# Ligand-receptor matchmaking: Signaling of the IDL proteins through the LRR-RLKs of the HAESA family

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# ABSTRACT

It was long believed that most cell to cell communication in plants occurred by non-peptide plant hormones. Peptide signaling in plants is a recent discovery that opens a new world of signaling in plants. One signaling peptide is IDA, INFLORESCENCE DEFICIENT IN ABSCISSION. It is believed to signal through the receptor-like kinases HAESA (HAE) and HAESA-LIKE2 (HSL2), mediating the floral organ abscission process in *Arabidopis*. A novel group of putative ligands in *Arabidopsis*, the IDA-LIKE (IDL) proteins was identified based on their similarities to IDA. They are thought to signal through receptors closely related to HAE, the HAESA-LIKE (HSL) proteins, where they regulate different cell separation processes. In this thesis it has been made an effort to identify novel putative receptor-ligand interactions by matching the expression pattern of *IDL* genes and *HSL* genes. The interaction between IDA's close relative IDL1 and IDA's native receptors, as well as a putative native receptor for IDL1, HSL1, has been investigated using a yeast two-hybrid assay. Furthermore, a genetic approach was used to investigate the interaction between IDL1, IDL2 and IDL3, and HAE/HSL2 in the floral organ AZ, and finally the roles of IDL1, HAE, HSL1 and HSL2 in root development were investigated by mutant studies.

Based on the overlapping expression pattern of promoter::reporter gene constructs, several novel putative ligand-receptor pairs were identified in this thesis. No direct, biochemical interaction between IDL1 and HAE, HSL1 or HSL2 could be identified. A genetic approach, however, revealed that IDL1, IDL2 and IDL3 were able to signal through HAE and HSL2 when expressed in the floral organ AZ. Preliminary results also indicate that IDL1 might signal through HSL1 and HSL2 in the root cap.

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# 1 INTRODUCTION

## 1.1 *Arabidopsis thaliana* as a model organism

*Arabidopsis thaliana* (*Arabidopsis*) is a small flowering dicoyledonous plant belonging to the *Brassicaceae* (mustard weed) family. This small plant has over the past 20 years become an excellent model for studying plant biology. *Arabidopsis* has several advantages as a model organism. It is small, requires simple growth conditions and has a short life cycle of ~7 weeks, thus making it easy to grow under laboratory conditions. The plant self-fertilizes and each plant produces thousands of seeds. *Arabidopsis* has the smallest genome with fewer repetitive sequences than any other known higher plant; 146 Mb (million base pairs) arranged into five chromosomes that contain ~26200 protein coding genes (Somerville and Koornneef, 2002; Bevan and Walsh, 2005). All of the features mentioned above make *Arabidopsis* the model system of choice for classical and molecular plant genetics, as well as for studying plant development, physiology and pathology (Page and Grossniklaus, 2002; Somerville and Koornneef, 2002). Sequencing of the *Arabidopsis* genome of the Columbia (Col) ecotype was completed at the end of year 2000 (The-Arabidopsis-Genome-Initiative, 2000) as the first plant genome sequenced. A collection of characterized mutations and transgenic plants is available, where genes involved in nearly every major biochemical pathway have been knocked out (Somerville and Koornneef, 2002).

### 1.1.1 T-DNA insertional mutagenesis

A key resource for studying the gene functions of *Arabidopsis* is the use of insertional mutagenesis. Common techniques includes the use of *Zea mays* transposable elements (Fedoroff, 1989) and *Agrobacterium tumefaciens* T-DNA (Koncz *et al.*, 1992; Azpiroz-Leehan and Feldmann, 1997). For this thesis T-DNA insertional mutants were used and novel gene constructs were introduced in *Arabidopsis* by *Agrobacterium* transformation. The T-DNA insertional mutagenesis techniques utilizes a portion of the tumor inducing plasmid from *A. tumefaciens*, which natural function is to induce crown galls by transferring T-DNA

into the nucleus of plant cells. When a plant is infected, T-DNA is transferred into the host cell and inserted into the nuclear genome (Binns, 2002)

SALK lines are T-DNA insertion lines generated by *A. tumefaciens* transformation of plants with the vector pBIN-pROK2. The lines are distributed by the Arabidopsis Biological Resource Center (ABRC) and the Nottingham Arabidopsis Stock Center (NASC). The T-DNA insertion sites are identified by the Salk Institute Genome Analysis Laboratory (SIGnAL) (Alonso *et al.*, 2003), and are available in the SIGnAL database using the *Arabidopsis* gene mapping tool, T-DNA Express. As the identification of insertion sites are high throughput operations the exact insertion sites have to be confirmed by sequencing the genomic region flanking the left border (LB) of the T-DNA.

