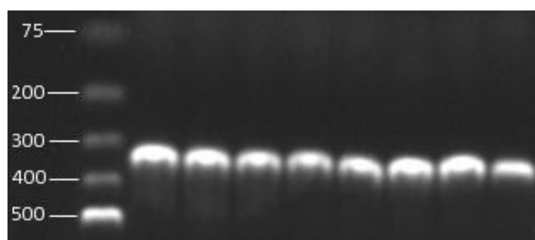

Figure 3.42 PCR fragments from bacterial PCR separated in gel shows desired PCR products of *IDL8* (325 bp). 8 different colonies of *A. tumefaciens* grown on LA with kanamycin, carbenicillin and rifampicin were investigated. Fermentas Gene Ruler™ 1 kb DNA ladder was used.

All PCR fragments were of expected size (325 bp) and therefore it was safe to use any of the cultures of *A. tumefaciens* for transforming *A. thaliana* by floral dipping.

Transformation of *A. thaliana* yielded T_0 seeds which were sown out on ½ x MS to select for successful transformants in the presence of basta (selection of seedlings with T-DNA insert) and claforan (to inhibit bacterial growth). Transformed T_1 plants were grown to harvest T_1 seeds which were sown out on ½ x MS with basta for segregation analysis to select for lines which show a 3:1 distribution of resistant:sensitive individuals. Table A8, Appendix D, shows the segregation analysis of *35S:IDL8* T_1 lines. A χ^2-test (Appendix C) was used to evaluate the null hypothesis "3:1 distribution" of resistant:sensitive individuals.

3.9.3 Segregation analyses of *pIDL8:GUS*

For analyses of promoter activity of *IDL8*, segregation analyses of T_1 *pIDL8:GUS* lines were performed. Table 3.8 shows the segregation analyses of seedlings grown on ½ x MS with hygromycin as a selection for inserted T-DNA. A χ^2-test (Appendix C) was used to evaluate the null hypothesis "3:1 distribution". The null hypothesis holds with at least 99% accuracy with a χ^2-value lower than 2.71 (shown in bold values).

Table 3.8 Segregation analysis of *pIDL8:GUS*. T_1 plants grown on ½ x MS containing hygromycin (Hyg) as a selection marker. Expected number of resistant (r) and sensitive (s) individuals was calculated according to the 3:1 distribution based on total number of seeds analyzed. A χ^2-test was used to evaluate the hypothesis; H_0 (3:1 distribution) and H_1 (not 3:1 distribution). Heterozygous lines showing approximate 3:1 distribution are shown in bold.

promoter:GUS line	Observed Hyg^r	Observed Hyg^s	Expected Hyg^r	Expected Hyg^s	χ^2
pIDL8:GUS 1	63	37	75	25	7.680
pIDL8:GUS 2	85	15	75	25	5.333
pIDL8:GUS 3	100	0	75	25	33.333
pIDL8:GUS 4	86	14	75	25	6.453
pIDL8:GUS 7	**79**	**21**	**75**	**25**	**0.853**
pIDL8:GUS 8	**76**	**24**	**75**	**25**	**0.053**
pIDL8:GUS 10	**73**	**27**	**75**	**25**	**0.213**
pIDL8:GUS 13	83	17	75	25	3.413
pIDL8:GUS 14	**79**	**21**	**75**	**25**	**0.853**
pIDL8:GUS 15	**77**	**23**	**75**	**25**	**0.213**
pIDL8:GUS 16	**79**	**21**	**75**	**25**	**0.853**
pIDL8:GUS 17	**78**	**22**	**75**	**25**	**0.480**
pIDL8:GUS 20	67	33	75	25	3.413
pIDL8:GUS 21	83	17	75	25	3.413
pIDL8:GUS 22	85	15	75	25	5.333
pIDL8:GUS 23	**75**	**25**	**75**	**25**	**0.000**
pIDL8:GUS 25	**75**	**25**	**75**	**25**	**0.000**
pIDL8:GUS 26	92	8	75	25	15.413
pIDL8:GUS 30	100	0	75	25	33.333
pIDL8:GUS 31	100	0	75	25	33.333

Nine of the *promoter:GUS* lines showed 3:1 distribution when grown on hygromycin (lines 7, 8, 10, 14, 15, 16, 17, 23 and 25).

4 Discussion

This thesis includes several different methods and separate experiments conducted on Wt *A. thaliana* as well as different transgenic lines. A phenotypic characterization was conducted to potentially reveal a specific phenotype linked to *IDL6* and *IDL7,* two genes encoding novel postulated peptides of the IDA peptide family. Mutant knockout lines and over-expression lines of the two genes were subjected to different stress treatments to investigate differences compared to Wt. GUS reporter lines of *IDL6* and *IDL7* promoters in *A. thaliana* were also subjected to different stress treatments to investigate promoter activity of the genes. Varying results led to a more thorough screen of the GUS reporter lines.

4.1 Phenotypic characterization

Identification of a phenotype linked to a certain genotype may be the first step towards understanding a certain gene function. This is the basis of a reverse genetic approach, which is the overall basis of the work presented here. It may not be easy to detect a phenotype if the certain phenotype is less pronounced or not present if it is not challenged. The differences in phenotype between Wt and a transgenic line may be so small that it is impossible to detect if not strictly controlled and analyzed. Boyes et al. (2001) provides a platform for analyzing minor deviation in phenotype between Wt *A. thaliana* and transgenic lines under both normal conditions or in environmentally stressed plants.

In the plate-based analyses of different transgenic lines of *A. thaliana* no major differences were observed between the transgenic lines and Wt. The minor significant deviations that were observed were notably only detected by statistical analyses and were not visual by just observing plants growing on ½ x MS under normal conditions. Results showed that there were no significant deviations in relation to developmental rate. However, some significant deviations in relation to total percentage of individuals reaching a certain developmental stage were detected by Student's t-test. Both *35S:IDL6 1.3* and *35S:IDL7 6.5* showed a significant tendency to have a higher total number of individuals reaching defined developmental stages during seedling development than Wt (Figure 3.3).

Differences in total percentage of individuals that will germinate, and subsequently total percentage of individuals that will reach later stages, may be caused by difference in seed quality. Seed quality is determined by several different factors, both during maturation of seeds, and during harvesting and storage of seeds. Seed moisture content upon harvesting and during storage may contribute to poor seed quality if the moisture content is high. High storage temperatures and long storage periods may also decrease the viability of seeds. *A. thaliana* seeds may fail to germinate if the seeds are dead or if the dormancy of the seeds is not broken (Rivero-Lepinckas et al., 2006). Dormancy is broken by subjecting seeds to a cold period for 2-4 days (stratification) (Weigel and Glazebrook, 2002) before they are placed under desired growing conditions for germination. If a mutant phenotype reveals itself

through lack of germination despite of subjection to stratifying period, this could be due to deficiency to break the dormancy. This has been shown to be related to a class of plant hormones known as gibberellins, but the level of abscisic acid (ABA) is also a major factor in controlling seed dormancy (Taiz and Zeiger, 2006).

Root growth analyses of plants grown on vertical square petri dishes also showed that over-expression lines grew longer roots than Wt (Figure 3.5). In addition to over-expression lines, all other lines (except *idl6xidl7 1.10*) showed significantly longer roots than Wt during these root growth analyses. *Idl6xidl7 1.10* showed significantly shorter roots than Wt. The great variation in root growth measurements may be caused by the sugar depleted growth medium that seeds were grown on. Sugar depleted medium was used to facilitate root growth measurements, but may also cause a stress factor related to osmotic balance. This factor may therefore cause artifacts when the intention is to analyze root growth under normal conditions, and especially if genes of mutant and over-expression lines potentially are linked to environmental stress signaling.

However, the differences observed in the plate-based analyses cannot be concluded to be real phenotypes connected to the different genotypes investigated. The differences may also be due to differences in seed quality of the different transgenic lines and Wt. Other factors may also contribute to differential growth patterns during analyses of these characters. Although effort was made to ensure as similar as possible growth conditions in the growth room by placing all plates at an equal distance from light source, it is possible that e.g. the most distal plants experienced a lower light intensity than plants directly below the light source. Small differences in temperature may also alter developmental rate and can occur due to different distance between plants and e.g. an air conditioner or another source of draft e.g. a door. However, in the plate-based analyses all plates were randomized throughout the shelf where they were placed, and t-test still gave satisfactory p-values to indicate that over-expression lines do in fact grow better than Wt, although the deviations are minor.

In soil-based analyses, some statistical deviations between Wt *A. thaliana* and the transgenic lines were detected. Wt showed better growth than some of the transgenic lines during several of the growth stages analyzed (Figure 3.6), whereas analyses of other growth stages did not reveal any significant differences (Figure 3.6 and 3.7). Limitations in number of individuals that could be analyzed in the soil-based analysis should also be considered when analyzing these results. Only 10 and 12 individuals from each line were analyzed in each biological replica due to restricted space in growth rooms. A low number of individuals in analyses of this kind make the data set especially vulnerable to experimental design. Even if all the plants from different lines and Wt were randomized in experiments conducted here, large variations may have occurred due to variation in light intensity due to uneven distribution of light in the growth room. Temperature differences may also occur in the growth rooms due to reasons already mentioned. A low number of sampling individuals may result in different growth and

development patterns due to this, rather than due to the mutation or over-expression the line in question harbors. Differences in light and temperature are also contributing factors to large standard deviations within lines and replicas; thus, a Student's t-test will not give significant deviations when sample number is low.

Two replicates are also too few to conclude anything about differences in height of primary shoot growth and development. The reason for not doing a third replica is the fact that no significant deviation was observed for the height measurements and the fact that large space and a lot of time is required to produce reliable data in these kinds of analyses.

4.2 Stress tolerance

Stress tolerance was analyzed through observations of growth and developmental patterns of several different knockout mutants and over-expression lines of *IDL6* and *IDL7* in *A. thaliana* when subjected to NaCl and mannitol. UV-B light was also used to potentially detect differences in ROS production in the different transgenic lines and expression analyses in Wt.

4.2.1 NaCl and mannitol tolerance

Large variations were observed in results from both NaCl and mannitol assays conducted here. This may be due to different shortcomings in the experimental design as discussed for the plate-based phenotypic characterization: Distance from light source and small differences in temperature may be a contributing factor to large standard deviations occurring in results from these kinds of experiments. To compensate for differences in light and temperature that may occur, all plates were placed on the same shelf with approximately the same distance from light source in the growth room, and they were randomized. In addition to initial randomization, all plates were randomly replaced to the shelf after registration of growth and development every day.

However, the randomizing of plates may not be sufficient to compensate for uneven light distribution and may even cause artifacts in the results. In e.g. relation to germination rate, the difference in amount of light a seed receives at day 1 after stratification cannot be compensated at day 2 (or later) and will contribute to undesired variation in germination rate.

This holds also for results concerning later growth stages. When e.g. some plants may have fully opened cotyledons and have access to more available light for energy conservation, this may give these plants an advantage so that they have the capacity to grow faster than plants with a lower amount of available light and/or less developed cotyledons.

In experiments conducted here, sufficient replicas were conducted to desirably exclude the problem of difference in distance to light source and minor temperature differences. Still, large deviations were observed in relations to some growth stages, e.g. in germination at 130 mM NaCl (Figure 3.10 h), and in development of fully opened cotyledons in 130 mM NaCl (Figure 3.12 g and h), and in 300 mM

mannitol (Figure3.17 e and f). These results show that average percentage of individuals within a certain line is different, but the standard deviations (Table A13, A15 and A20, Appendix D) are too large to support significant deviations by analyses with statistical tests.

In spite of this, there is reason to believe that these variations were not caused by differences in light or temperature as previously discussed. This is obvious from the fact that in results from plants grown on ½ x MS without NaCl or mannitol (controls), there were no large standard deviations in average total percentage of individuals reaching a certain growth stage. The same significant deviations were detected by statistical analyses between transgenic lines and Wt *A. thaliana* grown on ½ x MS without NaCl and mannitol as were detected in the plate-based phenotypic characterization as previously discussed.

The single knockout mutants *idl6 3.5* and *idl7 1.7* generally did not show enhanced or decreased tolerance to NaCl. The only exception is *idl7 1.4* in development of fully opened cotyledons, on 100 mM NaCl (Figure 3.12 d). Here, *idl7 1.4* showed to reach a higher total percentage of individuals to fully open the cotyledons than did Wt. The same tendency was not observed in earlier or in later growth stages. This may be due to the fact that this line had a small trend (no significant deviations) to have higher germination frequency than Wt and that the effect has been additive, creating room for significant validation of the characteristic at later growth stages. These results indicate that a mutation within *idl6* or *idl7* will not alter the NaCl tolerance of *A. thaliana* seedlings.

In contrast to this, *idl6 3.5* and *idl7 1.4* did in fact show some deviating growth and developmental patterns compared to Wt when grown in the presence of mannitol. *Idl6 3.5* showed minor significant differences in growth and development in respect to Wt when grown on ½ x MS (Figure 3.15 a, 3.16 a, 3.17 a, and 3.18 a) in mannitol control plates. *Idl6 3.5* grew slightly better than Wt on regular ½ x MS in mannitol controls, but this character was not altered when grown on medium containing mannitol. It still showed the same growth patterns in relation to Wt.

Idl7 1.4 showed a stronger deviation from Wt when grown on regular ½ x MS in mannitol control plates, and the tendency of this line to grow better than Wt was also present when grown in the presence of mannitol, except in 300 mM mannitol at later growth stages (Figures 3.15 a, d and e, 3.16 a, d and e, 3.17 a and d, and 3.18 a and d). No conclusion is possible to draw about *IDL7* in relation to increased mannitol tolerance, since the same growth patterns are observed on both regular ½ x MS and on medium containing mannitol.

The double knockout lines *idl6xidl7 1.9* and *idl6xidl7 1.10* showed the same growth patterns as Wt when grown on regular ½ x MS controls in the NaCl assay. In controls used in the mannitol assay, both lines showed some deviations from Wt (Figure 3.15 a, 3.16 a, 3.17 a, and 3.18 a). Notably,

idl6xidl7 1.9 and *idl6xidl7 1.10* grew better and poorer than Wt, respectively, in the presence of mannitol.

Idl6xidl7 1.9 was the only line that showed a higher germination rate than all other lines, including Wt, when grown on both medium containing NaCl and mannitol (Figure 3.10 and 3.15). However, this phenotype is not necessarily related to an increased NaCl tolerance. It may also be related to ABA deficiency or ABA insensitivity. ABA is well known to regulate germination through inhibiting it, or in other words prevent the breaking of seed dormancy (Taiz and Zeiger, 2006). Previous investigations have also revealed that mutants that were believed to be salt tolerant were in fact found to be ABA deficient or ABA insensitive mutants in stead (Quesada et al., 2000; Ruggiero et al., 2004; Verslues et al., 2006).

Further on, *idl6xidl7 1.9* grew significantly better than Wt in all concentrations of NaCl. This was also true for plants grown in the presence of mannitol. Even if some deviations from Wt were detected in control plates, the difference was enhanced in the presence of mannitol and strongly supported by statistics in high concentrations of mannitol. *idl6xidl7 1.10* showed the same or slightly better growth and development than Wt in NaCl but the same or slightly poorer growth and development than Wt in the presence of mannitol. As Figures 3.10, 3.11 and 3.12 shows, *idl6xidl7 1.9* and *idl6xidl71.10* showed a tendency to have better growth and development on NaCl supplemented growth medium. Although not significantly verified by statistical analyses in the case of the *idl6xidl7 1.10* line, the trend is evident.

The difference between double knockout lines and Wt was not observed for plants grown on ½ x MS in the NaCl tolerance analyses, supporting the indications that NaCl and mannitol affect growth and development in double knockout lines.

The increased tolerance to NaCl exhibited by the double knockout lines, is not observed for the single knockout lines. This indicates that both genes need to be non-functional in order to give any visible alterations related to NaCl tolerance. It can therefore be further deduced that the two gene products possibly are involved in stress tolerance, but that they may have overlapping functions. In other words, if one of the genes is dysfunctional the other will compensate for the loss of function in the mutated gene. Overlapping gene function is called genetic redundancy and has previously been proposed for both the CLE peptide family (Wang and Fiers, 2010) and for the IDA peptide family (Stenvik et al., 2008). The IDL6 and IDL7 peptides are very similar and they can possible act through the same receptors.

Genetic redundancy is often associated with genes involved in signal transduction, but also with genes involved in stress response in yeast (Kafri et al., 2009). Hanada et al. (2009) showed that *A. thaliana* followed the same patterns concerning genetic redundancy as yeast. In addition, Hanada and

coworkers showed that several redundant genes in *A. thaliana* only showed phenotypical changes when several of the redundant genes where knocked out simultaneously. This is highly comparable to the results obtained of phenotypical changes linked to *idl6* and *idl7* double knockouts here.

Moreover, the fact that it is the double knockout mutants that show increased tolerance supports a hypothesis that the IDL6 and IDL7 peptide may be involved in negative regulation of a defense response. Functional IDL6 and IDL7 peptides may potentially act as suppressors of defense response. A double knockout of the two genes may not be able to suppress the defense response and the defense response could possibly be constitutively activated in the double knockout mutants so that they will be able to tolerate a higher amount of NaCl. Suppression of defense response may be an important regulation mechanism in plants so that energy does not go to waste by defending the plant to non-harmful environmental conditions.

Some of the over-expression lines showed a small tendency to germinate better than Wt in 130 mM NaCl (Figure 3.10 i) but this was only supported on a 0.1 significance level. Despite the fact that this tendency was only barley visible in the control plates (Figure 3.10 c), it was evident in the plate-based phenotypic characterization, and can thus not be concluded to be a true characteristic of these lines. Further on no deviation was detected in later growth stages. According to these results, over-expression of either *IDL6* or *IDL7* will neither enhance nor decrease tolerance of NaCl or mannitol.

4.2.2 UV tolerance

As presented in Figure 3.19 and 3.20, only roots of plants subjected to UV-B irradiation showed detection of ROS by DAB assay. The aim of these experiments was to detect any difference in ROS production between mutant lines and Wt relative to UV dose. It is important to note that UV light also is a source of extensive heat generation and could provoke heat stress responses in the plant as well as stress provoked by UV-B light. When irradiating seedlings grown on ½ x MS, glass containers were used to increase the distance between the light source and the plants to prevent over heating. Another problem of analyzing plants grown in sealed containers is that plants are rapidly transferred from conditions of high humidity to lower humidity when removing covers to irradiate plants. This will subject the plants to water stress, and can lead to further artifacts in results. Plants grown in soil will not experience the same amount of water deficit stress as can be a problem with plants grown in sealed petri dishes, but the factor of generating excessive heat stress may still be a problem.

No difference was observed that could be related to UV-B dose compared to control treatments, and no indications that knockout mutants had increased or decreased ROS production relative to Wt were observed. It is possible that no specific DAB staining was detected by only analyzing the plants in a regular light microscope with relatively low magnification. UV-B light is a source of generating reactive oxygen species, and it would be expected that DAB staining would be visible in the light of this. Localization related patterns of DAB staining within the plant cells could possible be detected by

investigating leaves at a higher magnification. Attempts of this were not made during these experiments.

The lack of difference related to UV-B treatment holds also for experiments conducted for qPCR analyses. Sufficient controls are important to include for recognizinga stress response related to UV-B irradiation. In these experiments the control genes *HY5* and *PAD4* were included. The *Arabidopsis* eFP Browser show both genes to be highly up-regulated by UV-B exposure (BAR, 2007). In addition, *PAD4* has been shown to be involved in H_2O_2 metabolism (Rusterucci et al., 2001) and *HY5* has been shown by qPCR to be up-regulated upon UV-B treatment (Brown et al., 2005). It is thus expected that these two genes show to be up-regulated in qPCR analyses. No significant up-regulation of *HY5* and *PAD4* was detected in qPCR analyses in these experiments (Figure 3.21). The low expression of *PAD4* can be explained by the low reaction efficiency of this amplicon. The reason of these poor qPCR data can be explained by inexperience when this specific qPCR experiment was conducted. By contrast, up-regulation of *IDL6* and *IDL7* was detected in the same analyses, but since the UV-control genes did not seem to be affected by the UV treatment, and the fact that qPCR experiments clearly show shortcomings due to inexperience, no conclusions can be drawn related to these results.

Poor DAB staining after UV treatment and low increase in expression values after UV treatment may also indicate that the UV source employed did not function optimally. If the plants did not experience the given UV dose applied, no or low ROS production would have occurred, resulting in little or no DAB staining. For qPCR experiments; if not sufficient UV was applied, no up-regulation of control genes (*PAD4* and *HY5*) would occur, as observed. It would therefore be reasonable to think that the actual UV dose the plants experienced was insufficient to show up-regulation of *IDL6* and *IDL7* and control genes.

4.3 Promoter activity of *IDL6* and *IDL7*

Initially, promoter activity of *IDL6* and *IDL7* was investigated by the use of several different *promoter:GUS* lines and several different stress treatments to trigger the expression. Promoter activity was investigated through *GUS* expression in the plant tissue by GUS assay. Treatments were chosen on the basis of bioinformatics data obtained from the *Arabidopsis* eFP Browser (Table 3.1 and 3.2) (BAR, 2007). Since expression data obtained from the *Arabidopsis* eFP Browser indicated that several of the treatments would up-regulate the gene expression of *IDL6* and *IDL7* compared to negative controls, it was expected that these patterns could be verified or to some extent be recognized in plants of same age subjected to similar treatments.

4.3.1 Promoter activity upon aphid infestation

Aphids were used to investigate promoter activity of *IDL6* and *IDL7* upon insect infestation. *IDL6* and *IDL7* have previously been shown to be up-regulated in microarray experiments upon infestation with aphids (Kusnierczyk et al., 2008) and it was therefore expected that *promoter:GUS* lines of *IDL6* and

IDL7 would show a distinctive expression pattern upon aphid infestation. Results presented here (Figure 3.22 and 3.23) showed no distinct patterns.

A problem with experiments conducted to investigate promoter activity upon aphid infestation is the fact that aphids are mobile on the plant. The aphids feed of the plant phloem by penetrating through the plant tissue with their mouth parts (Kusnierczyk et al., 2007). Since they are mobile on the plant, the actual feeding site of the aphids and for how long they had been feeding on a certain location could not be determined in these experiments. Attempts were made to design specialized cages to restrict the feeding area on the plant. Cages made of thick double-sided tape with a circular hole (5 mm in diameter) and a fine net to prevent aphids from escaping turned out to be toxic to aphids.

The experiments that were conducted with aphids suffered from negative controls with the same amount of GUS staining as the infested tissue; thus, no conclusions can be made about promoter activity as an effect of aphid infestation.

4.3.2 Promoter activity upon H_2O_2 and Pst infiltration

Treatments of 5 weeks old plants by infiltration of H_2O_2 or Pst by a syringe gave somewhat varying results. The infiltration patterns in control treatment could indicate that mechanical wounding caused by infiltration by the syringe could induce *GUS* expression (Figure 3.25 and 3.26 a and b). However, the same expression patterns were not observed in leaves treated with H_2O_2 or Pst (Figure 3.25 and 3.26 c and d), which showed a more dispersed *GUS* expression pattern. It would be expected that if the infiltration caused damage to the tissue, which could lead to altered *GUS* expression, the same pattern would be observed in all infiltrated leaves independent of the solution the leaves were infiltrated with. Results presented in Figure 3.25 a and b and 3.26 a and b also indicate that *GUS* expression caused by wounding during infiltration decreases with time, since less *GUS* expression was observed after one hour compared to 0.5 hours. This was also evident in leaves harvested 24 hours after treatment (Figure 3.27 a and b). However, one of these control leaves showed extensive *GUS* expression. The plant from which this leaf was harvested may have been stressed in some other way that has caused this expression pattern.

GUS expression in leaves treated with H_2O_2 or Pst was more dispersed throughout the leaf, possibly indicating rapid signaling throughout the leaf upon H_2O_2 or Pst treatment.

Both Pst and H_2O_2 are known to trigger HR and SAR; spreading of signal to neighboring tissue related to infiltration was therefore expected 24 hours after treatment. The presence of *IDL7* promoter activity in neighboring leaves (Figure 3.27) could therefore indicate that *IDL7* is activated downstream of SAR induction.

In silico data from the Arabidopsis eFP Browser showed that *IDL7* is highly up-regulated 3 h after treatment with methyl jasmonate (data not shown). Methyl jasmonate is a precursor of the hormone

signal jasmonic acid (JA). JA is one of the main signaling molecules in systemic signaling (Taiz and Zeiger, 2006). Promoter activity of *IDL7* in non-treated neighboring leaves after 24 hours (Figure 3.27) could indicate that *IDL7* may be directly regulated by JA or indirectly downstream of the JA signaling in systemic signaling.

In a separate experiment, other transgenic lines were used as controls of *GUS* expression. These lines (*pPDF 1.2:GUS, pPR1:GUS* and *pPR1:GUS/ndr1-1*) have previously been shown to show distinct *GUS* expression upon treatment with microbes and other treatments related to HR and SAR such as H_2O_2 and Pst (Manners et al., 1998; Zhang and Shapiro, 2002). These GUS lines would therefore give reliable indications of treatment specific *GUS* expression in these experiments in addition to possibly indicating links between *IDL6* and *IDL7* to HR or SAR if expression patterns are coherent with control lines.

No difference in *GUS* expression was observed in the *pPDF 1.2:GUS* line. Extensive *GUS* expression was observed (Figure 3.28) in all treatments. Manners et al. (1998) showed that mechanical wounding did not induce expression of *pPDF 1.2*, so the expression observed might be caused by the solutions that the leaves were treated with. Another possible reason for the constant *GUS* expression may be that *pPDF 1.2* is constitutively expressed at this growth stage. Expression data from the *Arabidopsis* eFP Browser (BAR, 2007) do in fact show that *pPDF 1.2* is relatively higher expressed in older rosette leaves than in other tissue (data not shown).

Some GUS staining was observed for treatment with MQ-water and H_2O_2 in leaves harvested from the *pPR1:GUS* control plants (Figure 3.29 a and c). However, no staining of tissue treated with $MgCl_2$ or Pst was observed in this experiment (Figure 3.29 b and d). It was expected that since H_2O_2 is a trigger of HR and systemic defense responses, and thus the up-regulation of *PR1* expression, the promoter activity in H_2O_2 treated plants would be higher than in treatment with MQ-water.

Moreover the *PR1* is down-regulated by jasmonic acid (JA). This could in fact be a reason for less expression in treated tissue. If treatment of H_2O_2 induces JA, it would suppress the expression of *PR1* and thus promoter activity and expression of *UidA*. H_2O_2 triggers programmed cell death by HR which can activate systemic defense responses with the involvement of JA (Taiz and Zeiger, 2006). Thus, the possibility that H_2O_2 treatment can decrease *PR1* expression is likely.

Further on, this may also explain the low promoter activity of *PR1* in Pst treated tissue since Pst also is known to trigger HR and systemic defense responses. However, another explanation of the absent promoter activity in Pst treated tissue and partially in H_2O_2 treated tissue could be that 1 h is not sufficient to induce *PR1* promoter activity. *PR1* is as previously noted induced by HR and SAR, it would be reasonable to believe that induction of HR and subsequently the SAR and salisylic acid

accumulation would require more than 1 h to give visible promoter activity of *PR1* in *pPR1:GUS* lines.

Unfortunately, *GUS* expression caused by wounding in the *promoter:GUS* lines of *IDL6* and *IDL7* (Figures 3.30 and 3.31), as previously discussed, is probably masking the potential treatment-specific *GUS* expression. No links between *IDL6* and *IDL7* and HR and SAR can thus be detected in relations to the *PDF1.2* and *PR1* expression patterns from these results.

4.4 Screening of *promoter:GUS* lines

As results presented here show (Figures 3.22-3.32), all *promoter:GUS* lines of *IDL6* and *IDL7* showed highly variable expression patterns independent of treatment and length of treatment. *In silico* data show that both *IDL6* and *IDL7* have low expression profiles under normal conditions but are up-regulated under stressful conditions. Remarkably many of the negative controls and mock treatments did also show a broad extent of *GUS* expression and thus promoter activity of the *IDL6* and *IDL7* genes. Without sufficient negative controls or any difference from negative controls, it is impossible to deduce anything about the effect of the given treatment upon GUS activity.

This fact lead to the more thorough screening of several of the *promoter:GUS* lines of *IDL6* and *IDL7* promoters. Especially, young plants showed extensive promoter activity in GUS assays (Figure 3.33), whereas older plants showed less GUS activity in rosette leaves (Figure 3.34). Inflorescences of 35 days old plants were also analyzed, and GUS activity was high in flowers of plants of this age (Figure 3.35). This was surprising, since expression profiles show that *IDL6* and *IDL7* have low expression under normal conditions, as previously noted.

IDL6 and *UidA* in *pIDL6:GUS* lines are controlled by the same promoter. This is also the case for *UidA* and *IDL7* in *pIDL7:GUS* lines. Since the promoter region of a gene controls the transcription of a gene in a strict manner, it would be expected that *UidA* and the respective *IDL*-gene will be expressed at the same level in *promoter:GUS* lines. qPCR analyses of different GUS lines of *A. thaliana* were therefore performed to investigate the expression of the *UidA* amplicon and the *IDL6* and *IDL7* amplicons. However, from expression analyses from qPCR (Tables 3.3- 3.5) *IDL6, IDL7* and *UidA* showed to have remarkably different expression profiles despite the fact that they are governed by the same promoter in the respective GUS lines.

From these results it is reason to believe that *IDL6* and *IDL7* expression may be regulated by rapid degradation of the mRNAs potentially signaled by the gene sequence of *IDL6* and *IDL7* itself. Although the genes may be transcribed, they might not be translated into the peptide sequences. The mRNA molecule may be degraded rapidly as it is produced if no signal is present that indicate that the peptide sequence is necessary and thus translation of the RNA molecule should be initiated. This is supported by the high mRNA levels of *UidA* that was detected. *UidA* mRNA is clearly not degraded

possibly because a degradation signal is not present in the *UidA* sequence despite it being controlled by the *IDL6* or *IDL7* promoter.

As a consequence, a lot of GUS staining will be produced and indicate promoter activity at sites were the blue stain is observed in the plant after GUS assay. This may be the reason for observing the seemingly contradictive results in experiments involving *promoter:GUS* lines described here. While *in silico* data describes *IDL6* and *IDL7* as genes with very low expression patterns during normal conditions, GUS experiments seemingly tell a different story. All GUS experiments on plants grown under normal conditions here show an extensive promoter activity, and thus expression of the genes. However, the reason this is not detected in *in silico* data or qPCR analyses conducted here may be that the mRNA produced by transcription have a short life-time and may be degraded soon after transcription if degradation is not prevented by additional signals.

When observing higher mRNA content of *IDL6* and *IDL7* in plants which are subjected to stress (*in silico* data, BAR) and qPCR analysis conducted here, it is further reason to believe that environmental signals may be involved in regulation of the life-time of mRNAs of *IDL6* and *IDL7*.

Cycloheximide was used to further investigate the stability of *IDL6* and *IDL7* mRNAs. Cycloheximide inhibits the protein synthesis and serves as a stabilizer of mRNA (Ohh and Takei, 1995). The stabilization of mRNA is mediated through dissolving of a decapping complex which is involved in decapping of mRNA molecules (Xu et al., 2006). Results from qPCR clearly showed that expression levels of *IDL6* and *IDL7* were highly increased upon cycloheximide treatment (Figure 3.37). Cycloheximide has previously been used to detect highly labile and rapidly degraded mRNA molecules in both mammalian studies and in studies concerning *A. thaliana* (Ohh and Takei, 1995; Itani et al., 2003; Saul et al., 2009).

Results presented here clearly indicate that mRNAs of *IDL6* and *IDL7* are highly labile, and are rapidly degraded post-transcriptionally. Instable mRNA molecules facilitate fast changes in mRNA levels, which are required in many processes in an organism (Gutierrez et al., 2002). A rapid change in mRNA levels may be crucial for a plant to respond quickly to environmental changes and in stress response.

Several different means of degrading mRNAs are known in plants. mRNA can be degraded by ribonucleases which recognize specific destabilizing sequence elements often contained in the 3' untranslated region (3'UTR). One such stabilizing element is the AUUUA repeat element (ARE). These are AU rich elements with repeating units of the AUUUA consensus sequence. It is postulated that mRNAs with high similarity to the consensus ARE are more unstable than mRNA with lower degree of similarity to the ARE (Clark, 2005).

Degradation of mRNA may also occur by post-transciptional gene silencing (PTGS) in plants. PTGS is analogous to RNA interference (RNAi) in animals. PTGS is often related to defense against viral attack where viral RNA molecules are recognized and destroyed (Jana et al., 2004; Vaucheret, 2006).

Several previous studies have been done to investigate unstable transcripts in *A. thaliana* (Gutierrez et al., 2002; Narsai et al., 2007). Narsai et al. (2007) presented three features of genes that displayed a short half-life of their corresponding mRNA molecules: 1) Absence of introns in gene sequence, 2) presence of specific sequence elements and 3) mRNAs that are microRNA (miRNA) targets.

Interestingly, both *IDL6* and *IDL7* are intron-less genes, and thus they are (according to Narasi et al., 2007) likely to have mRNA molecules with short half-life further supporting the results presented here. However, the mechanism of degradation of *IDL6* and *IDL7* mRNAs remains unknown.

4.5 *IDL8* transgenic lines

The *idl8 1.1* mutant line was shown to have the T-DNA inserted to the promoter region of the *IDL8* gene. The promoter region directs the transcription of the gene it governs, and a disruption in this region is highly likely to prevent transcription of the gene in question.

4.6 Recommendations for further work

The work presented here opens opportunities for further investigations of *IDL6, IDL7* and *IDL8.* This can lead to identification of a phenotype linked to the different genes, but it can also lead to new insights into post-transcriptional gene regulation and the importance of this related to plant defense against stressful environmental conditions.

4.6.1 Further search of a phenotype linked to *IDL6* and *IDL7*

Plate-based analyses and analyses of stress tolerance related to NaCl and mannitol have the possibility of revealing miniscule phenotypes, which do not reveal themselves unless carefully analyzed. In these types of analyses it is important to analyze seeds that have the same potential of growth and development. It is therefore recommended that all lines that are used in these kinds of analyses are sown out and grown to harvest seeds of all lines at the same time. This will ensure that all seeds have been treated in exactly the same manner, and will give more reliable results. It should also be considered to make new double mutant lines by doing new crossings between single mutant lines, since results here show that some of these lines in fact behave in the opposite manner of each other.

Separate experiments on germination rate may also be conducted to further study the increased germination rate upon NaCl that was observed here. Plants should be grown in the presence of ABA to verify whether increased germination rate is due to increased NaCl tolerance or due to ABA deficiency.

In relation to the possible functional redundancy of *IDL6* and *IDL7,* it would be particularly interesting to investigate if knocking out of one of these genes will cause an up-regulation of the other one, as observed for redundant genes in other eukaryotic organisms (Kafri et al., 2009).

Further on, it is recommended that analyses of plants grown on soil are performed in large scale, with many sampling individuals. This will ultimately eliminate large variation in data due to potential variations in light and temperature in growth rooms as previously discussed. This is both extremely time and space consuming, but should be considered if it is desired to potentially find a phenotype linked to mutation in *idl6* or *idl7,* or linked to over-expression of the *IDL6* and *IDL7*.

4.6.2 Further work related to regulation of gene expression of *IDL6* and *IDL7*

The potential roles of *IDL6* and *IDL7* in plant defense still remain unknown, but the new insights into regulation of expression of these genes provides a basis for further work, both related to post-transcriptional gene regulation and in relations to plant defense. Further analyses of nucleotide sequences may possibly give interesting findings of sequence elements that have been shown to be characteristic for labile mRNA molecules. This may also give indications of which mechanisms that govern the rapid degradation of these mRNA molecules.

It would also be interesting to analyze the exact half-life of these mRNA molecules. At this moment it is not known whether the half-life is in the magnitude of minutes or hours. Determining the exact half-life can give hints about the possible involvement of these genes in an extremely rapid signaling process or a more moderate fast signaling process. Promising results may also be obtained by determining changes in half-life of mRNA molecules levels in response to different stress treatments. Changes in half-lives may possibly provide evidences if these genes are involved in plant defense.

Analyses of the peptides in a protein assay (e.g. western blot) to verify that no protein is present in the plant tissue despite the high mRNA levels could also give interesting results and further support the hypothesis that the mRNAs of *IDL6* and *IDL7* are not translated. Unfortunately, this is very difficult when studying small proteins like the peptides of the IDA-family.

4.6.3 Further work related to the *IDL8* gene

Segregation analyses of *pIDL8:GUS* lines performed here identified several single insertion T-DNA lines. This provide stable *pIDL8:GUS* lines which should be subjected to a GUS screen to investigate possible expression patterns of the *IDL8* gene.

The over-expression lines of *IDL8* that was constructed in the work of this thesis should also be screened to find a potential over-expression phenotype. The *idl8* mutant line that was showed to have T-DNA inserted in the promoter region could also be used directly in a screen for detection of visible phenotypes, but it is highly recommended that the expression of *IDL8* is analyzed by qPCR to ensure that this line is a true knockout mutant line.