### 1.1.2 Reporter gene systems

It is possible to investigate a gene's function by examining when and where the gene is expressed, both in the cell and in the entire organism. This is done by cloning the promoter region of the gene of interest in front of a reporter gene. The reporter gene can be a fluorescing protein or an enzyme, whose activity can easily be monitored. Two such reporter gene systems are the GUS (*β-glucuronidase*) system and the YFP system.

#### 1.1.2.1 The GUS reporter gene system

The *β-glucuronidase* (*gusA*) gene is a frequently used reporter gene in genetically modified plants. This gene was first isolated from *Escherichia coli* and encoded the GUS enzyme, which catalyses the hydrolysis of several different glucuronides (Jefferson, 1989). This ability is utilized when the gene is used as a reporter gene to study and monitor gene expression, mainly the tissue specificity of promoter sequences. It splits the histochemical substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) into a blue end-product, staining the tissue blue and hence visualizing the activity of the gene of interest. GUS is absent in many organisms other than vertebrates, and this is a major advantage, making it possible to visualize small quantities of GUS activity without having to consider background signaling (Jefferson, 1989). When fused to a promoter, the promoter will regulate the expression of the

*gusA* gene, and *gusA* will adopt the expression pattern of the gene originally regulated by the promoter.

#### 1.1.2.2 The YFP reporter gene system

After the discovery of green fluorescent protein (GFP) (Shimomura *et al.*, 1962; Morin and Hastings, 1971; Morise *et al.*, 1974), a variety of fluorescing proteins have been discovered that can function as reporter-genes, including red fluorescent protein (Matz *et al.*, 1999) and yellow fluorescent protein (YFP) (Macheroux *et al.*, 1987). The gene encoding YFP was first isolated from *Vibrio fischeri* (Macheroux *et al.*, 1987). When cloning the promoter of the gene of interest in front of YFP, the YFP gene will adopt the expression pattern of the gene of interest. An advantage with using YFP, like GFP, is that it only requires blue light, so that availability of substrates is not a limiting factor. Another advantage is that it is possible to visualize the YFP expression in live plants. This makes it a good choice for monitoring gene expression.

## 1.2 Cell separation in plants

Plant cells are joined together by an adhesive matrix that cements the cells together. However several events in a plants life cycle are dependent on breakdown of this adhesion between the cells. The loss of adhesion is accomplished by the process of cell separation, as a part of the programmed development of the plant or as a response to environmental stress (Taylor and Whitelaw, 2001). Cell separation facilitates penetration of the primary root through the soil, lateral root emergence, expansion of cotyledons and leaves, release of pollen from the anthers and softening of fruit, as well as shedding of flowers or floral organs (Roberts *et al.*, 2002) (figure 1.1). Common to all these processes is the degradation of the cell wall.