5 Conclusion

From research done to investigate potential phenotypes linked to a specific genotype in *A. thaliana* here, it is evident that strict growing conditions are crucial to give valid results. This is especially important when analyzing phenotypes that may only have minor differences from wild type *A. thaliana*. However, since small variations in light conditions and temperature were considered and plates were randomized, these factors have likely not been the cause of the observed differences between Wt and over-expression lines in the plate-based analyses. Seed quality may however be a contributing factor on the observed difference; it is therefore very important to use seeds that are treated in the same manner when doing analyses like the ones presented here (e.g. same growth conditions during seed maturation, same harvesting time of seeds, same storage conditions for seeds and seeds of same age).

Because of these potential artifacts in experiments conducted here, a phenotype linked to mutation in *idl6* or *idl7,* or linked to over-expression of *IDL6* or *IDL7* seems to be difficult to identify. However, a tendency of the double knockout lines of *idl6* and *idl7* to tolerate higher concentrations of NaCl better than Wt and other transgenic lines possibly indicates a functional overlap and the involvement of these genes in suppressing defense response in *A. thaliana*.

mRNA levels of *IDL6* and *IDL7* have been shown to deviate from expected levels based on promoter activity of the *IDL6* and *IDL7* promoters. High mRNA instability and rapid degradation of mRNA are the likely source of this observed difference. This may possibly be an intricate way of rapidly regulating gene expression in *A. thaliana* and has been reported for several other genes previously. This type of post-transcriptional regulation enables the plant to rapidly regulate the expression of genes upon a stimulating signal. It is crucial for the plant to respond rapidly to environmental signals or a pathogen attack. mRNAs are continually being produced by transcription and continually degraded (possibly by being decapped and/or recognized by ribonucleases that respond to specific elements in the mRNA molecule or by PTGS) but if a signal suddenly inhibits the degradation process, synthesized mRNAs of *IDL6* or *IDL7* will be ready for translation without the necessity of initiation of the transcription process.

Analyses and work on *IDL8* transgenic lines were successfully conducted. A mutant lines was verified as a true knockout line; over-expression lines and GUS reporter lines of *IDL8* have been constructed and analyzed, respectively, and can be used for further analyses of the *IDL8* gene in *A. thaliana.*

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Appendix A: Growth mediums, gels and buffers

A 1 Growth medium for plants and bacteria

A 1.1 ½ x MS

Half strength Murashige and Skoogs (½ x MS) medium was used for growing of *A. thaliana* on agar plates, liquid ½ x MS is prepared by the same recipe without agar:

2.15 g/L MS
20 g/L sucrose
6 g/L 3:1 phyto agar: bacto agar

pH = 5.8, autoclaved at 121°C for 20 minutes.

½ x MS for square plates was made with some modifications to make the medium more solid for vertical orientation by raising the agar concentration from 6 g/L to 10 g/L. No supplemental sugar was added for root growth analysis.

A 1.2 0.1 % agarose for spreading of seeds

0.1 % agarose was prepared by autoclaving desired amount of 1 g agarose for routine use in lab per L MQ-water.

A 1.3 LB/ LA medium

Luria-Bertani (LB)-medium was used to grow *E. coli* and *A. tumefaciens*. In medium for plates (LA) additional 14 g/L agar was added:

10 g/L trypton
5 g/L NaCl
5 g/L yeast extract Medium was autoclaved at 121°C for 20 minutes.

A 1.4 Kings B

Kings B medium was used to grow *Pseudomonas syringae* pv. *tomato.* In medium for agar plates additional 15 g/L agar was added:

20 g/L proteose peptone number 3
1.5 g/L K_2HPO_4
1.5 g/L $MgSO_4 \cdot H_2O$
10 mL/L glycerol Medium was autoclaved at 121°C for 20 minutes.

A 2 Reaction buffers

A 2.1 DAB reaction buffer

1 mg 3.3'- diaminobenzidine (DAB) was solved per mL MQ-water.

pH adjusted with HCl to pH 3.8.

A 2.2 GUS reaction buffer

100 mM Na-P buffer (pH 7.0- 7.7) (described below).

10 mM Na-EDTA

1 mM Ferricyanide

1 mM Ferrocyanide

1% Triton X-100

1 mM X-Gluc

The solution was shaken for 30-60 minutes.

A 2.3 Phosphate buffer for GUS assay

0.1 M Na-P- buffer was prepared by mixing 90.5 mL 0.2 M Na_2HPO_4 and 10.5 mL 0.2 M NaH_2PO_4 and water to a final volume of 200 mL. The buffer was pH adjusted to pH between 7.0 and 7.7.

A 3 Gels and running buffers for electrophoresis

A 3.1 1 % agarose gel for standard electrophoresis

1 % agarose gel for electrophoresis was prepared by solving 1 g of agarose for routine use in lab per 100 mL of TAE buffer by heating in a microwave oven. For staining of nucleic acids in the gel using Gel Red, 0.5 µL Gel Red/ 10 mL agarose mixture was added before gel casting.

A 3.2 TAE running buffer

242 g Tris base

57.1 mL Glacial acetic acid

100 mL 0.5M EDTA

MQ-water to 1L.

A 3.3 FA gel for denaturing electrophoresis

1.2% FA agarose gel for FA gel electrophoresis was prepared by solving 0.6 g of agarose for routine use in lab with 5 mL 10 x FA gel buffer and a total volume of 50 mL with MQ-water by heating in a microwave oven. The solution was cooled to 65°C and 900 µL 37% formaldehyde and 0.5 µL ethidium bromide was added before gel casting.

A 3.4 FA running buffer

The 10 x FA gel buffer contained the following ingredients in MQ-water:

200 mM MOPS

50 mM sodium acetate

10 mM EDTA

pH 7.0, adjusted with NaOH.

Appendix B: Vector maps

Figures A1, A2, and A3 display the different vectors used for cloning experiments described here. Selection markers are also indicated in the respective maps.

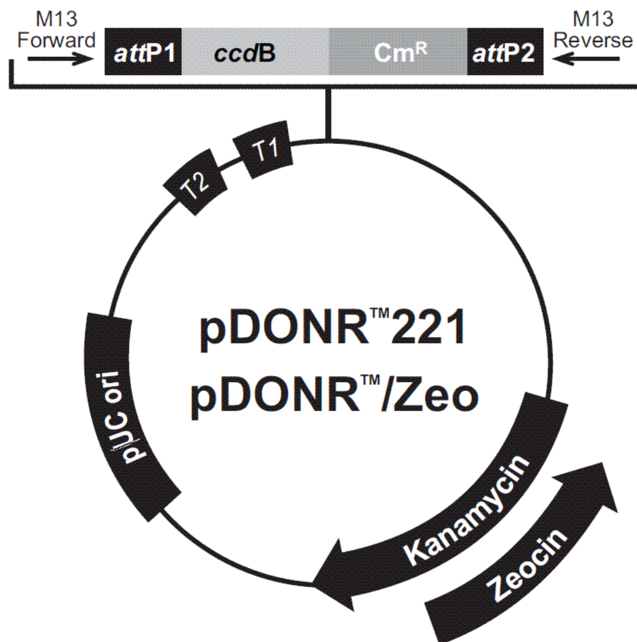

Figure A1 **pDONR™ / Zeo plasmid map shows major features of the vector (Invitrogen, 2004).**

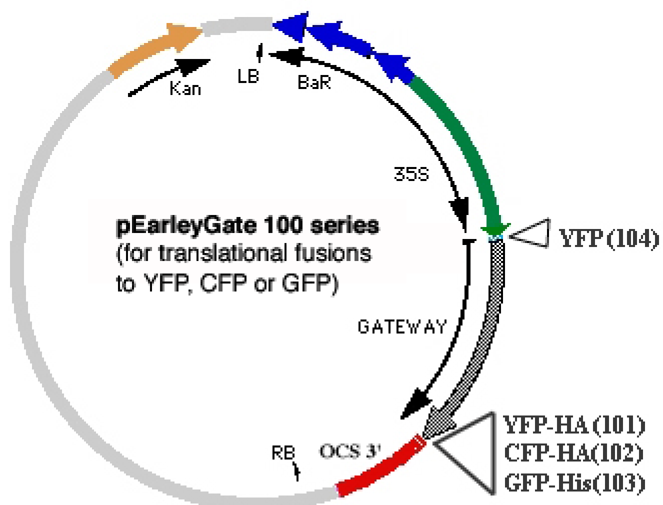

Figure A2 **pEarlyGate (pEG) 100 plasmid map shows major features of the vector (Earley et al., 2006).**

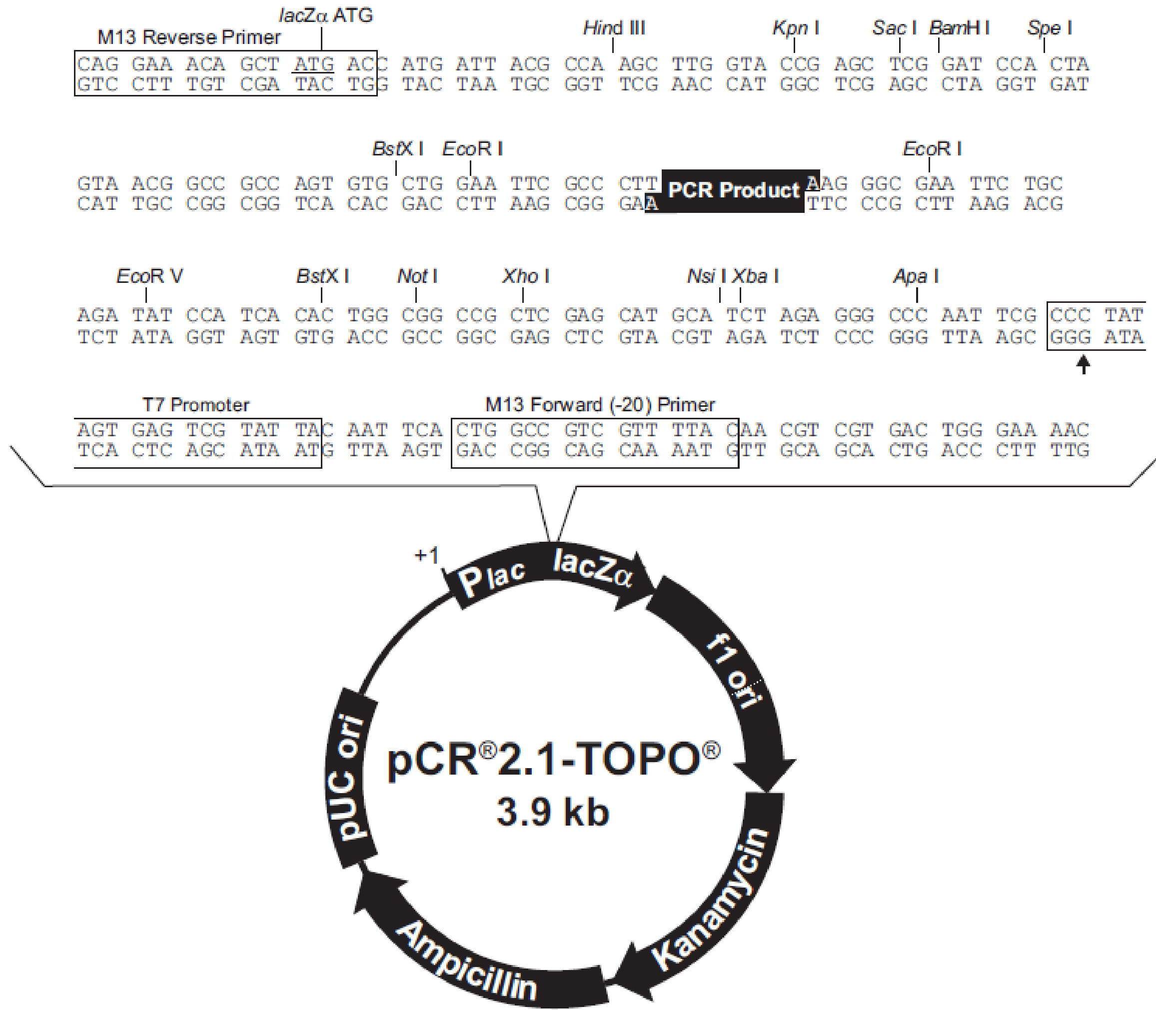

Figure A3 **pCR®2.1-TOPO® plasmid map show major features of the vector and restriction sites for cleaving with restriction enzymes (Invitrogen, 2006).**

Appendix C: Statistical tests

C 1 Student's *t*-test

The Student's *t*-test was used to evaluate the differences between to sample populations. The *t*-test determines if the differences between to sample populations are large enough in relations to the variance in the biological material.

t = (average - expectation value) / (standard deviation / √number of samples)

C 2 χ^2-test

The χ^2-test was used to evaluate data that exists in the form of frequencies. The t-test was performed by evaluating two hypothesis defined as the null hypothesis (H_0) and the alternative hypothesis (H_1). If the χ^2 -value is lower than a certain value derived from a χ^2-table one of the two hypotheses may be rejected.

$\chi^2 = \Sigma (O - E)^2 / E$

Where O is observed frequency and E is expected frequency.

Appendix D: Output data from statistical analyses and qPCR analyses

D 1 Segregation analyses of *IDL6* and *IDL7* over-expression lines

Table A1 shows segregation analysis of over-expression lines *35S:IDL6* and *35S:IDL7*. A χ^2-test was used to evaluate the null hypothesis "3:1 distribution" and the null hypothesis "1:0 distribution" for heterozygous and homozygous lines respectively. The null hypothesis holds with at least 99% accuracy with a χ^2-value lower than 2.71 (derived from a χ^2-table) (shown in bold values). Homozygous individuals are also indicated in Table A1 (underlined values). χ^2-values for testing 1:0 distribution of homozygous individuals are not shown here for simplicity of the table.

Table A1 Segregation analysis of over-expression lines (OE) of *IDL6* and *IDL7*. T_2 plants were grown on ½ x MS containing hygromycin as a selection marker. Expected number of resistant and sensitive individuals was calculated according to the 3:1 distribution based on total number of seeds analyzed. A χ^2-test was used to evaluate the hypothesis; H_0 (3:1 distribution) and H_1 (not 3:1 distribution). Heterozygous lines showing approximate 3:1 distribution are shown in bold. Homozygous lines showing approximate 1:0 distribution are indicated with underlined values.

OE line	Observed Hyg^r	Observed Hyg^s	Expected Hyg^r	Expected Hyg^s	χ^2
35S:IDL6 1-3	70	0	52.5	17.5	23.333
35S:IDL6 2-1	60	9	51.75	17.25	5.261
35S:IDL6 2-3	**56**	**14**	**52.5**	**17.5**	**0.933**
35S:IDL6 2-6	**52**	**17**	**51.75**	**17.25**	**0.005**
35S:IDL7 2-6	60	10	52.5	17.5	4.286
35S:IDL7 3-1	**52**	**16**	**51**	**17**	**0.078**
35S:IDL7 4-4	**53**	**15**	**51**	**17**	**0.314**
35S:IDL7 6-5	67	3	52.5	17.5	16.019

Segregation analysis of the T_3 seeds of *35S:IDL6* and *35S:IDL7* is shown in Table A2 and A3 respectively. A χ^2-test was used to evaluate the null hypothesis "1:0 distribution of homozygous resistant individuals" (underlined values). The null hypothesis holds with at least 99% accuracy with a χ^2-value lower than 2.71 (derived from a χ^2-table). Heterozygous individuals showing a 3:1 distribution are shown in bold. χ^2-values for testing 3:1 distribution are not shown here for simplicity of the table.

Table A2 **Segregation analysis of over-expression (OE) lines of *IDL6*. T_3 plants were grown on ½ x MS containing hygromycin as a selection marker. Expected number of resistant and sensitive individuals was calculated according to the 1:0 distribution of homozygous resistant individuals based on total number of seeds analyzed. A χ^2-test was used to evaluate the hypothesis; H_0 (1:0 distribution) and H_1 (not 1:0 distribution). Homozygous lines showing approximate 1:0 distribution are shown in underlined values. Heterozygous lines showing approximate 3:1 distribution are indicated in bold.**

OE line	Observed Hyg^r	Observed Hyg^s	Expected Hyg^r	Expected Hyg^s	χ^2
35S:IDL6 2-1 3	**69**	**31**	**100**	**0**	**909.091**
35S:IDL6 2-1 5	99	1	100	0	1.010
35S:IDL6 2-1 6	94	6	100	0	25.253
35S:IDL6 2-1 7	89	11	100	0	101.010
35S:IDL6 2-1 8	90	10	100	0	81.818
35S:IDL6 2-1 9	100	0	100	0	0.000
35S:IDL6 2-1 10	**78**	**22**	**100**	**0**	**445.455**
35S:IDL6 2-3 1	**81**	**19**	**100**	**0**	**327.273**
35S:IDL6 2-3 2	**77**	**23**	**100**	**0**	**488.889**
35S:IDL6 2-3 3	100	0	100	0	0.000
35S:IDL6 2-3 4	100	0	100	0	0.000
35S:IDL6 2-3 5	**68**	**32**	**100**	**0**	**970.707**
35S:IDL6 2-3 6	**72**	**28**	**100**	**0**	**736.364**
35S:IDL6 2-3 7	**80**	**20**	**100**	**0**	**364.646**
35S:IDL6 2-3 8	**77**	**23**	**100**	**0**	**488.889**
35S:IDL6 2-3 9	**76**	**24**	**100**	**0**	**534.343**
35S:IDL6 2-3 10	**79**	**21**	**100**	**0**	**404.040**
35S:IDL6 2-3 12	100	0	100	0	0.000
35S:IDL6 2-6 1	**70**	**30**	**100**	**0**	**849.495**
35S:IDL6 2-6 2	**73**	**27**	**100**	**0**	**682.828**
35S:IDL6 2-6 4	**76**	**24**	**100**	**0**	**534.343**
35S:IDL6 2-6 6	99	1	100	0	1.010
35S:IDL6 2-6 7	**73**	**27**	**100**	**0**	**682.828**
35S:IDL6 2-6 8	98	2	100	0	1.810
35S:IDL6 2-6 9	100	0	100	0	0.000
35S:IDL6 2-6 10	**80**	**20**	**100**	**0**	**364.646**

Table A3 **Segregation analysis of over-expression lines (OE) of *IDL7*. T_3 plants were grown on ½ x MS containing hygromycin as a selection marker. Expected number of resistant and sensitive individuals was calculated according to the 1:0 distribution of homozygous resistant individuals based on total number of seeds analyzed. A χ^2-test was used to evaluate the hypothesis; H_0 (1:0 distribution) and H_1 (not 1:0 distribution). Homozygous lines showing approximate 1:0 distribution are shown in underlined values. Heterozygous lines showing approximate 3:1 distribution are indicated in bold.**

OE line	Observed Hyg^r	Observed Hyg^s	Expected Hyg^r	Expected Hyg^s	χ^2
35S:IDL7 2-6 1	**69**	**31**	**100**	**0**	**909.091**
35S:IDL7 2-6 3	**75**	**25**	**100**	**0**	**581.818**
35S:IDL7 2-6 4	**72**	**28**	**100**	**0**	**736.364**
<u>*35S:IDL7 2-6 5*</u>	<u>99</u>	<u>1</u>	<u>100</u>	<u>0</u>	<u>1.010</u>
35S:IDL7 2-6 6	**87**	**13**	**100**	**0**	**145.455**
<u>*35S:IDL7 3-1 1*</u>	<u>99</u>	<u>1</u>	<u>100</u>	<u>0</u>	<u>1.010</u>
35S:IDL7 3-1 2	**75**	**25**	**100**	**0**	**581.818**
35S:IDL7 3-1 3	**74**	**26**	**100**	**0**	**631.313**
35S:IDL7 3-1 4	**73**	**27**	**100**	**0**	**682.828**
35S:IDL7 3-1 5	**75**	**25**	**100**	**0**	**581.818**
35S:IDL7 3-1 6	**72**	**28**	**100**	**0**	**736.364**
35S:IDL7 3-1 7	**72**	**28**	**100**	**0**	**736.364**
35S:IDL7 3-1 8	**69**	**31**	**100**	**0**	**909.091**
35S:IDL7 3-1 9	**72**	**28**	**100**	**0**	**736.364**
35S:IDL7 3-1 10	**74**	**26**	**100**	**0**	**631.313**
35S:IDL7 4-4 1	66	34	100	0	1100.000
35S:IDL7 4-4 2	64	36	100	0	1237.374
35S:IDL7 4-4 3	60	40	100	0	1536.364
35S:IDL7 4-4 4	53	47	100	0	2137.374
35S:IDL7 4-4 5	58	42	100	0	1697.980
35S:IDL7 4-4 6	50	50	100	0	2425.253
35S:IDL7 4-4 7	**81**	**19**	**100**	**0**	**327.273**
35S:IDL7 4-4 8	27	73	100	0	5236.364
35S:IDL7 4-4 9	51	49	100	0	2327.273
35S:IDL7 4-4 10	26	74	100	0	5382.828

D 2 Segregation analyses of *IDL6* and *IDL7 promoter:GUS* lines

Table A4 and A5 show segregation analysis of seedlings grown on ½ x MS with hygromycin for selection of lines containing T-DNA. A χ^2-test was used to evaluate the null hypothesis "3:1 distribution". The null hypothesis holds with at least 99% accuracy with a χ^2-value lower than 2.71 (derived from a χ^2-table) (shown in bold values). Homozygous individuals are also indicated in Table A4 (underlined values). χ^2-values for testing 1:0 distribution of homozygous individuals are not shown here for simplicity of the table.

Table A4 Segregation analysis of *pIDL6:GUS*. T_2 plants were grown on ½ x MS containing hygromycin as a selection marker. Expected number of resistant and sensitive individuals was calculated according to the 3:1 distribution based on total number of seeds analyzed. A χ^2-test was used to evaluate the hypothesis; H_0 (3:1 distribution) and H_1 (not 3:1 distribution). Heterozygous lines showing approximate 3:1 distribution are shown in bold. Homozygous lines showing approximate 1:0 distribution are indicated with underlined values.

promoter:GUS line	Observed Hyg^r	Observed Hyg^s	Expected Hyg^r	Expected Hyg^s	χ^2
pIDL6:GUS 4.1	**44**	**24**	**51**	**25**	**1.001**
pIDL6:GUS 4.3	70	0	52.5	25	30.833
pIDL6:GUS 18.4	70	0	52.5	24.75	30.583
pIDL6:GUS 25.3	**52**	**17**	**51.75**	**24.75**	**2.428**
pIDL6:GUS 25.4	**51**	**18**	**51.75**	**24.75**	**1.852**

Table A5 Segregation analysis of *pIDL7:GUS*. T_1 plants were grown on ½ x MS containing hygromycin as a selection marker. Expected number of resistant and sensitive individuals was calculated according to the 3:1 distribution based on total number of seeds analyzed. A χ^2-test was used to evaluate the hypothesis; H_0 (3:1 distribution) and H_1 (not 3:1 distribution). Heterozygous lines showing approximate 3:1 distribution are shown in bold.

promoter:GUS line	Observed Hyg^r	Observed Hyg^s	Expected Hyg^r	Expected Hyg^s	χ^2
pIDL7:GUS 1	0	76	57	19	228.000
pIDL7:GUS 2	**77**	**22**	**74.25**	**24.75**	**0.407**
pIDL7:GUS 3	**71**	**29**	**75**	**25**	**0.853**
pIDL7:GUS 4	**77**	**22**	**74.25**	**24.75**	**0.407**
pIDL7:GUS 5	**67**	**27**	**70.5**	**23.5**	**0.695**
pIDL7:GUS 6	**75**	**24**	**74.25**	**24.75**	**0.030**
pIDL7:GUS 7	**72**	**26**	**73.5**	**24.5**	**0.122**
pIDL7:GUS 8	94	6	75	25	19.253
pIDL7:GUS 9	**74**	**26**	**75**	**25**	**0.053**
pIDL7:GUS 10	67	32	74.25	24.75	2.832
pIDL7:GUS 11	**77**	**24**	**75.75**	**25.25**	**0.083**
pIDL7:GUS 12	**79**	**21**	**75**	**25**	**0.853**
pIDL7:GUS 13	**70**	**29**	**74.25**	**24.75**	**0.973**
pIDL7:GUS 14	82	19	75.75	25.25	2.063
pIDL7:GUS 15	**71**	**29**	**75**	**25**	**0.853**
pIDL7:GUS 16	**73**	**27**	**75**	**25**	**0.213**
pIDL7:GUS 17	90	10	75	25	12.000
pIDL7:GUS 18	**71**	**28**	**74.25**	**24.75**	**0.569**
pIDL7:GUS 19	**68**	**31**	**74.25**	**24.75**	**2.104**
pIDL7:GUS 20	**77**	**22**	**74.25**	**24.75**	**0.407**

A second segregation analysis was conducted during a thorough screening of GUS lines. Tables A6 and A7 show segregation analyses of *pIDL6:GUS* and *pIDL7:GUS* lines respectively.

A χ^2-test was used to evaluate the null hypothesis "1:0 distribution of homozygous resistant individuals" (underlined values). The null hypothesis holds with at least 99% accuracy with a χ^2-value

lower than 2.71 (derived from a χ^2- table). Heterozygous individuals showing a 3:1 distribution are shown in bold. χ^2-values for testing 3:1 distribution are not shown here for simplicity of the tables.

Table A6 Segregation analysis of *pIDL6:GUS*. T_2 plants were grown on ½ x MS containing hygromycin as a selection marker. Expected number of resistant and sensitive individuals was calculated according to the 1:0 distribution based on total number of seeds analyzed. A χ^2-test was used to evaluate the hypothesis; H_0 (1:0 distribution) and H_1 (not 1:0 distribution). Homozygous lines showing approximate 1:0 distribution are shown with underlined values. Heterozygous lines showing approximate 3:1 distribution are indicated in bold.

Promoter:GUS line	Observed Hyg^r	Observed Hyg^s	Expected Hyg^r	Expected Hyg^s	χ^2
pIDL6:GUS 4.1	**30**	**16**	**46**	**0**	**230.000**
pIDL6:GUS 4.2	31	19	50	0	330.612
pIDL6:GUS 4.4	**33**	**13**	**46**	**0**	**147.200**
pIDL6:GUS 4.5	49	1	50	0	1.020
pIDL6:GUS 8.2	**33**	**17**	**50**	**0**	**261.224**
pIDL6:GUS 8.3	28	22	50	0	450.000
pIDL6:GUS 9.2	**33**	**17**	**50**	**0**	**261.224**
pIDL6:GUS 9.3	30	20	50	0	368.367
pIDL6:GUS 15.5	**33**	**17**	**50**	**0**	**261.224**
pIDL6:GUS 15.8	50	0	50	0	0.000
pIDL6:GUS 16.1	**37**	**13**	**50**	**0**	**146.939**
pIDL6:GUS 16.3	**43**	**7**	50	0	**36.735**
pIDL6:GUS 18.1	**37**	**13**	**50**	**0**	**146.939**
pIDL6:GUS 18.4	50	0	50	0	0.000
pIDL6:GUS 22.2	**32**	**18**	**50**	**0**	**294.898**
pIDL6:GUS 22.3	30	20	50	0	368.367
pIDL6:GUS 25.1	**38**	**12**	**50**	**0**	**123.469**
pIDL6:GUS 25.3	**42**	**8**	**50**	**0**	**50.000**
pIDL6:GUS 25.4	**35**	**11**	**47**	**0**	**102.630**

Table A7 Segregation analysis of *pIDL7:GUS*. T_2 plants were grown on ½ x MS containing hygromycin as a selection marker. Expected number of resistant and sensitive individuals was calculated according to the 1:0 distribution based on total number of seeds analyzed. A χ^2-test was used to evaluate the hypothesis; H_0 (1:0 distribution) and H_1 (not 1:0 distribution). Homozygous lines showing approximate 1:0 distribution are shown with underlined values. Heterozygous lines showing approximate 3:1 distribution are indicated in bold.

Promoter:GUS line	Observed Hyg^r	Observed Hyg^s	Expected Hyg^r	Expected Hyg^s	χ^2
pIDL7:GUS 2.1	28	22	50	0	450.000
pIDL7:GUS 2.2	**36**	**14**	**50**	**0**	**172.449**
pIDL7:GUS 3.1	48	2	50	0	1.020
pIDL7:GUS 3.2	50	0	50	0	0.000
pIDL7:GUS 3.5	**70**	**26**	**96**	**0**	**631.579**
pIDL7:GUS 4.3	**36**	**14**	**50**	**0**	**172.449**
pIDL7:GUS 4.5	**42**	**8**	**50**	**0**	**50.000**
pIDL7:GUS 5.2	**36**	**14**	**50**	**0**	**172.449**
pIDL7:GUS 5.5	50	0	50	0	0.000
pIDL7:GUS 6.3	**33**	**17**	**50**	**0**	**261.224**
pIDL7:GUS 6.5	49	1	50	0	1.020
pIDL7:GUS 7.1	**40**	**10**	**50**	**0**	**82.653**
pIDL7:GUS 7.3	**40**	**10**	**50**	**0**	**82.653**
pIDL7:GUS 9.2	**40**	**10**	**50**	**0**	**82.653**
pIDL7:GUS 9.5	50	0	50	0	0.000
pIDL7:GUS 10.1	31	19	50	0	330.612
pIDL7:GUS 10.5	**33**	**17**	**50**	**0**	**261.224**
pIDL7:GUS 12.1	49	1	50	0	1.020
pIDL7:GUS 12.4	**38**	**12**	**50**	**0**	**123.469**
pIDL7:GUS 14.3	48	2	50	0	1.020
pIDL7:GUS 14.4	**32**	**18**	**50**	**0**	**294.898**
pIDL7:GUS 15.1	**39**	**11**	**50**	**0**	**102.041**
pIDL7:GUS 15.5	**37**	**13**	**50**	**0**	**146.939**
pIDL7:GUS 16.1	**40**	**10**	**50**	**0**	**82.653**
pIDL7:GUS 16.3	**37**	**13**	**50**	**0**	**146.939**
pIDL7:GUS 18.1	**38**	**12**	**50**	**0**	**123.469**
pIDL7:GUS 18.2	**38**	**12**	**50**	**0**	**123.469**
pIDL7:GUS 19.1	**32**	**18**	**50**	**0**	**294.898**
pIDL7:GUS 19.2	50	0	50	0	0.000
pIDL7:GUS 20.4	50	0	50	0	0.000
pIDL7:GUS 20.5	50	0	50	0	0.000

D 3 Segregation analyses of *IDL8* over-expression lines

Transformed T_1 plants were grown to harvest T_1 seeds which were sown out on ½ x MS with basta for segregation analysis to select for lines which show a 3:1 distribution of resistant:sensitive individuals. Table A8 show the segregation analysis of *35S:IDL8* T_1 lines. A χ^2-test was used to evaluate the null

hypothesis "3:1 distribution". The null hypothesis holds with at least 99% accuracy with a χ^2-value lower than 2.71 (derived from a χ^2-table) (shown in bold values).

Table A8 Segregation analysis of *35S:IDL8*. T_1 plants were grown on ½ x MS containing hygromycin as a selection marker. Expected number of resistant and sensitive individuals was calculated according to the 3:1 distribution based on total number of seeds analyzed. A χ^2-test was used to evaluate the hypothesis; H_0 (3:1 distribution) and H_1 (not 3:1 distribution). Heterozygous lines showing approximate 3:1 distribution are shown in bold.

OE line	Observed Hyg^r	Observed Hyg^s	Expected Hyg^r	Expected Hyg^s	χ^2
35S:IDL8 1	82	14	72	24	5.556
35S:IDL8 3	86	10	72	24	10.889
35S:IDL8 4	**69**	**27**	**72**	**24**	**0.500**
35S:IDL8 6	91	5	72	24	20.056
35S:IDL8 7	89	11	75	25	10.453
35S:IDL8 8	89	11	75	25	10.453
35S:IDL8 9	**73**	**23**	**72**	**24**	**0.056**
35S:IDL8 11	**75**	**25**	**75**	**25**	**0.000**
35S:IDL8 12	50	50	75	25	33.333
35S:IDL8 13	**76**	**24**	**75**	**25**	**0.053**
35S:IDL8 14	93	7	75	25	17.280
35S:IDL8 15	**67**	**29**	**72**	**24**	**1.389**
35S:IDL8 16	62	38	75	25	9.013
35S:IDL8 17	**79**	**21**	**75**	**25**	**0.853**
35S:IDL8 18	**76**	**20**	**72**	**24**	**0.889**
35S:IDL8 19	94	6	75	25	19.253
35S:IDL8 21	91	9	75	25	13.653
35S:IDL8 22	38	62	75	25	73.013
35S:IDL8 23	0	100	75	25	300.000
35S:IDL8 25	96	4	75	25	23.520
35S:IDL8 26	**74**	**26**	**75**	**25**	**0.053**
35S:IDL8 27	83	17	75	25	3.413
35S:IDL8 28	**74**	**26**	**75**	**25**	**0.053**
35S:IDL8 29	0	100	75	25	300.000
35S:IDL8 30	**79**	**20**	**74.25**	**24.75**	**1.215**
35S:IDL8 31	**72**	**28**	**75**	**25**	**0.480**
35S:IDL8 32	0	100	75	25	300.000
35S:IDL8 33	**74**	**26**	**75**	**25**	**0.053**
35S:IDL8 34	**71**	**29**	**75**	**25**	**0.853**
35S:IDL8 35	92	5	72.75	24.25	20.375

D 4 P-values from t-tests

Statistical analyses was used to evaluate the difference between Wt *A. thaliana* and transgenic lines in respect to growth and development in phenotypic characterization analyses and in analyses of plants grown on medium containing NaCl or mannitol.

Table A9 **Student's t-test was used to evaluate the actual difference in percentage of seedlings from different transgenic lines of *A. thaliana* and Wt *A. thaliana* that reached a specific growth stage when grown on ½ x MS. P-values ≤ 0.050 indicate true difference between the respective lines and Wt with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no true difference between the lines at a specific growth stage.**

	Day 4		Day 5		Day 6		Day 7		Day 8	
Germination	**% ± SD**	**P**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	96.4 ± 2.1		96.6 ± 2.0		97.2 ± 1.5		97.4 ± 1.3		97.5 ± 1.6	
idl6 3.5	98.0 ± 2.6	0.479	98.0 ± 2.6	0.520	98.0 ± 2.6	0.673	98.0 ± 2.6	0.746	98.0 ± 2.6	0.826
idl7 1.4	98.1 ± 1.7	0.339	98.8 ± 1.3	0.182	99.1 ± 0.8	0.131	99.1 ± 0.8	0.133	99.6 ± 0.6	0.105
idl6xidl7 1.10	98.3 ± 2.9	0.413	98.7 ± 2.3	0.309	98.9 ± 1.9	0.291	98.9 ± 1.9	0.323	98.9 ± 1.9	0.406
35S:IDL6 1.3	99.4 ± 1.0	0.091	99.4 ± 1.0	0.089	99.4 ± 1.0	0.094	99.4 ± 1.0	0.092	100.0 ± 0.0	0.057
35S:IDL7 6.5	100.0 ± 0.0	0.045	100.0 ± 0.0	0.041	100.0 ± 0.0	0.032	100.0 ± 0.0	0.026	100.0 ± 0.0	0.057

	Day 4		Day 5		Day 6		Day 7		Day 8	
Hypoc. and cotyl. emerg.	**% ± SD**	**P**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	96.4 ± 2.1		96.4 ± 2.1		96.4 ± 2.1		97.0 ± 1.7		97.2 ± 2.0	
idl6 3.5	97.6 ± 2.2	0.554	97.6 ± 2.2	0.554	97.6 ± 2.2	0.554	97.6 ± 2.2	0.736	97.6 ± 2.2	0.821
idl7 1.4	97.6 ± 2.5	0.576	97.9 ± 2.0	0.429	98.3 ± 1.7	0.295	99.1 ± 0.8	0.135	99.1 ± 0.8	0.205
idl6xidl7 1.10	96.9 ± 4.5	0.893	98.0 ± 3.5	0.543	98.9 ± 1.9	0.295	98.9 ± 1.9	0.275	98.9 ± 1.9	0.345
35S:IDL6 1.3	99.4 ± 1.0	0.091	99.4 ± 1.0	0.091	99.4 ± 1.0	0.295	99.4 ± 1.0	0.099	99.4 ± 1.0	0.152
35S:IDL7 6.5	99.4 ± 1.0	0.092	100.0 ± 0.0	0.045	100.0 ± 0.0	0.295	100.0 ± 0.0	0.040	100.0 ± 0.0	0.071

	Day 4		Day 5		Day 6		Day 7		Day 8	
Cotyledons fully opened	**% ± SD**	**P**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	82.7 ± 17.4		88.7 ± 11.9		93.8 ± 5.0		96.4 ± 2.1		96.6 ± 2.4	
idl6 3.5	94.8 ± 7.1	0.328	95.9 ± 5.1	0.387	97.0 ± 3.2	0.402	97.0 ± 3.2	0.801	97.6 ± 2.2	0.634
idl7 1.4	95.4 ± 6.2	0.302	96.9 ± 3.7	0.318	97.8 ± 2.5	0.291	98.5 ± 1.7	0.258	98.5 ± 1.7	0.327
idl6xidl7 1.10	90.2 ± 7.5	0.533	94.0 ± 5.6	0.523	96.5 ± 2.7	0.466	98.0 ± 2.6	0.479	98.1 ± 2.7	0.510
35S:IDL6 1.3	96.1 ± 6.7	0.283	99.4 ± 1.0	0.192	99.4 ± 1.0	0.130	99.4 ± 1.0	0.091	99.4 ± 1.0	0.134
35S:IDL7 6.5	96.0 ± 6.8	0.285	99.4 ± 1.0	0.193	100.0 ± 0.0	0.100	100.0 ± 0.0	0.045	100.0 ± 0.0	0.073