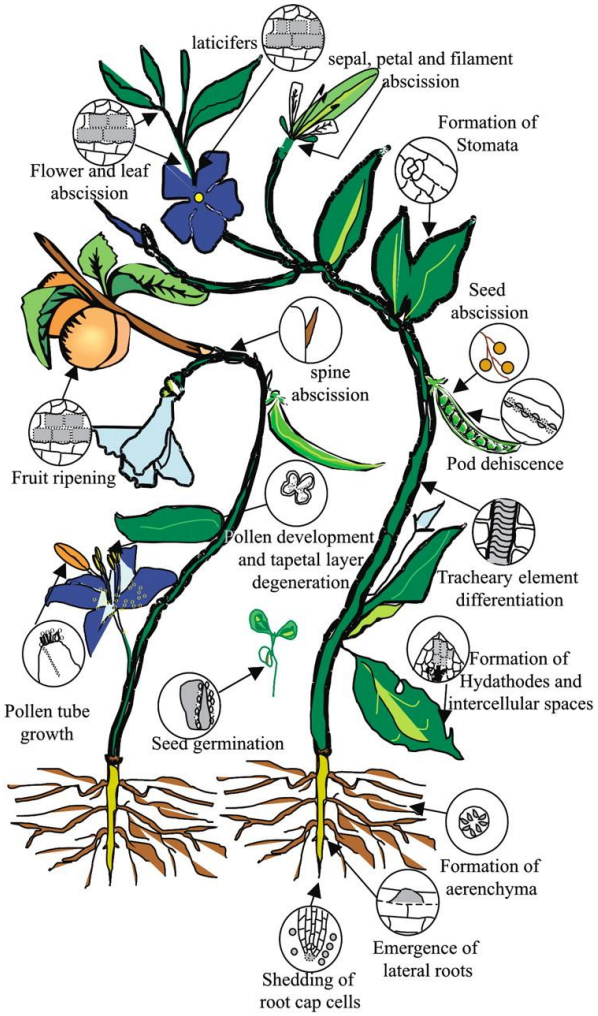


**Figure 1.1 Sites of cell separation** (Roberts *et al.*, 2002).

### 1.2.1 Abscission

Abscission is a developmentally determined program of cell separation that results in the shedding of organs. Entire organs are shed to secure dispersal or propagation, aid pollination, as a defense mechanism against pathogens or, when the organ no longer serves a function or is damaged (Patterson, 2001). The sites of abscission, termed abscission zones (AZs), are normally highly predictable (Taylor and Whitelaw, 2001) and AZs are often located in stems between the organ to be abscised and the body of the plant (Bleecker and Patterson, 1997; Patterson, 2001). It has long been recognized that the timing of the abscission process is determined by the balance between the plant hormones ethylene and auxin, where ethylene

has been recognized as the inducing agent and auxin as the break (Taylor and Whitelaw, 2001). When exposed to the appropriate stimulus, the cells in the AZ enlarge and the middle lamella dissolves (Bleecker and Patterson, 1997). After shedding of the organ a continued enlargement of the AZ cells and differentiation of a protective layer follow (Bleecker and Patterson, 1997; Patterson, 2001).

*Arabidopsis* does not display leaf or fruit abscission, but it does abscise floral organs and seeds. Several *Arabidopsis* genes have been shown to be involved in the abscission of floral organs. Of interest for this thesis is the gene *IDA*, that encodes a small putative peptide ligand involved in the control of floral organ abscission (Butenko *et al.*, 2003). The *ida* mutant was first characterized in 2003 (Butenko *et al.*, 2003). The mutant shows no floral organ abscission; hence the sepals, petals and stamens remains attached to the plant body, and the affected gene was named *INFLORESCENCE DEFICIENT IN ABSCISSION*. The IDA protein is necessary for abscission of floral organs in *Arabidopsis*. Over-expression of the IDA protein results in earlier abscission and ectopic expression leads to abscission of organs that are not normally shed (Stenvik *et al.*, 2006). The double mutant *haehsl2* displays the same abscission-defective phenotype as the *ida*-mutant, indicating that the receptor-like kinases HAESA (HAE) and HAESA-LIKE 2 (HSL2) of the HAESA family of LRR-RLKs also are involved in the regulation of floral organ abscission in *Arabidopsis* (Cho *et al.*, 2008).

### 1.2.2 Sloughing

Sloughing is a programmed cell-to-cell separation process that takes place in the outer layers of the root cap and results in the shedding of live cells (del Campillo *et al.*, 2004). Shedding of the root cap is a process similar to abscission in that it involves the activity of cellulases and pectolytic enzymes (Uheda *et al.*, 1997). The root cap serves as a protective layer in front of the root meristem, and shields it against damage from soil particles (Bengough and McKenzie, 1997). The living cells are continuously shed from the root tip while secreting a slimy mucilage, thus creating a sheath decreasing the friction at the soil-root interface (Bengough and McKenzie, 1997; Roberts *et al.*, 2002). This facilitates rapid growth in compacted soils (Iijima *et al.*, 2003) and serves as a barrier against pathogen attack (Vicre *et al.*, 2005). *Promoter::GUS* expression of *IDL1* (*IDA-LIKE1*), a close relative of *IDA*, has

been shown to have a strong expression in the columella cap of the primary root, and is proposed to have a function in this cell-separation process (Stenvik *et al.*, 2008).