	Day 6		Day 7		Day 8		Day 9		Day 10	
2 rosette leaves > 1 mm	**% ± SD**	**P**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	15.4 ± 25.2		85.9 ± 12.8		93.0 ± 4.3		96.4 ± 2.1		96.4 ± 2.1	
idl6 3.5	7.2 ± 8.6	0.636	94.4 ± 6.8	0.366	97.6 ± 2.2	0.177	97.6 ± 2.2	0.554	97.6 ± 2.2	0.554
idl7 1.4	14.6 ± 23.9	0.973	97.0 ± 3.4	0.220	97.6 ± 2.5	0.186	97.6 ± 2.5	0.576	97.6 ± 2.5	0.576
idl6xidl7 1.10	22.6 ± 30.9	0.768	92.2 ± 4.8	0.471	96.0 ± 2.4	0.348	97.4 ± 3.6	0.707	97.4 ± 3.6	0.707
35S:IDL6 1.3	9.4 ± 8.6	0.719	97.2 ± 4.8	0.226	99.4 ± 1.0	0.064	99.4 ± 1.0	0.091	99.4 ± 1.0	0.091
35S:IDL7 6.5	21.0 ± 29.3	0.814	98.3 ± 2.9	0.177	100.0 ± 0.0	0.047	100.0 ± 0.0	0.045	100.0 ± 0.0	0.045

	Day 7		Day 8		Day 9		Day 10	
4 rosette leaves > 1 mm	**% ± SD**	**P**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0.7 ± 1.3		26.6 ± 37.6		82.0 ± 14.2		90.2 ± 6.4	
idl6 3.5	0.0	0.374	21.5 ± 22.3	0.849	90.7 ± 3.6	0.359	95.5 ± 2.2	0.244
idl7 1.4	0.0	0.374	22.2 ± 23.4	0.872	90.6 ± 5.3	0.383	96.9 ± 1.7	0.153
idl6xidl7 1.10	0.4 ± 0.6	0.674	32.6 ± 30.8	0.842	88.5 ± 4.6	0.491	95.2 ± 4.3	0.329
35S:IDL6 1.3	0.0	0.374	21.9 ± 24.9	0.864	85.0 ± 6.4	0.756	93.7 ± 5.5	0.515
35S:IDL7 6.5	0.7 ± 1.3	1.000	24.5 ± 35.4	0.948	90.8 ± 8.1	0.401	96.3 ± 3.2	0.212

Table A10 **Student's t-test was used to evaluate the actual difference in root length between Wt *A. thaliana and* different transgenic lines of *A. thaliana* based on mean root length (mm) from vertical growing seedlings in sugar depleted ½ x MS, and standard deviation (SD). P-values ≤ 0.050 are underlined and indicate true difference between the respective lines and Wt with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference in respect to root length at certain time points at day 5 through 10.**

	Day 5		Day 6		Day 7		Day 8		Day 10	
	mm±SD	p	mm±SD	p	mm±SD	p	mm±SD	p	mm±SD	p
Wt	8.1±3.8		11.6±5.1		14.3±7.3		19.7±9.5		27.9±13.6	
idl6 3.5	9.4±2.6	3.21x10^{-5}	13.1±3.9	0.002	16.4±3.9	6.22x10^{-4}	22.3±6.7	4.80x10^{-4}	31.6±10.9	0.007
idl7 1.4	10.1±2.2	2.84x10^{-12}	13.7±3.4	3.62x10^{-6}	18.7±3.8	2.54x10^{-12}	23.9±5.6	3.73x10^{-9}	32.9±8.4	2.69x10^{-5}
idl6xidl7 1.10	8.5±3.9	0.350	10.4±5.6	0.025	12.1±6.5	0.002	18.8±10.0	0.264	24.1±15.0	0.010
35S:IDL6 1.3	9.4±2.3	5.94x10^{-6}	13.0±3.5	0.003	16.3±4.4	0.002	22.1±5.7	9.45x10^{-4}	30.4±8.6	0.035
35S:IDL7 6.5	11.7±2.6	3.38x10^{-30}	15.6±3.7	1.17x10^{-16}	19.4±4.6	3.77x10^{-15}	26.0±6.3	3.07x10^{-17}	34.9±9.3	1.06x10^{-8}

Table A11 **Student's t-test was used to evaluate the actual difference in root length between all transgenic lines of *A. thaliana* and Wt *A. thaliana* growing in vertical standing sugar depleted ½ x MS plates at day 10. P-values ≤ 0.050 are underlined and indicate true difference between the respective lines and Wt with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference in respect to root length at day 10.**

	Wt	*idl6 3.5*	*idl7 1.4*	*idl6xidl7 1.10*	*35S:IDL6 1.3*
idl6 3.5	0,007				
idl7 1.4	2.694 x 10-5	0,142			
idl6xidl7 1.10	0,011	1.368 x 10-7	1.056 x 10-11		
35S:IDL6 1.3	0,036	0,326	0,006	4.803 x 10-7	
35S:IDL7 6.5	1.063 x 10-8	0,001	0,028	6.437 x 10 -16	4.948 x 10-6

Table A12 **Student's t-test was used to evaluate the actual difference between Wt *A. thaliana* and different transgenic lines of *A. thaliana* grown on soil. P-values ≤ 0.050 are underlined and indicate true difference between the respective lines and Wt with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.050 are underlined while p-values ≥ 0.100 indicate that there is no difference in respect to the different growth stages. Average number of days a specific line takes to reach a specific developmental stage ± standard deviation (SD) is also listed.**

	4 rosette leaves		**8 rosette leaves**			
	Average ± SD	**p**	**Average ± SD**	**p**		
Wt	12.4 ± 1.1		17.7 ± 0.8			
idl6 3.5	12.6 ± 0.6	0.059	18.2 ± 0.1	0.049		
idl7 1.4	12.7 ± 0.4	0.033	17.9 ± 0.4	0.241		
idl6xidl7 1.9	12.6 ± 0.6	0.089	17.8 ± 0.3	0.210		
35S:IDL6 1.3	12.7 ± 0.6	0.035	18.3 ± 0.4	0.124		
35S:IDL6 2.6	12.9 ± 0.6	0.019	18.4 ± 0.2	0.013		
35S:IDL7 3.1	12.9 ± 0.4	0.010	18.2 ± 0.6	0.098		
35S:IDL7 6.5	12.6 ± 0.6	0.059	17.9 ± 0.5	0.275		
	First flower buds visble		**First flower open**		**Secondary shoots**	
	Average ± SD	**p**	**Average ± SD**	**p**	**Average ± SD**	**p**
Wt	23.1 ± 1.2		24.6 ± 8.7		28.0 ± 2.3	
idl6 3.5	22.6 ± 0.3	0.733	27.8 ± 1.3	0.353	28.1 ± 1.0	0.900
idl7 1.4	22.5 ± 0.4	1.000	28.1 ± 0.7	0.018	28.0 ± 0.9	1.000
idl6xidl7 1.9	22.5 ± 0.6	0.712	27.7 ± 0.7	0.200	28.0 ± 0.5	1.000
35S:IDL6 1.3	22.7 ± 0.1	0.451	28.7 ± 1.1	0.005	29.4 ± 1.0	0.096
35S:IDL6 2.6	23.4 ± 2.2	0.476	27.3 ± 0.9	0.926	27.5 ± 1.3	0.550
35S:IDL7 3.1	22.6 ± 1.1	0.476	27.8 ± 0.8	0.147	28.6 ± 1.4	0.487
35S:IDL7 6.5	21.6 ± 0.1	0.357	28.7 ± 2.7	0.150	28.5 ± 1.8	0.589

Table A13 Student's t-test was used to evaluate the actual difference in germination between different transgenic lines of *A. thaliana* and Wt *A. thaliana* grown in the presence of different concentrations of NaCl. Average percentage of seeds that germinated ± standard deviation (SD) is given. P-values ≤ 0.050 indicate true difference between the respective lines and Wt *A. thaliana* with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference between the lines in respect to total number of germinating individuals at a given time point.

	Day 1		Day 3		Day 5		Day 6		Day 7	
0 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	34.4 ± 13.3		94.5 ± 3.2		95.9 ± 1.5		97.0 ± 1.7		97.3 ± 1.4	
idl6 3.5	47.7 ± 42.4	0.966	96.0 ± 1.4	0.647	96.7 ± 2.1	0.884	96.7 ± 2.1	0.641	96.7 ± 2.1	0.496
idl7 1.4	53.3 ± 47.0	0.923	97.3 ± 0.9	0.964	97.9 ± 1.2	0.618	98.4 ± 0.5	0.520	98.4 ± 0.5	0.637
sos 1.1	10 ± 9.4	0.345	99.2 ± 1.2	0.588	99.2 ± 1.2	0.371	99.2 ± 1.2	0.414	99.2 ± 1.2	0.455
sos 2.1	14.2 ± 13.0	0.395	98.3 ± 0.0	0.763	100 ± 0.0	0.227	100 ± 0.0	0.223	100 ± 0.0	0.221
idl6xidl7 1.9	69.5 ± 33.6	<u>0.092</u>	98.3 ± 2.4	0.167	98.3 ± 2.4	0.186	98.3 ± 2.4	0.541	98.3 ± 2.4	0.598
idl6xidl7 1.10	79.5 ± 10.6	0.408	95.7 ± 0.6	0.528	97.0 ± 1.0	0.361	97.4 ± 0.7	0.941	97.6 ± 08	0.983
35S:IDL6 1.3	26.7 ± 40.6	0.265	98.9 ± 1.0	0.117	98.9 ± 1.0	0.197	98.9 ± 1.0	0.226	98.9 ± 1.0	0.276
35S:IDL6 2.6	15.0 ± 26.0	0.163	99.4 ± 1.0	<u>0.099</u>	99.4 ± 1.0	0.159	99.2 ± 1.4	0.210	99.2 ± 1.4	0.254
35S:IDL7 3.1	16.7 ± 20.8	0.191	98.3 ± 1.7	0.144	99.4 ± 1.0	0.159	100 ± 0.0	0.131	100 ± 0.0	0.158
35S:IDL7 6.5	16.7 ± 23.3	0.241	98.9 ± 2.0	0.126	100 ± 0.0	0.124	100 ± 0.0	0.131	100 ± 0.0	0.158
	Day 1		**Day 3**		**Day 5**		**Day 6**		**Day 7**	
100 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	2.7 ± 3.3		77.6 ± 12.2		87.9 ± 8.1		88.9 ± 7.6		89.5 ± 7.4	
idl6 3.5	2.4 ± 2.5	0.412	89.1 ± 13.1	0.855	94.1 ± 8.0	0.902	94.1 ± 8.0	0.718	94.4 ± 7.4	0.649
idl7 1.4	5.7 ± 9.0	0.924	90.2 ± 2.6	0.728	96.7 ± 2.1	0.184	96.9 ± 2.0	0.612	97.2 ± 2.5	0.821
sos 1.1	21.7 ± 30.6	0.433	87.5 ± 1.2	0.115	90.0 ± 4.7	0.161	90.8 ± 5.9	0.242	90.8 ± 5.9	0.227
sos 2.1	35.8 ± 50.7	0.358	70.4 ± 27.7	0.404	90.8 ± 1.1	<u>0.012</u>	92.5 ± 3.5	0.251	92.5 ± 3.5	0.245
idl6xidl7 1.9	0.8 ± 1.2	0.639	98.3 ± 0.0	<u>0.069</u>	99.2 ± 1.2	<u>0.100</u>	99.2 ± 1.2	<u>0.101</u>	99.2 ± 1.2	<u>0.105</u>
idl6xidl7 1.10	25.3 ± 29.0	0.160	91.1 ± 7.0	0.147	96.2 ± 3.2	0.127	97.0 ± 1.7	<u>0.094</u>	97.0 ± 1.7	<u>0.101</u>
35S:IDL6 1.3	0.6 ± 1.0	0.561	89.4 ± 8.4	0.187	97.8 ± 3.9	0.215	98.3 ± 2.9	0.240	98.3 ± 2.9	0.243
35S:IDL6 2.6	0 ± 0	0.374	93.3 ± 6.0	0.135	100 ± 0.0	0.170	100 ± 0.0	0.202	100 ± 0.0	0.203
35S:IDL7 3.1	0 ± 0	0.374	89.4 ± 14.2	0.215	98.3 ± 1.7	0.200	98.3 ± 1.7	0.238	98.9 ± 1.0	0.227
35S:IDL7 6.5	0 ± 0	0.374	95.0 ± 4.4	0.117	98.3 ± 1.7	0.200	98.3 ± 1.7	0.238	98.3 ± 1.7	0.241
	Day 1		**Day 3**		**Day 5**		**Day 6**		**Day 7**	
130 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0 ± 0		70.2 ± 19.6		81.9 ± 12.3		85.1 ± 11.1		85.9 ± 10.5	
idl6 3.5	0.3 ± 0.6	0.374	62.7 ± 18.7	0.177	81.2 ± 19.7	0.532	83.9 ± 20.2	0.655	84.8 ± 19.9	0.654
idl7 1.4	0 ± 0	-	67.6 ± 22.9	0.379	92.8 ± 6.2	0.379	93.9 ± 6.0	0.329	94.6 ± 5.8	0.366
sos 1.1	7.5 ± 10.6	0.272	65.0 ± 25.9	0.342	90.8 ± 5.9	0.652	91.7 ± 4.7	0.578	92.5 ± 5.9	0.662
sos 2.1	10.0 ± 14.1	0.272	59.2 ± 48.3	0.460	90.8 ± 3.5	0.548	90.8 ± 3.5	0.696	91.7 ± 2.4	0.725
idl6xidl7 1.9	0 ± 0	-	98.3 ± 0	0.119	99.2 ± 1.2	0.124	99.2 ± 1.2	0.184	99.2 ± 1.2	0.179
idl6xidl7 1.10	6.4 ± 8.6	0.268	88.4 ± 8.9	0.219	93.2 ± 5.8	0.227	94.3 ± 3.8	0.299	94.9 ± 2.8	0.266
35S:IDL6 1.3	0 ± 0	-	57.2 ± 26.8	0.689	93.9 ± 4.2	<u>0.091</u>	96.1 ± 3.5	0.110	98.3 ± 1.7	<u>0.098</u>
35S:IDL6 2.6	0 ± 0	-	60.6 ± 3.5	0.475	95.6 ± 2.6	<u>0.076</u>	97.2 ± 1.9	<u>0.097</u>	97.2 ± 1.9	0.110
35S:IDL7 3.1	0 ± 0	-	62.2 ± 26.1	0.545	91.1 ± 5.4	0.123	94.4 ± 4.2	0.132	96.1 ± 1.9	0.123
35S:IDL7 6.5	0 ± 0	-	75.4 ± 20.3	0.239	97.2 ± 3.5	<u>0.066</u>	98.9 ± 1.9	<u>0.082</u>	98.9 ± 1.9	<u>0.093</u>

Table A14 **Student's t-test was used to evaluate the actual difference in development of hypocotyls and cotyledons between different transgenic lines of *A. thaliana* and Wt *A. thaliana* grown in the presence of different concentrations of NaCl. Average percentage of seedlings that developed hypocotyls and cotyledons ± standard deviation (SD) is given. P-values ≤ 0.050 indicate true difference between the respective lines and Wt *A. thaliana* with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference between the lines at a given time point.**

	Day 2		Day 4		Day 6		Day 8		Day 10	
0 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	61.6 ± 12.8		96.0 ± 3.3		96.5 ± 1.9		97.1 ± 1.5		97.2 ± 1.4	
idl6 3.5	55.2 ± 42.4	0.990	95.3 ± 1.5	0.530	96.7 ± 2.1	0.990	96.7 ± 2.1	0.641	96.7 ± 2.1	0.641
idl7 1.4	60.4 ± 45.2	0.903	97.0 ± 1.7	0.865	97.9 ± 1.2	0.530	98.4 ± 0.5	0.520	99.0 ± 1.0	0.349
sos 1.1	50.8 ± 24.7	0.899	99.2 ± 1.2	0.344	99.2 ± 1.2	0.344	99.2 ± 1.2	0.414	99.2 ± 1.2	0.414
sos 2.1	30.0 ± 30.6	0.532	99.2 ± 1.2	0.344	100 ± 0.0	0.219	100 ± 0.0	0.223	100 ± 0.0	0.223
idl6xidl7 1.9	90.8 ± 3.5	0.057	97.5 ± 1.2	0.597	98.3 ± 2.4	0.399	98.3 ± 2.4	0.506	98.3 ± 2.4	0.520
idl6xidl7 1.10	64.8 ± 35.9	0.892	93.6 ± 2.2	0.347	96.9 ± 0.2	0.731	97.2 ± 0.7	0.875	97.6 ± 0.8	0.690
35S:IDL6 1.3	40.0 ± 43.7	0.702	98.9 ± 1.0	0.104	98.9 ± 1.0	0.193	98.9 ± 1.0	0.227	98.9 ± 1.0	0.276
35S:IDL6 2.6	30.6 ± 45.9	0.527	99.4 ± 1.0	0.086	99.2 ± 1.4	0.180	99.2 ± 1.4	0.210	99.2 ± 1.4	0.254
35S:IDL7 3.1	45.6 ± 38.1	0.812	97.8 ± 1.0	0.155	99.4 ± 1.0	0.153	100 ± 0.0	0.127	100 ± 0.0	0.158
35S:IDL7 6.5	45.6 ± 38.0	0.813	99.4 ± 1.0	0.086	100 ± 0.0	0.116	100 ± 0.0	0.127	100 ± 0.0	0.158
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
100 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	8.3 ± 3.3		70.6 ± 16.3		85.1 ± 10.2		88.0 ± 8.8		89.0 ± 7.7	
idl6 3.5	0.7 ± 1.2	0.304	79.7 ± 9.9	0.727	91.9 ± 9.5	1.000	94.1 ± 8.0	0.765	94.4 ± 7.4	0.720
idl7 1.4	1.0 ± 1.7	0.345	70.5 ± 21.9	0.753	95.7 ± 3.1	0.112	96.6 ± 2.5	0.578	96.6 ± 2.5	0.828
sos 1.1	0 ± 0	0.374	0 ± 0	-	1.7 ± 0	-	1.7 ± 0	-	1.7 ± 0	-
sos 2.1	0 ± 0	0.374	6.8 ± 0.0	-	15.3 ± 0	-	16.9 ± 0	-	16.9 ± 0	-
idl6xidl7 1.9	17.6 ± 3.4	0.056	98.3 ± 0.0	0.107	99.2 ± 1.2	0.162	99.2 ± 1.2	0.187	99.2 ± 1.2	0.175
idl6xidl7 1.10	9.0 ± 7.8	0.898	78.8 ± 17.2	0.626	93.6 ± 4.6	0.258	95.9 ± 3.7	0.223	96.8 ± 3.0	0.178
35S:IDL6 1.3	2.8 ± 4.8	0.561	65.0 ± 21.7	0.608	90.6 ± 4.2	0.338	95.6 ± 1.9	0.283	97.2 ± 1.9	0.264
35S:IDL6 2.6	0 ± 0	0.374	58.3 ± 29.5	0.824	86.7 ± 21.7	0.542	96.1 ± 6.7	0.284	99.4 ± 1.0	0.214
35S:IDL7 3.1	0 ± 0	0.374	57.2 ± 26.7	0.849	92.8 ± 3.5	0.285	96.7 ± 1.7	0.257	97.8 ± 1.0	0.250
35S:IDL7 6.5	0 ± 0	0.374	81.1 ± 23.0	0.283	95.5 ± 4.8	0.235	98.3 ± 1.7	0.223	98.3 ± 1.7	0.238
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
130 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0.6 ± 0.9		40.9 ± 17.0		70.0 ± 15.2		80.3 ± 12.8		84.5 ± 11.2	
idl6 3.5	0 ± 0	0.374	16.7 ± 11.8	0.228	67.6 ± 24.1	0.456	79.2 ± 20.1	0.464	83.1 ± 19.0	0.555
idl7 1.4	0.7 ± 1.2	0.374	20.8 ± 27.1	0.554	74.9 ± 18.7	0.713	89.1 ± 7.8	0.929	94.2 ± 5.4	0.296
sos 1.1										
sos 2.1										
idl6xidl7 1.9	0 ± 0	0.374	90.0 ± 2.4	0.031	97.5 ± 1.2	0.094	99.2 ± 1.2	0.143	99.2 ± 1.2	0.177
idl6xidl7 1.10	1.0 ± 1.7	0.559	67.6 ± 20.1	0.205	89.6 ± 9.9	0.135	92.3 ± 6.4	0.220	94.5 ± 2.7	0.205
35S:IDL6 1.3	0 ± 0	0.374	27.8 ± 29.5	0.991	53.9 ± 20.6	0.945	73.9 ± 8.6	0.597	87.8 ± 8.6	0.270
35S:IDL6 2.6	0 ± 0	0.374	8.9 ± 8.2	0.294	56.1 ± 21.0	0.859	86.1 ± 8.2	0.228	91.7 ± 3.3	0.164
35S:IDL7 3.1	0 ± 0	0.374	8.9 ± 10.2	0.304	51.7 ± 15.9	0.964	78.9 ± 9.2	0.414	88.3 ± 7.6	0.250
35S:IDL7 6.5	0 ± 0	0.374	38.5 ± 28.4	0.643	75.4 ± 15.6	0.256	92.2 ± 5.1	0.129	97.2 ± 2.6	0.094

Table A15 **Student's t-test was used to evaluate the actual difference in development of fully opened cotyledons between Wt *A. thaliana* and different transgenic lines grown in the presence of different concentrations of NaCl. Average percentage of seedlings that developed fully opened cotyledons ± standard deviation (SD) is given. P-values ≤ 0.050 indicate true difference between the respective lines and Wt with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference at a given time point.**

	Day 2		Day 4		Day 6		Day 8		Day 10	
0 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0.2 ± 0.3		86.2 ± 6.4		95.4 ± 2.5		96.4 ± 2.1		96.7 ± 1.6	
idl6 3.5	1.0 ± 1.7	0.468	87.3 ± 0.9	0.755	95.6 ± 3.0	0.765	96.7 ± 2.1	0.990	96.7 ± 2.1	0.990
idl7 1.4	0.7 ± 1.2	0.523	92.1 ± 5.4	0.605	97.2 ± 2.0	0.485	97.9 ± 1.2	0.530	99.0 ± 1.0	0.256
sos 1.1	0 ± 0	0.495	95.0 ± 4.7	0.523	97.5 ± 3.5	0.563	97.5 ± 3.5	0.783	98.3 ± 2.4	0.546
sos 2.1	0 ± 0	0.495	91.7 ± 2.4	0.613	97.5 ± 3.5	0.563	100 ± 0	0.219	100 ± 0	0.219
idl6xidl7 1.9	0 ± 0	0.495	95.8 ± 1.1	0.137	97.5 ± 1.2	0.374	97.5 ± 1.2	0.553	97.5 ± 1.2	0.588
idl6xidl7 1.10	0.3 ± 0.6	0.528	87.8 ± 8.7	0.818	94.8 ± 1.3	0.721	96.0 ± 1.0	0.783	97.6 ± 0.8	0.440
35S:IDL6 1.3	0 ± 0	0.495	94.4 ± 5.4	0.222	98.9 ± 1.0	0.098	98.9 ± 1.0	0.150	98.9 ± 1.0	0.169
35S:IDL6 2.6	0 ± 0	0.495	98.9 ± 1.0	0.081	99.2 ± 1.4	0.093	99.2 ± 1.4	0.141	99.2 ± 1.4	0.157
35S:IDL7 3.1	0 ± 0	0.495	95.6 ± 1.0	0.148	97.5 ± 0.8	0.185	98.9 ± 1.0	0.150	99.4 ± 1.0	0.127
35S:IDL7 6.5	0 ± 0	0.495	94.9 ± 6.1	0.215	99.4 ± 1.0	0.076	100 ± 0	0.087	100 ± 0	0.089
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
100 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0 ± 0		37.6 ± 27.4		67.8 ± 12.9		76.8 ± 9.1		79.8 ± 13.6	
idl6 3.5	0 ± 0	-	16.2 ± 22.9	0.989	74.7 ± 15.6	0.498	85.7 ± 12.5	0.616	88.7 ± 13.1	0.830
idl7 1.4	0 ± 0	-	25.5 ± 36.1	0.772	86.1 ± 7.0	0.078	92.3 ± 5.5	0.109	94.3 ± 4.0	0.063
sos 1.1										
sos 2.1										
idl6xidl7 1.9	0 ± 0	-	90.8 ± 3.5	0.081	97.5 ± 1.2	0.054	99.2 ± 1.2	0.047	99.2 ± 1.2	0.152
idl6xidl7 1.10	0 ± 0	-	33.8 ± 17.2	0.877	79.7 ± 5.0	0.212	90.3 ± 4.7	0.085	92.9 ± 5.4	0.195
35S:IDL6 1.3	0 ± 0	-	23.3 ± 36.2	0.871	62.2 ± 28.3	0.828	73.3 ± 19.6	0.764	75.0 ± 25.9	0.783
35S:IDL6 2.6	0 ± 0	-	5.0 ± 8.7	0.358	55.6 ± 42.2	0.987	65.0 ± 40.5	0.965	65.8 ± 48.3	0.974
35S:IDL7 3.1	0 ± 0	-	13.3 ± 20.2	0.575	63.3 ± 25.0	0.789	72.8 ± 21.1	0.787	75.0 ± 25.9	0.783
35S:IDL7 6.5	0 ± 0	-	21.4 ± 22.1	0.713	79.4 ± 17.5	0.406	85.5 ± 18.0	0.457	96.7 ± 2.3	0.382
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
130 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0 ± 0		5.3 ± 4.8		30.8 ± 14.2		39.6 ± 15.2		45.9 ± 16.2	
idl6 3.5	0 ± 0	-	0 ± 0	0.423	16.0 ± 22.2	0.847	23.4 ± 30.1	0.850	26.2 ± 32.3	0.831
idl7 1.4	0 ± 0	-	0.5 ± 0.7	1.000	21.4 ± 15.2	0.902	38.7 ± 15.0	0.472	44.9 ± 16.6	0.459
sos 1.1										
sos 2.1										
idl6xidl7 1.9	0 ± 0	-	35.0 ± 0.0	0.004	80.8 ± 1.2	0.018	87.5 ± 1.2	0.024	89.2 ± 1.2	0.037
idl6xidl7 1.10	0 ± 0	-	2.0 ± 2.9	0.462	38.0 ± 21.4	0.651	59.8 ± 18.4	0.217	64.1 ± 13.4	0.209
35S:IDL6 1.3	0 ± 0	-	3.3 ± 2.9	0.743	29.4 ± 31.2	0.484	36.1 ± 33.1	0.682	43.3 ± 44.8	0.994
35S:IDL6 2.6	0 ± 0	-	0 ± 0	0.374	6.7 ± 11.6	0.293	15.0 ± 23.0	0.439	30.0 ± 44.8	0.784
35S:IDL7 3.1	0 ± 0	-	0 ± 0	0.374	11.7 ± 14.5	0.440	20.0 ± 23.1	0.559	28.3 ± 35.4	0.758
35S:IDL7 6.5	0 ± 0	-	1.1 ± 1.0	0.477	25.7 ± 6.8	1.000	37.4 ± 11.3	0.744	48.7 ± 11.3	0.881

Table A16 Student's t-test was used to evaluate the actual difference in development of green tissue between different transgenic lines of *A. thaliana* and Wt *A. thaliana* grown in the presence of different concentrations of NaCl. Average percentage of seedlings that developed green tissue ± standard deviation (SD) is given. P-values ≤ 0.050 indicate true difference between the respective lines and Wt *A. thaliana* with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference at a given time point.

	Day 2		Day 4		Day 6		Day 8		Day 10	
0 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	61.6 ± 12.8		93.7 ± 4.1		96.2 ± 2.0		96.6 ± 1.8		96.7 ± 1.6	
idl6 3.5	55.2 ± 42.4	0.990	93.2 ± 0.2	0.927	96.3 ± 2.3	0.920	96.7 ± 2.1	0.990	96.7 ± 2.1	0.990
idl7 1.4	60.4 ± 45.2	0.903	96.0 ± 1.4	0.685	97.9 ± 1.2	0.445	98.4 ± 0.5	0.351	99.0 ± 1.0	0.256
sos 1.1	50.8 ± 24.7	0.899	99.2 ± 1.2	0.469	99.2 ± 1.2	0.334	99.2 ± 1.2	0.344	99.2 ± 1.2	0.344
sos 2.1	30.0 ± 30.6	0.532	98.3 ± 2.4	0.525	99.2 ± 1.2	0.334	100 ± 0	0.219	100 ± 0	0.219
idl6xidl7 1.9	90.8 ± 3.5	0.057	96.6 ± 0.0	0.409	98.3 ± 2.4	0.357	98.3 ± 2.4	0.402	98.3 ± 2.4	0.408
idl6xidl7 1.10	64.8 ± 35.9	0.892	89.8 ± 5.7	0.380	96.0 ± 1.0	0.886	96.9 ± 0.2	0.774	97.6 ± 0.8	0.440
35S:IDL6 1.3	40.0 ± 43.7	0.702	97.8 ± 1.0	0.119	98.9 ± 1.0	0.150	98.9 ± 1.0	0.121	98.9 ± 1.0	0.169
35S:IDL6 2.6	30.6 ± 45.9	0.527	99.4 ± 1.0	0.072	99.2 ± 1.4	0.141	99.2 ± 1.4	0.115	99.2 ± 1.4	0.157
35S:IDL7 3.1	45.6 ± 38.1	0.812	97.8 ± 1.0	0.119	98.9 ± 1.0	0.150	99.4 ± 1.0	0.090	99.4 ± 1.0	0.127
35S:IDL7 6.5	45.6 ± 38.2	0.813	98.9 ± 2.0	0.093	100 ± 0.0	0.087	100 ± 0.0	0.061	100 ± 0.0	0.089
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
100 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	8.3 ± 3.3		52.9 ± 22.2		63.9 ± 4.8		46.5 ± 11.3		42.1 ± 13.6	
idl6 3.5	0.7 ± 1.2	0.304	38.9 ± 7.9	0.764	80.2 ± 4.7	0.11	60.5 ± 15.4	0.830	59.3 ± 18.3	0.803
idl7 1.4	1.0 ± 1.7	0.345	46.2 ± 25.2	0.653	87.0 ± 0.7	0.04	53.1 ± 8.0	0.655	49.1 ± 10.5	0.589
sos 1.1										
sos 2.1										
idl6xidl7 1.9	17.6 ± 3.4	0.056	95.8 ± 3.5	0.083	73.1 ± 4.4	0.117	56.3 ± 4.2	0.341	59.7 ± 6.7	0.200
idl6xidl7 1.10	9.0 ± 7.8	0.898	51.0 ± 8.5	0.920	67.1 ± 10.0	0.638	47.0 ± 2.6	0.944	46.8 ± 6.7	0.624
35S:IDL6 1.3	2.8 ± 4.8	0.561	48.9 ± 35.3	0.867	72.2 ± 15.8	0.567	66.7 ± 13.0	0.238	65.6 ± 16.4	0.301
35S:IDL6 2.6	0 ± 0	0.374	33.3 ± 40.6	0.762	62.8 ± 40.2	0.941	43.3 ± 26.8	0.863	43.9 ± 16.4	0.950
35S:IDL7 3.1	0 ± 0	0.374	42.2 ± 34.3	0.964	67.8 ± 22.8	0.748	61.1 ± 24.3	0.487	57.8 ± 27.4	0.588
35S:IDL7 6.5	0 ± 0	0.374	68.8 ± 29.6	0.409	86.1 ± 17.0	0.252	63.1 ± 28.5	0.472	58.7 ± 25.8	0.550
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
130 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0.6 ± 0.9		19.0 ± 16.7		31.0 ± 6.6		20.9 ± 7.3		15.6 ± 5.7	
idl6 3.5	0 ± 0	0.374	0 ± 0	0.145	22.9 ± 20.5	0.896	9.5 ± 10.2	0.705	7.3 ± 7.6	0.632
idl7 1.4	0.7 ± 1.2	0.374	2.5 ± 3.5	0.764	35.3 ± 5.2	0.531	20.4 ± 17.7	0.544	14.9 ± 12.4	0.622
sos 1.1										
sos 2.1										
idl6xidl7 1.9	0 ± 0	0.374	70.0 ± 4.7	0.028	63.3 ± 2.4	0.008	39.2 ± 1.2	0.044	28.3 ± 4.7	0.081
idl6xidl7 1.10	1.0 ± 1.7	0.770	12.0 ± 11.3	0.646	31.0 ± 26.2	0.998	17.0 ± 17.1	0.733	14.6 ± 15.6	0.918
35S:IDL6 1.3	0 ± 0	0.374	20.6 ± 27.0	0.870	36.1 ± 30.0	0.762	27.2 ± 19.5	0.812	30.0 ± 25.9	0.777
35S:IDL6 2.6	0 ± 0	0.374	1.1 ± 1.9	0.313	11.1 ± 14.9	0.342	9.4 ± 13.5	0.344	15.0 ± 18.9	0.768
35S:IDL7 3.1	0 ± 0	0.374	3.3 ± 5.8	0.389	14.4 ± 16.4	0.443	16.7 ± 22.0	0.705	21.7 ± 30.6	0.989
35S:IDL7 6.5	0 ± 0	0.374	12.3 ± 11.1	0.774	33.5 ± 11.6	0.782	24.5 ± 13.4	0.930	26.8 ± 11.6	0.819

Table A17 Student's t-test was used to evaluate the actual difference in development of white tissue between different transgenic lines of *A. thaliana* and Wt *A. thaliana* grown in the presence of different concentrations of NaCl. Average percentage of seedlings that developed white tissue ± standard deviation (SD) is given. P-values ≤ 0.050 indicate true difference between the respective lines and Wt *A. thaliana* with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference at a given time point.

	Day 6		Day 7		Day 8		Day 9		Day 10	
100 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	10.4 ± 9.0		26.6 ± 16.3		32.3 ± 14.4		36.6 ± 16.2		35.4 ± 22.9	
idl6 3.5	8.3 ± 7.6	0.940	19.2 ± 15.4	0.890	25.7 ± 18.4	0.882	28.2 ± 15.4	0.884	29.4 ± 13.6	0.878
idl7 1.4	8.7 ± 8.1	0.981	25.9 ± 12.4	0.620	39.2 ± 2.3	0.255	40.9 ± 5.1	0.294	45.2 ± 6.6	0.213
sos 1.1										
sos 2.1										
idl6xidl7 1.9	25.2 ± 4.5	0.128	38.6 ± 1.9	0.396	42.8 ± 3.1	0.401	41.2 ± 3.1	0.733	39.5 ± 5.5	0.828
idl6xidl7 1.10	13.7 ± 11.8	0.719	34.1 ± 9.5	0.526	43.9 ± 6.4	0.271	45.9 ± 6.7	0.408	48.4 ± 9.1	0.410
35S:IDL6 1.3	1.1 ± 1.0	0.591	5.0 ± 5.0	0.242	11.1 ± 6.9	0.274	13.9 ± 3.5	0.109	13.3 ± 4.7	0.303
35S:IDL6 2.6	0 ± 0	0.289	9.4 ± 10.7	0.628	23.9 ± 13.6	0.757	28.9 ± 12.1	0.649	25.0 ± 11.8	0.797
35S:IDL7 3.1	0 ± 0	0.289	6.7 ± 5.0	0.327	13.3 ± 4.4	0.347	18.3 ± 8.8	0.416	24.2 ± 13.0	0.863
35S:IDL7 6.5	0 ± 0	0.289	15.1 ± 13.1	0.903	23.0 ± 17.0	0.847	27.4 ± 13.7	0.787	23.6 ± 12.2	0.895
	Day 6		**Day 7**		**Day 8**		**Day 9**		**Day 10**	
130 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	5.5 ± 5.4		14.3 ± 9.3		19.5 ± 9.2		25.5 ± 12.6		28.3 ± 11.6	
idl6 3.5	2.0 ± 2.9	0.895	8.2 ± 11.4	0.925	13.6 ± 19.8	0.896	16.2 ± 21.6	0.875	18.9 ± 25.0	0.902
idl7 1.4	2.7 ± 3.8	0.964	11.2 ± 4.0	0.535	20.4 ± 12.3	0.600	25.8 ± 9.9	0.491	30.0 ± 9.5	0.478
sos 1.1										
sos 2.1										
idl6xidl7 1.9	16.7 ± 4.7	<u>0.098</u>	43.3 ± 0.0	<u>0.025</u>	48.3 ± 2.4	<u>0.026</u>	55.8 ± 3.5	<u>0.050</u>	60.8 ± 5.9	<u>0.038</u>
idl6xidl7 1.10	10.3 ± 10.0	0.499	29.0 ± 12.2	0.172	46.1 ± 21.1	0.115	49.2 ± 16.3	0.117	49.5 ± 15.0	0.125
35S:IDL6 1.3	1.1 ± 1.0	0.519	7.8 ± 10.7	0.904	10.6 ± 12.9	0.836	11.1 ± 13.9	0.943	9.4 ± 16.4	0.742
35S:IDL6 2.6	0 ± 0	0.205	1.1 ± 1.9	0.272	6.7 ± 11.6	0.585	7.2 ± 12.5	0.428	8.9 ± 15.4	0.710
35S:IDL7 3.1	0 ± 0	0.205	1.1 ± 1.0	0.267	3.9 ± 2.5	0.347	5.0 ± 4.4	0.266	4.4 ± 5.1	0.431
35S:IDL7 6.5	0.6 ± 1.0	0.352	7.3 ± 2.6	0.808	12.9 ± 3.6	0.984	19.6 ± 2.6	0.863	14.6 ± 12.6	0.989

Table A18 Student's t-test was used to evaluate the actual difference in germination between different transgenic lines of *A. thaliana* and Wt *A. thaliana* grown in the presence of different concentrations of mannitol. Average percentage of seeds that germinated ± standard deviation (SD) is given. P-values ≤ 0.050 indicate true difference between the respective lines and Wt *A. thaliana* with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference between the lines in respect to total number of individuals with white tissue at a given time point.