## 1.3 Signaling in plants

Cell-to-cell interactions are in general essential for differentiation, organization and function of most organ systems. How cells in multi-cellular organisms communicate is therefore a central question in biology. Responses, like differentiation, growth and development, are strictly regulated and coordinated through signaling between cells. Intracellular communication in plants was for many years explained on the basis of signaling by the five non-peptide plant-hormones: auxin; cytokinin, ethylene, abscisic acid (Kende and Zeevaart, 1997) and brassinolides (Mandava, 1988). Later it has become clear that plant cell communication also makes use of small peptide signals and specific receptors (Matsubayashi, 2003; Ryan *et al.*, 2007). To date, only a few ligand-receptor pairs have been identified in plants, but the number is increasing (Butenko *et al.*, 2009).

### 1.3.1 The receptor-like kinases

Representing almost 2.5 % of the plant's protein coding genes one of the largest gene families in the *Arabidopsis* genome is the receptor-like kinases (RLKs) with its 625 members (Shiu and Bleecker, 2001a, 2001b, 2003). Characteristic of the RLKs are an N-terminal signal peptide (SP), a ligand binding extracellular domain (ECD) and a cytoplasmic, C-terminal serine/threonine domain (Walker, 1994; Torii, 2000). The RLK gene family can further be divided into 44 subfamilies, based on their kinase domains (Shiu and Bleecker, 2001b). A fraction of these are lacking the ECD, and are referred to as receptor-like cytoplasmic kinases (RLCKs). RLKs possessing an extracellular domain are thought to function in the cell membrane, where they recognize extracellular ligands, following an activation of the intracellular domain and the subsequent transduction of a downstream signaling pathway (Torii, 2004). The ECD varies greatly in the RLKs and they have been shown to participate in protein-protein interactions, binding of carbohydrate substrates, including plant and microbial cell-wall components, glycoproteins or steroids (Shiu and Bleecker, 2001b).

The RLKs with leucine-rich repeat (LRR) ECDs are most frequent in *Arabidopsis* with 216 genes (Shiu and Bleecker, 2001b). LRR domains are involved in protein-protein or protein-peptide recognition processes (Kobe and Deisenhofer, 1994), and members of the LRR-RLK subfamily have been found to regulate various developmental processes, phytohormone perception and defense responses (Shiu and Bleecker, 2001a). Developmental regulators in *Arabidopsis* include the proteins ERECTA (specifies organ shape) (Torii *et al.*, 1996), CLAVATA1 (controls meristem cell fate) (Clark *et al.*, 1997) and HAESA (HAE) (involved in floral organ abscission) (Jinn *et al.*, 2000; Cho *et al.*, 2008).

### 1.3.2 Signaling peptides in plants

In contrast to the RLKs, very few ligands have been identified. A reason why so few functional signal peptides have been identified to date might be the small size of the peptide molecules and their complementary DNA (cDNA). Small cDNAs are often not represented in cDNA libraries and peptide gene tagging by insertional mutagenesis is often not an option. From a bioinformatic perspective these small signal peptides (many less than 100 amino acids long) are difficult to discover, as the programs used to search for putative peptides are set to a minimum of 100 amino acids). However, more and more signaling peptides are identified. Many of these putative ligands are thought to interact with a receptor and trigger a downstream signaling pathway (e.g. the MAPK pathway) (Shiu and Bleecker, 2001a).

The putative ligands SCR (S-LOCUS CYSTEINE-RICH) (Schopfer *et al.*, 1999), PSK (PHYTOSULFOKINE) (Yang *et al.*, 2001), NCR (NODULE-SPECIFIC CYSTEINE RICH) (Mergaert *et al.*, 2003) and IDA (INFLORESCENCE DEFICIENT IN ABSCISSION) (Butenko *et al.*, 2003) (see section 1.4.1) all have an N-terminal signal sequence. The signal sequence is thought to act as a signal for transport through a secretory pathway to the extracellular space. Proteolytic processing is a common way of activating signaling peptides. These peptides are therefore though to be processed into smaller active peptides, which may interact with the LRR-domain of the LRR-RLK receptor (Butenko *et al.*, 2009).