	Day 1		**Day 3**		**Day 5**		**Day 6**		**Day 7**	
0 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	37.5 ± 31.4		95.5 ± 1.0		97.2 ± 1.2		97.4 ± 0.9		97.4 ± 0.9	
idl6 3.5	48.0 ± 30.1	0.676	98.0 ± 1.2	0.029	98.3 ± 1.7	0.333	98.3 ± 1.7	0.378	98.3 ± 1.7	0.378
idl7 1.4	23.9 ± 20.7	0.546	98.0 ± 1.2	0.029	98.0 ± 1.2	0.413	98.6 ± 0.4	0.094	99.1 ± 0.8	0.050
idl6xidl7 1.9	79.2 ± 13.0	0.161	98.3 ± 0.0	0.02	98.3 ± 0.0	0.266	98.3 ± 0.0	0.237	98.3 ± 0.0	0.237
idl6xidl7 1.10	36.9 ± 20.6	0.977	92.0 ± 4.8	0.207	96.2 ± 2.6	0.527	96.6 ± 2.8	0.585	97.1 ± 1.8	0.789
35S:IDL6 1.3	42.5 ± 45.2	0.639	99.6 ± 0.8	0.003	99.6 ± 0.8	0.043	99.6 ± 0.8	0.034	99.6 ± 0.8	0.034
35S:IDL7 6.5	41.7 ± 36.2	0.668	98.8 ± 2.5	0.119	98.8 ± 2.5	0.498	98.8 ± 2.5	0.560	98.8 ± 2.5	0.560
	Day 1		**Day 3**		**Day 5**		**Day 6**		**Day 7**	
100 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	11.8 ± 12.2		96.4 ± 1.6		96.6 ± 2.0		97.3 ± 1.4		97.3 ± 1.4	
idl6 3.5	12.4 ± 9.4	0.938	98.2 ± 0.2	0.110	99.4 ± 1.0	0.075	99.4 ± 1.0	0.064	99.4 ± 1.0	0.064
idl7 1.4	1.2 ± 0.1	0.205	98.9 ± 1.0	0.063	99.4 ± 1.0	0.075	99.4 ± 1.0	0.064	100 ± 0.0	0.018
idl6xidl7 1.9	56.7 ± 0.0	0.008	95.0 ± 4.7	0.593	95.0 ± 4.7	0.554	95.0 ± 4.7	0.379	95.8 ± 3.5	0.483
idl6xidl7 1.10	14.3 ± 14.0	0.804	92.0 ± 3.5	0.072	96.9 ± 2.0	0.869	96.9 ± 2.0	0.785	97.4 ± 1.3	0.858
35S:IDL6 1.3	7.0 ± 12.0	0.204	98.7 ± 0.8	0.095	98.7 ± 0.8	0.205	98.7 ± 0.8	0.237	98.7 ± 0.8	0.237
35S:IDL7 6.5	0.4 ± 0.8	0.182	97.5 ± 5.0	0.925	99.2 ± 1.7	0.190	99.2 ± 1.7	0.239	99.2 ± 1.7	0.239
	Day 1		**Day 3**		**Day 5**		**Day 6**		**Day 7**	
300 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0 ± 0	-	49.2 ± 23.6		85.2 ± 3.2		87.8 ± 2.6		90.0 ± 2.7	
idl6 3.5	0 ± 0	-	84.1 ± 6.6	0.059	96.7 ± 1.7	0.003	97.3 ± 0.9	0.002	97.3 ± 0.9	0.006
idl7 1.4	0 ± 0	-	79.1 ± 8.6	0.095	96.1 ± 1.0	0.003	98.9 ± 1.0	0.001	98.9 ± 1.0	0.003
idl6xidl7 1.9	1.7 ± 0.0	0.000	88.3 ± 0.0	0.092	90.8 ± 1.2	0.084	90.8 ± 1.2	0.205	90.8 ± 1.2	0.692
idl6xidl7 1.10	0 ± 0	-	67.7 ± 5.7	0.251	84.3 ± 2.0	0.714	87.6 ± 2.1	0.921	89.0 ± 2.4	0.644
35S:IDL6 1.3	0 ± 0	-	55.9 ± 36.6	0.771	91.5 ± 13.6	0.659	95.4 ± 6.1	0.142	95.8 ± 5.3	0.210
35S:IDL7 6.5	0 ± 0	-	67.1 ± 45.5	0.810	87.1 ± 23.6	0.863	93.8 ± 10.3	0.529	94.6 ± 9.8	0.635

Table A19 Student's t-test was used to evaluate the actual difference in development of hypocotyls and cotyledons between different transgenic lines of *A. thaliana* and Wt *A. thaliana* grown in the presence of different concentrations of mannitol. Average percentage of seedlings that developed hypocotyls and cotyledons ± standard deviation (SD) is given. P-values ≤ 0.050 indicate true difference between the respective lines and Wt *A. thaliana* with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference between the lines in respect to total number of individuals with emerging hypocotyls and cotyledons at a given time point.

	Day 2		Day 4		Day 6		Day 8		Day 10	
0 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	73.5 ± 18.7		95.4 ± 0.8		96.5 ± 0.5		97.0 ± 0.7		97.0 ± 0.7	
idl6 3.5	83.1 ± 10.1	0.463	97.7 ± 0.9	<u>0.014</u>	98.0 ± 1.2	<u>0.073</u>	98.3 ± 1.7	0.194	98.3 ± 1.7	0.194
idl7 1.4	75.2 ± 16.2	0.908	98.0 ± 1.2	<u>0.016</u>	98.6 ± 0.4	<u>0.002</u>	99.1 ± 0.8	<u>0.013</u>	99.1 ± 0.8	<u>0.013</u>
idl6xidl7 1.9	90.8 ± 3.5	0.286	98.3 ± 0.0	<u>0.007</u>	98.3 ± 0.0	<u>0.008</u>	98.3 ± 0.0	<u>0.057</u>	98.3 ± 0.0	<u>0.057</u>
idl6xidl7 1.10	54.3 ± 18.5	0.189	90.3 ± 3.2	<u>0.019</u>	94.2 ± 5.2	0.359	96.2 ± 2.6	0.514	97.1 ± 1.8	0.793
35S:IDL6 1.3	70.8 ± 0.7	0.638	99.0 ± 1.3	<u>0.001</u>	99.6 ± 0.8	<u>0.003</u>	99.6 ± 0.8	<u>0.010</u>	99.6 ± 0.8	<u>0.010</u>
35S:IDL7 6.5	70.8 ± 41.0	0.628	98.8 ± 2.5	<u>0.098</u>	98.8 ± 2.5	0.261	98.8 ± 2.5	0.396	99.2 ± 1.7	0.119
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
100 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	50.2 ± 30.1		96.0 ± 1.3		97.0 ± 1.7		97.3 ± 1.4		97.3 ± 1.4	
idl6 3.5	56.8 ± 26.1	0.776	98.2 ± 1.7	<u>0.104</u>	99.4 ± 1.0	<u>0.079</u>	99.4 ± 1.0	<u>0.064</u>	99.4 ± 1.0	<u>0.064</u>
idl7 1.4	45.1 ± 27.8	0.828	98.9 ± 1.0	<u>0.022</u>	99.4 ± 1.0	<u>0.079</u>	100 ± 0.0	<u>0.018</u>	100 ± 0.0	<u>0.018</u>
idl6xidl7 1.9	80.8 ± 22.4	0.281	94.2 ± 5.9	0.528	95.0 ± 4.7	0.442	95.0 ± 4.7	0.379	95.8 ± 3.5	0.483
idl6xidl7 1.10	37.8 ± 19.5	0.564	85.2 ± 4.7	<u>0.006</u>	92.6 ± 3.1	<u>0.054</u>	94.2 ± 1.5	<u>0.039</u>	95.9 ± 0.8	0.189
35S:IDL6 1.3	17.5 ± 29.6	0.314	98.3 ± 1.4	0.109	98.7 ± 0.8	0.251	98.7 ± 0.8	0.237	98.7 ± 0.8	0.237
35S:IDL7 6.5	40.4 ± 45.9	0.408	97.5 ± 5.0	0.837	98.3 ± 3.3	0.741	99.2 ± 1.7	0.239	99.2 ± 1.7	0.239
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
300 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0 ± 0		15.0 ± 15.4		36.5 ± 17.2		49.5 ± 17.5		62.2 ± 16.4	
idl6 3.5	0 ± 0	-	37.5 ± 8.3	0.165	66.0 ± 4.9	<u>0.037</u>	82.0 ± 5.9	<u>0.029</u>	93.3 ± 2.4	<u>0.035</u>
idl7 1.4	0 ± 0	-	20.0 ± 11.8	0.728	50.9 ± 7.9	0.242	72.6 ± 8.1	<u>0.091</u>	86.7 ± 4.7	<u>0.070</u>
idl6xidl7 1.9	20.8 ± 29.5	0.178	74.2 ± 17.7	<u>0.028</u>	78.3 ± 11.8	<u>0.039</u>	81.7 ± 7.1	<u>0.075</u>	85.0 ± 4.7	<u>0.073</u>
idl6xidl7 1.10	0.3 ± 0.6	0.286	20.0 ± 7.1	0.707	42.3 ± 8.1	0.613	60.7 ± 9.0	0.363	71.7 ± 7.1	0.350
35S:IDL6 1.3	0 ± 0	-	4.5 ± 3.5	0.312	40.0 ± 24.8	0.763	56.3 ± 31.7	0.888	62.4 ± 25.5	0.991
35S:IDL7 6.5	0 ± 0	-	7.2 ± 8.6	0.488	35.8 ± 27.6	0.626	51.3 ± 31.1	0.759	57.2 ± 26.4	0.794

Table A20 Student's t-test was used to evaluate the actual difference in development of fully opened cotyledons between different transgenic lines of *A. thaliana* and Wt *A. thaliana* grown in the presence of different concentrations of mannitol. Average percentage of seedlings that developed fully opened cotyledons ± standard deviation (SD) is given. P-values ≤ 0.050 indicate true difference between the respective lines and Wt *A. thaliana* with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference between the lines in respect to total number of individuals with fully opened cotyledons at a given time point.

	Day 2		Day 4		Day 6		Day 8		Day 10	
0 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0.9 ± 1.1		90.8 ± 1.0		95.7 ± 1.0		96.8 ± 0.9		97.0 ± 0.7	
idl6 3.5	0.3 ± 0.6	0.437	89.7 ± 3.4	0.547	97.7 ± 0.9	0.039	98.01.2	0.181	98.01.2	0.210
idl7 1.4	0 ± 0	0.206	95.7 ± 3.3	0.035	98.0 ± 1.2	0.036	98.6 ± 0.4	0.025	99.1 ± 0.8	0.013
idl6xidl7 1.9	23.3 ± 33.0	0.192	96.7 ± 0.0	0.002	97.51.2	0.111	97.5 ± 1.2	0.441	98.3 ± 0.0	0.057
idl6xidl7 1.10	0 ± 0	0.206	63.3 ± 7.6	0.000	90.0 ± 5.5	0.071	93.1 ± 4.2	0.139	95.9 ± 2.5	0.428
35S:IDL6 1.3	0 ± 0	0.206	99.2 ± 1.2	0.593	100 ± 0.0	0.769	100 ± 0.0	0.709	100 ± 0.0	0.896
35S:IDL7 6.5	0 ± 0	0.206	99.4 ± 1.0	0.016	99.6 ± 0.8	0.140	99.6 ± 0.8	0.345	99.6 ± 0.8	0.396
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
100 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0.2 ± 0.4		90.3 ± 4.3		95.8 ± 1.5		96.6 ± 2.0		97.3 ± 1.4	
idl6 3.5	0 ± 0	0.432	85.8 ± 15.3	0.644	94.8 ± 2.7	0.566	99.4 ± 1.0	0.075	99.4 ± 1.0	0.064
idl7 1.4	0 ± 0	0.432	96.7 ± 0.0	0.139	98.9 ± 1.0	0.026	99.4 ± 1.0	0.075	100 ± 0.0	0.018
idl6xidl7 1.9	11.7 ± 16.5	0.751	92.5 ± 8.2	0.708	94.2 ± 5.9	0.601	95.0 ± 4.7	0.554	95.0 ± 4.7	0.379
idl6xidl7 1.10	0 ± 0	0.432	60.0 ± 4.7	0.005	85.6 ± 4.2	0.006	90.8 ± 2.9	0.024	92.4 ± 1.3	0.006
35S:IDL6 1.3	0 ± 0	0.432	96.6 ± 0.0	0.124	98.9 ± 1.0	0.034	98.9 ± 1.0	0.205	98.9 ± 1.0	0.237
35S:IDL7 6.5	0 ± 0	0.432	97.2 ± 3.5	0.805	99.6 ± 0.8	0.370	99.6 ± 0.8	0.391	99.6 ± 0.8	0.530
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
300 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0 ± 0	-	0 ± 0		12.4 ± 10.7		20.9 ± 15.2		23.9 ± 20.4	
idl6 3.5	0 ± 0	-	0.8 ± 1.2	0.272	19.1 ± 14.0	0.504	41.3 ± 14.8	0.136	51.7 ± 21.2	0.238
idl7 1.4	0 ± 0	-	0 ± 0	-	14.1 ± 7.5	0.826	32.1 ± 8.4	0.308	46.7 ± 9.4	0.250
idl6xidl7 1.9	0 ± 0	-	21.7 ± 21.2	0.148	59.2 ± 20.0	0.017	69.2 ± 15.3	0.022	71.7 ± 16.5	0.072
idl6xidl7 1.10	0 ± 0	-	0 ± 0	-	11.8 ± 1.7	0.925	20.4 ± 2.1	0.960	28.3 ± 0.0	0.789
35S:IDL6 1.3	0 ± 0	-	0 ± 0	-	15.0 ± 17.4	0.225	36.1 ± 19.2	0.814	34.2 ± 20.0	0.949
35S:IDL7 6.5	0 ± 0	-	0 ± 0	-	2.9 ± 2.5	0.177	20.4 ± 21.4	0.358	20.0 ± 14.2	0.714

Table A21 Student's t-test was used to evaluate the actual difference in development of green tissue between different transgenic lines of *A. thaliana* and Wt *A. thaliana* grown in the presence of different concentrations of mannitol. Average percentage of seedlings that developed green tissue ± standard deviation (SD) is given. P-values ≤ 0.050 indicate true difference between the respective lines and Wt *A. thaliana* with 95% accuracy. P-values ≤ 0.100 indicate difference with 90% certainty, all p-values ≤ 0.100 are underlined. P-values ≥ 0.100 indicate that there is no difference between the lines in respect to total number of individuals with green tissue at a given time point.

	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
0 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	73.5 ± 18.7		94.2 ± 0.6		96.5 ± 0.5		96.8 ± 0.9		97.0 ± 0.7	
idl6 3.5	83.1 ± 10.1	0.463	96.0 ± 2.7	0.242	98.0 ± 1.2	0.073	98.0 ± 1.2	0.181	98.3 ± 1.7	0.194
idl7 1.4	75.2 ± 16.2	0.908	97.5 ± 0.8	0.002	98.0 ± 1.2	0.073	98.6 ± 0.4	0.025	99.1 ± 0.8	0.013
idl6xidl7 1.9	90.8 ± 3.5	0.286	96.7 ± 0.0	0.006	98.3 ± 0.0	0.008	98.3 ± 0.0	0.082	98.3 ± 0.0	0.057
idl6xidl7 1.10	36.7 ± 23.5	0.068	75.4 ± 7.5	0.004	92.6 ± 5.1	0.172	95.9 ± 2.5	0.531	96.2 ± 2.6	0.586
35S:IDL6 1.3	70.8 ± 35.0	0.638	99.0 ± 1.3	0.000	99.6 ± 0.8	0.003	99.6 ± 0.8	0.013	99.6 ± 0.8	0.010
35S:IDL7 6.5	70.8 ± 41.0	0.628	98.8 ± 2.5	0.035	98.8 ± 2.5	0.261	98.8 ± 2.5	0.345	99.2 ± 1.7	0.119
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
100 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	50.2 ± 30.1		94.5 ± 1.7		96.6 ± 2.0		97.3 ± 1.4		97.3 ± 1.4	
idl6 3.5	56.8 ± 26.1	0.776	94.9 ± 1.8	0.760	99.4 ± 1.0	0.075	99.4 ± 1.0	0.064	99.4 ± 1.0	0.064
idl7 1.4	45.1 ± 27.8	0.828	98.1 ± 0.5	0.017	99.4 ± 1.0	0.075	100 ± 0.0	0.018	100 ± 0.0	0.018
idl6xidl7 1.9	80.8 ± 22.4	0.281	93.3 ± 7.1	0.751	95.0 ± 4.7	0.554	95.0 ± 4.7	0.379	95.0 ± 4.7	0.379
idl6xidl7 1.10	37.8 ± 19.5	0.564	73.6 ± 1.7	0.000	89.1 ± 4.6	0.029	93.1 ± 2.5	0.036	95.3 ± 0.6	0.073
35S:IDL6 1.3	17.5 ± 29.6	0.314	97.9 ± 1.6	0.055	98.7 ± 0.8	0.205	98.7 ± 0.8	0.237	98.7 ± 0.8	0.237
35S:IDL7 6.5	40.4 ± 45.9	0.408	97.5 ± 5.0	0.489	98.3 ± 3.3	0.622	98.8 ± 2.5	0.530	98.8 ± 2.5	0.530
	Day 2		**Day 4**		**Day 6**		**Day 8**		**Day 10**	
300 mM	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**	**% ± SD**	**p**
Wt	0 ± 0		10.8 ± 11.8		23.4 ± 15.5		34.9 ± 20.9		40.6 ± 28.0	
idl6 3.5	0 ± 0	-	19.2 ± 5.9	0.436	48.1 ± 15.1	0.088	59.2 ± 18.2	0.170	71.7 ± 18.9	0.271
idl7 1.4	0 ± 0	-	12.5 ± 13.0	0.890	39.8 ± 13.2	0.202	59.3 ± 14.0	0.144	72.5 ± 5.9	0.227
idl6xidl7 1.9	20.8 ± 29.5	0.286	65.0 ± 21.2	0.032	73.3 ± 14.1	0.019	77.5 ± 10.6	0.060	79.2 ± 10.6	0.172
idl6xidl7 1.10	0.3 ± 0.6	0.286	10.8 ± 3.5	1.000	23.7 ± 10.0	0.979	31.9 ± 12.2	0.834	40.8 ± 8.2	0.990
35S:IDL6 1.3	0 ± 0	-	1.7 ± 1.7	0.252	34.2 ± 20.9	0.777	46.7 ± 28.0	0.780	46.2 ± 31.6	0.829
35S:IDL7 6.5	0 ± 0	-	4.4 ± 6.3	0.454	29.6 ± 26.4	0.886	38.3 ± 29.0	0.852	35.6 ± 33.3	0.852

D 5 LinReg and REST output from analyses of qPCR data

All data obtained by qPCR was analyzed in LinReg and REST2009. The LinReg software was used to define Ct-values and reaction efficiency through linear regression and REST2009 software was used to calculate relative expression and statistical significance of difference between amplicons in sample groups and control groups.