## 1.4 Ligand-receptor pairs

Due to few loss-of-function mutants for the small signal peptides is that it has been difficult to identify ligand-receptor pairs in plants. However, eight ligand-receptor systems has been identified in plants so far, where the ligand-receptor interaction has been confirmed by either genetic or biochemical evidence (Butenko *et al.*, 2009).

As a response to wounding, Tomato systemin is released and recognized by SR160, a typical LRR-RLK (Scheer and Ryan, 2002). AtPEP1 is also involved in defense, amplifying innate defense responses upon interaction with the LRR-RLK PEPR1 (PEP RECEPTOR 1) (Huffaker *et al.*, 2006; Huffaker and Ryan, 2007). Other systems regulate cellular proliferation and differentiation, such as PSK1 (PHYTOSULFOKINE 1) that interacts with PSKR (PSK RECEPTOR 1) (Yang *et al.*, 2001) and PSY1 that interacts through the LRR-RLK At1g72300 (Amano *et al.*, 2007). The peptide ligand SCR (S-LOCUS CYSTEINE-RICH) induces self-incompatibility response upon binding of the receptor SRK (S-LOCUS RECEPTOR KINASE) (Vanoosthuyse *et al.*, 2001; Mishima *et al.*, 2003) whereas tapetum TAPETUM DETERMINANT (TPD1) binds MICROSPOROCYTES 1 (EMS1) to determine the cell fate in anthers (Yang *et al.*, 2003). The CLAVATA system consists of the extracellular peptide CLV3 and a receptor complex consisting of CLV1, CLV2 (Jeong *et al.*, 1999) and CRN (CORYNE) (Muller *et al.*, 2008). The LRR-RLK CLV2 is structurally similar to CLV1, but is lacking an intracellular kinase domain. To compensate for the lacking kinase domain in CLV2, the heterodimer constitutes a functional unit with the extracellular LRR domains of CLV1 and CLV2 and the intracellular domain of CRN (Muller *et al.*, 2008). Biochemical evidence support the interaction of CLV3 and the extracellular domain of CLV1 in a recent publication (Ogawa *et al.*, 2008) and it is clear that CLV3 together with the CLV1-CLV2-CRN receptor complex plays an important role in maintaining the plant meristem, regulating the balance between meristem stem cell proliferation and differentiation (Matsubayashi, 2003). The IDA-HAE-HSL2 system is the latest ligand-receptor pair to be identified in plants (Butenko *et al.*, 2009). The peptide IDA is signaling through the LRR-RLKs HAE and HSL2 to regulate floral abscission in *Arabidopsis* (Cho *et al.*, 2008; Stenvik *et al.*, 2008).

### 1.4.1 IDA and the IDA-likes – a family of peptide ligands

The *IDA* gene encodes a small protein of 77 amino acids with an N-terminal signal peptide of 26 amino acids. C-terminally you find the extended PIP domain (EPIP), which is thought to encompass the active peptide (Stenvik *et al.*, 2008). As mentioned above the protein is involved in the cell separation process of floral organ abscission, and has been localized to the extracellular space using an onion epidermis assay (Butenko *et al.*, 2003).

*IDA* and the *IDA-LIKE* genes constitute a family of 6 members in *Arabidopsis*, all encoding proteins with less than 100 amino acids (Butenko *et al.*, 2003). They are expressed in different tissues where cell separation events take place (Stenvik *et al.*, 2008). Common for the IDA and IDL proteins is an N-terminal hydrophobic signal peptide, a variable region and a 20 amino acid conserved C-terminal EPIP motif (Stenvik *et al.*, 2008). The function of the variable region is not clear, but might assist the IDL EPIP-C domain in binding the receptor (Stenvik *et al.*, 2008) (figure 1.2).