D 5.1 Verification of *idl6xidl7* double knockout lines in *A. thaliana*

Table A22 Mean Ct-values from three replicas normalized in LinReg and reaction efficiency of qPCR of tissue from Wt *A. thaliana* and four different double knockout lines of *IDL6* and *IDL7*. *TIP41*-like and *PP2A* were used as reference genes.

Amplicon	Wt	*idl6xidl7 1.9*	*idl6xidl7 1.10*	*idl6xidl7 2.20*	*idl6xidl7 5.14*	Reaction efficiency
IDL6	25.43 ± 0.15	31.46 ± 0.19	29.90 ± 0.49	29.56 ± 1.56	24.48 ± 0.21	1.858
IDL7	24.40 ± 0.45	40.15 ± 0.32	61.02 ± 32.24	40.30 ± 0.53	39.01 ± 1.73	1.745
PP2A	24.70 ± 0.24	24.28 ± 0.05	25.06 ± 0.23	24.57 ± 0.03	24.44 ± 0.13	1.859
TIP41-like	24.48 ± 0.23	24.30 ± 0.05	25.14 ± 0.21	24.45 ± 0.08	24.52 ± 0.11	1.897

D 5.2 Expression analyses of UV treated tissue from Wt *A. thaliana*

Table A23 qPCR data of UV treated tissue from Wt *A. thaliana* analyzing expression of *IDL6* and *IDL7*. Tissue was harvested 0.5 h, 1 h, 3 h, 6 h and 24 h after 15 min treatment of UV-B light (365nm). Normalized crossing threshold (Ct) and reaction efficiency was calculated from fluorescence history by LinReg. Mean Ct of three replicas is presented together with relative expression (RE) and p-values from statistical analyses in REST2009. *HY5* and *PAD4* were used as control genes of stress response and *TIP41*-like and *PP2A* were used as reference genes.

Amplicon	Mean Ct ± SD (Control)	Mean Ct ± SD (UV treated)	Reaction efficiency	RE	RE standard error	p-values
			0.5 h after treatment			
IDL6	34.42 ± 2.4	33.07 ± 1.8	1.786	1.247	0.570 - 3.882	0.615
IDL7	30.07 ± 2.3	29.99 ± 0.9	1.708	0.607	0.303 - 1.159	0.292
HY5	26.07 ± 0.7	25.59 ± 0.6	1.801	0.757	0.422 - 1.055	0.618
PAD4	40.00 ± 0.0	40.00 ± 0.0	1.000	-	-	-
TIP41-like	25.55 ± 0.9	25.22 ± 0.3	1.798	0.686		
PP2A	26.45 ±1.9	24.84 ± 0.8	1.787	1.457		
			1 h after treatment			
IDL6	35.37 ± 1.5	30.85 ± 0.8	1.771	3.776	1.803 - 6.862	0
IDL7	31.86 ± 1.8	27.3 ± 1.0	1.733	3.551	2.724 - 4.411	0.033
HY5	26.81 ± 0.8	25.52 ± 0.1	1.831	0.621	0.420 - 0.895	0.158
PAD4	40.00 ± 0.0	40.00 ± 0.0	1.670	0.283	0.133 - 0.651	0.158
TIP41-like	26.38 ± 1.1	24.98 ± 0.3	1.807	0.635		
PP2A	28.04 ± 1.9	25.11 ± 0.7	1.807	1.574		
			3 h after treatment			
IDL6	29.95 ± 1.4	29.69 ± 1.4	1.870	1.12	0.881 - 1.492	0.547
IDL7	35.79 ± 1.9	34.51 ± 1.9	1.660	1.827	1.224 - 2.436	0.052
HY5	26.01 ± 0.7	25.93 ± 0.5	1.857	1	0.603 - 1.714	0.947
PAD4	40.00 ± 0.0	40.00 ± 0.0	1.745	0.953	0.418 - 2.935	0.931
TIP41-like	26.28 ± 1.1	26.03 ± 0.6	1.853	1.117		
PP2A	27.29 ± 1.7	27.39 ± 1.9	1.879	0.895		
			6 h after treatment			
IDL6	29.37 ± 0.4	29.02 ± 1.0	1.885	1.316	0.778 - 1.987	0.352
IDL7	35.18 ± 1.1	34.63 ± 1.3	1.678	1.396	0.753 - 2.999	0.428
HY5	25.41 ± 0.2	25.59 ± 0.2	1.877	0.937	0.889 - 0.983	0.184
PAD4	40.00 ± 0.0	40.00 ± 0.0	1.757	0.895	0.523 - 1.480	0.751
TIP41-like	25.83 ± 0.3	25.99 ± 0.3	1.882	1.052		
PP2A	26.65 ± 0.6	26.93 ± 1.1	1.889	0.95		
			24 h after treatment			
IDL6	31.44 ± 0.7	28.94 ± 0.4	1.885	3.522	2.730 - 4.941	0.037
IDL7	31.31 ± 1.6	27.86 ± 0.4	1.778	5.269	2.606 - 10.883	0.037
HY5	26.47 ± 0.1	26.21 ± 0.1	1.926	0.86	0.758 - 0.929	0
PAD4	40.00 ± 0.0	40.00 ± 0.0	1.780	0.724	0.594 - 0.831	0.03
TIP41-like	26.10 ± 0.1	25.74 ± 0.0	1.874	0.911		
PP2A	27.28 ± 0.4	26.63 ± 0.1	1.899	1.098		

D 5.3 Expression analysis of *promoter:GUS* lines in *A. thaliana*

Table A24 qPCR of 10 days old *pIDL6:GUS* and *pIDL7:GUS* lines grown on ½ x MS under normal conditions to investigate the relative expression of *IDL6, IDL7* and *UidA*. Reaction efficiency was calculated by linear regression in the exponential phase by LinReg software. *TIP41*-like and *PP2A* were used as reference genes.

pIDL6:GUS lines		*pIDL7:GUS* lines	
Amplicon	**Reaction efficiency**	**Amplicon**	**Reaction efficiency**
UidA	1.865	*UidA*	1.865
IDL6	1.846	*IDL7*	1.823
PP2A	1.857	*PP2A*	1.867
TIP41 like	1.853	*TIP41* like	1.859

Table A25 qPCR of 23 days old *pIDL6:GUS* and *pIDL7:GUS* lines grown on soil under normal conditions to investigate the relative expression of *IDL6, IDL7* and *UidA*. Reaction efficiency was calculated by linear regression in the exponential phase by LinReg software. *TIP41* like and *PP2A* were used as reference genes.

pIDL6:GUS lines		*pIDL7:GUS* lines	
Amplicon	**Reaction efficiency**	**Amplicon**	**Reaction efficiency**
UidA	1.879	*UidA*	1.877
IDL6	1.848	*IDL7*	1.818
PP2A	1.879	*PP2A*	1.854
TIP41-like	1.926	*TIP41*-like	1.912

Table A26 qPCR of 35 days old *pIDL6:GUS* and *pIDL7:GUS* lines grown on soil under normal conditions to investigate the relative expression of *IDL6, IDL7* and *UidA*. Reaction efficiency was calculated by linear regression in the exponential phase by LinReg software. *TIP41* like and *PP2A* were used as reference genes.

pIDL6:GUS lines		*pIDL7:GUS* lines	
Amplicon	**Reaction efficiency**	**Amplicon**	**Reaction efficiency**
UidA	1.871	*UidA*	1.886
IDL6	1.876	*IDL7*	1.850
PP2A	1.846	*PP2A*	1.880
TIP41-like	1.901	*TIP41*-like	1.926

D 5.4 Expression analysis of cycloheximide treated tissue

Table A27 qPCR of *pIDL6:GUS* and *pIDL7:GUS* lines treated with cycloheximide (CHX) in liquid ½ x MS for three hours were performed on 10 days old plants of *A. thaliana* to investigate the relative expression of *IDL6, IDL7* and *UidA*. Seedlings only embedded in liquid ½ x MS, and seedlings treated with DMSO were used as negative controls. Reaction efficiency was calculated by linear regression in the exponential phase by LinReg software. *TIP41*-like and *PP2A* were used as reference genes.

Amplicon	½ x MS	DMSO MOCK	CHX
UidA	1.875	1.893	1.881
IDL6	1.886	1.868	1.837
IDL7	1.803	1.791	1.810
TIP41-like	1.932	1.927	1.910
PP2A	1.887	1.867	1.844

Table A28 qPCR of *pIDL6:GUS* and *pIDL7:GUS* lines treated with cycloheximide (CHX) in liquid ½ x MS for three hours were performed on 10 days old plants of *A. thaliana* to investigate the relative expression of *IDL6, IDL7* and *UidA* compared to DMSO treated tissue as control group. Relative expression and statistical analysis were obtained by REST2009. REST2009 consider p-values ≤0.05 as statistically significant.

	UidA		*IDL6*		*IDL7*	
	Relative expression	REST p-value	Relative expression	REST p-value	Relative expression	REST p-value
pIDL6:GUS 4.5	1.812	0.058	301.202	0.046		
pIDL6:GUS 18.4	2.325	0.057	140.889	0.022		
pIDL7:GUS 3.2	3.985	0.039			179.108	0.037
pIDL7:GUS 20.4	0.908	0.815			180.642	0.001

Appendix E: Sequence alignments

MACAW sequence alignment of *IDL8* obtained from TOPO vector and the pDONR vector are displayed in Figure A4 and A5, respectively.

```
AT5G02600gen   atgaaagaaaaaaaattcccctgcattgtggttaaaatgtgaatagcttttatggcctaa  2100
IDL8cDNA       ------------------------------------------------------------     0
U-01           ------------------------------------------------------------   265

AT5G02600gen   ttgaaacgtgtttcttctgcttggttcctacggattggattggaacgtTTTGTTGGAATT  2160
IDL8cDNA       ------------------------------------------------------------     0
U-01           ------------------------------------------------TTTGTTGGAATT   277

AT5G02600gen   GGATCATTTTTAGTCAACATAGCTTTTATGAAGAAGAAAAATATGAATCAATTTACAAAA  2220
IDL8cDNA       ------------------------------------------------------------     0
U-01           GGATCATTTTTAGTCAACATAGCTTTTATGAAGAAGAAAAATATGAATCAATTTACAAAA   337

AT5G02600gen   TAATTTTGTTTACTACTAAAAAGAAAAATATTTATTGACAAGATAAAGACATCCAAAAGT  2280
IDL8cDNA       ------------------------------------------------------------     0
U-01           TAATTTTGTTTACTACTAAAAAGAAAAATATTTATTGACAAGATAAAGACATCCNAAAGT   397

AT5G02600gen   TTATATAAACCTTCCAAACCATATCAACTTGTGTCAGTGTGTACAACAAAGTAATATTGT  2340
IDL8cDNA       ----------------------------------CAGTGTGTACAACAAAGTATTATTGT    26
U-01           TTATATAAACCTTCCAAACCATATCAACTTGTGTCAGTGTGTACAACAAAGTAATATTGT   457

AT5G02600gen   GATGGCGAAATCAACTTATGTTCTTGTGGTAATTTCTTTTGGCCTCCTCTTCGCTTGTGT  2400
IDL8cDNA       GATGGCGAAATCAACTTATGTTCTTGTGGTAATTTCTTTTGGCCTCCTCTTCGCTTGTGT    86
U-01           GATGGCGAAATCAACTTATGTTCTTGTGGTAATTTCTTTTGGCCTCCTCTTCGCTTGTGT   517

AT5G02600gen   CATTGGAACCACTCAAGATGAGACCAGCCGTTTGCTCTGGTCTAGACCATGGGCTAGAGG  2460
IDL8cDNA       CATTGGAACCACTCAAGATGAGACCAGCCGTTTGCTCTGGTCTAGACCATGGGCTAGAGG   146
U-01           CATTGGAACCACTCAAGATGAGACCAGCCGTTTGCTCTGGTCTAGACCATGGGCTAGAGG   577

AT5G02600gen   ACTGGCTGATTCACCACCACAAGATCCACATAAACCAACCATCTTCGGCTTAAAACCATG  2520
IDL8cDNA       ACTGGCTGATTCACCACCACAAGATCCACATAAACCAACCATCTTCGGCTTAAAACCATG   206
U-01           ACTGGCTGATTCACCACCACAAGATCCACATAAACCAACCATCTTCGGCTTAAAACCATG   637

AT5G02600gen   GTCACCATCACAACGCCTGATTTTCAGAATGTTACCAAAAAACGTTCCAATCCCGCCATC  2580
IDL8cDNA       GTCACCATCACAACGCCTGATTTTCAGAATGTTACCAAAAAACGTTCCAATCCCGCCATC   266
U-01           GTCACCATCACAACGCCTGATTTTCAGAATGTTACCAAAAAACGTTCCAATCCCGCCATC   697

AT5G02600gen   GGGACCAAGCCGAAAAGAAACTCCTCCATCTCCGCCTAGATCCGTATGATACTCGTTAAA  2640
IDL8cDNA       GGGACCAAGCCGAAAAGAAACTCCTCCATCTCCGCCTAGATCCGTATGATACTCGTTAAA   326
U-01           GGGACCAAGCCGAAAAGAAACTCCTCCATCTCCGCCTAGATCCGTATGATACTCGTTAAA   757

AT5G02600gen   CTTGCTGTAGTAAGGATCGCTTATTTATAATTCAATTCTACATGTTGTAAAATTTGGTAT  2700
IDL8cDNA       CTTGCTGTAGTAAGGATCGCTTATTTATAATTCAATTCTACATGTTGTAAAATTTGGTAT   386
U-01           CTTGCTGTAGTAAGGATCGCTTATTTATNATTCAATTCTACATGTNGTAAAATTTGGTAT   817

AT5G02600gen   GTGAATCTGATGCAAAGCTTGTAAAATGGTAGTAGAATAATTTCG---------------  2745
IDL8cDNA       GTGAATCTGATGCAAAGCTTGTAAAATGGTAGTAGAATAATTTCGcagtgatcacaaata   446
U-01           GTGAATCTGATGCNNNGCTTGNNAgggcgaattccagcncactggcggccgttncnnntg   877

AT5G02600gen   ------------------------------------------------------------  2745
IDL8cDNA       aaagttcaaagtcacatttctaaacaaaaaa-----------------------------   477
U-01           gatccgagctcggnaccnagcttggcgtannnnnggncatagctgtttcctgtgtgaaat   937
```

Figure A4 Sequence alignments of *IDL8* coding sequence and upstream promoter sequence against the postulated mutated *idl8* (U-01) obtained from TOPO vector show that the vector encompassed the *IDL8* gene without any shifts or mutations, however the T-DNA insert may be positioned in the promoter region about 150 bp upstream of coding sequence.

```
IDL8cDNA        ------------------------------------------------------------     0
U-05            gnnnannnnagtcttagctcggnnnnnataatgattttattttgactganagtgaccggt    60

IDL8cDNA        ------------------------------------------------------------     0
U-05            tggttgcagnaaanggacgtccaatgcggttttataatgccagctttgtacaaaaaagca   120

IDL8cDNA        ------ATGGCGAAATCAACTTATGTTCTTGTGGTAATTTCTTTTGGCCTCCTCTTCGCT    54
U-05            gggtttATGGCGAAATCAACTTATGTTCTTGTGGTAATTTCTTTTGGCCTCCTCTTCGCT   180

IDL8cDNA        TGTGTCATTGGAACCACTCAAGATGAGACCAGCCGTTTGCTCTGGTCTAGACCATGGGCT   114
U-05            TGTGTCATTGGAACCACTCAAGATGAGACCAGCCGTTTGCTCTGGTCTAGACCATGGGCT   240

IDL8cDNA        AGAGGACTGGCTGATTCACCACCACAAGATCCACATAAACCAACCATCTTCGGCTTAAAA   174
U-05            AGAGGACTGGCTGATTCACCACCACAAGATCCACATAAACCAACCATCTTCGGCTTAAAA   300

IDL8cDNA        CCATGGTCACCATCACAACGCCTGATTTTCAGAATGTTACCAAAAAACGTTCCAATCCCG   234
U-05            CCATGGTCACCATCACAACGCCTGATTTTCAGAATGTTACCAAAAAACGTTCCAATCCCG   360

IDL8cDNA        CCATCGGGACCAAGCCGAAAAGAAACTCCTCCATCTCCGCCTAGATCCGTATGA------   288
U-05            CCATCGGGACCAAGCCGAAAAGAAACTCCTCCATCTCCGCCTAGATCCGTATGAgaccca   420

IDL8cDNA        ------------------------------------------------------------   288
U-05            gctttcttgtacaaagttggcattataagaaagcattgcttatcaatttgttgcaacgaa   480

IDL8cDNA        ------------------------------------------------------------   288
U-05            caggtcactatcagtcaaaataaaatcattatttggggcccgngcganagaccggncgnn   540

IDL8cDNA        ------------------------------------------------------------   288
U-05            gnntnnnatngnngggncnggnnnnnnncnggngancnctggcccgtgtcnnnaaatcnc   600

IDL8cDNA        ---------------------------------                              288
U-05            tgatgttacattgcacaananaaaaatatatnn                              633
```

Figure A5 **Sequence alignments of *IDL8* coding sequence against *IDL8* (U-05) obtained from pDONR vector show that the pDONR vector encompassed the *IDL8* gene without any shifts or mutations.**