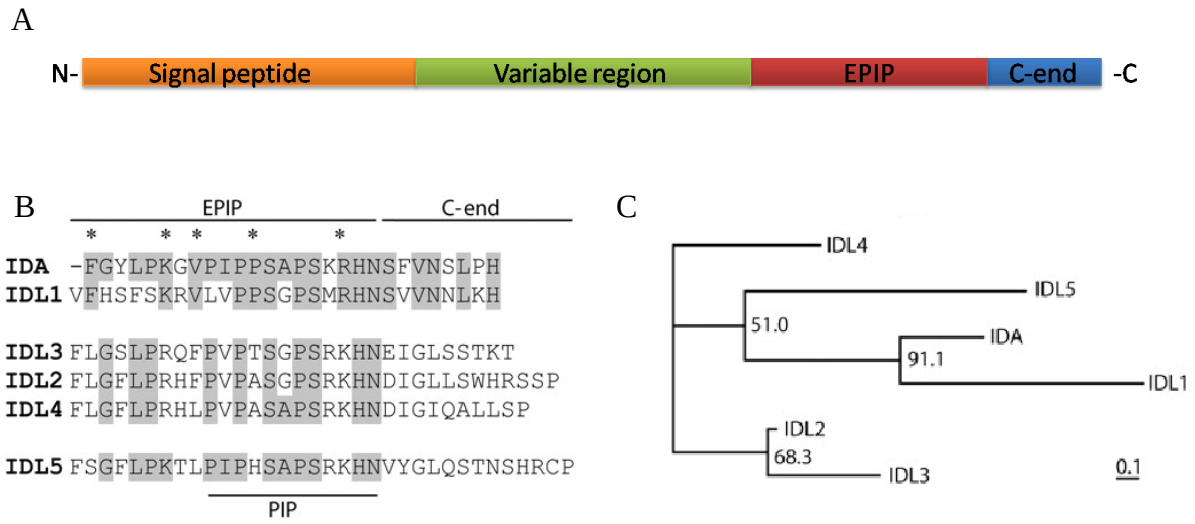

**Figure 1.2 IDA and the IDLs.** (A) A schematic view of the IDA and IDL proteins. (B) Alignment of the C-terminus of IDA and the IDL proteins. IDL1 is the one most similar to IDA, and most able to substitute for IDAs function. The middle cluster partially substitutes for IDA, and IDL5 at the bottom does not substitute for IDA. Amino acids in the EPIP-Cs of IDLs identical to the IDA sequence are shaded grey; an asterisk indicates the EPIP residues common to IDA and IDL1, but not any of the other IDLs. (C) A phylogenetic tree illustrating the relationship between IDA and the IDL proteins. The tree was constructed using maximum likelihood analysis after alignment of the full-length protein sequences. Bootstrap values are indicated as percentages. (Stenvik *et al.*, 2008)

The *IDL* genes differ in expression pattern, but over-expression of the genes result in phenotypes similar to the over-expression of *IDA*, indicating that the IDL proteins are able to initiate the same cellular response as IDA, early abscission (Butenko *et al.*, 2003; Stenvik *et al.*, 2006; Stenvik *et al.*, 2008). Therefore it is thought that they might function through common mechanisms when expressed in the floral organ AZ, as well as when expressed in their native positions. HAE and HSL2 are proposed to be the receptors of IDA in the floral organ AZ (Stenvik *et al.*, 2008) and IDL1, the closest relative of IDA (Butenko *et al.*, 2003), is thought to be able to act through the receptors of IDA in the AZ (Stenvik *et al.*, 2008). The phenotypic similarities resulting from over-expression of the *IDL* genes suggest that the receptors of the IDL proteins most likely are those closely related to HAE and HSL2, thus indicating that the HSL proteins might be the receptors of the IDL peptides.

### 1.4.2 HAESA and the HAESA-likes

HAE and HSL2 are members of a family of LRR kinases (Shiu and Bleecker, 2001a). They are plasma membrane serine/threonine protein kinases expressed at the base of petioles, base of pedicels and the floral organ AZ (Jinn *et al.*, 2000; Cho *et al.*, 2008). They are thought to either homodimerize or heterodimerize with each other and the HAE/HSL2 complex is proposed to be the receptor complex of IDA (Cho *et al.*, 2008; Stenvik *et al.*, 2008). Both the *ida*- and the *haehsl2*-mutants lack floral organ abscission (Butenko *et al.*, 2003; Cho *et al.*, 2008; Stenvik *et al.*, 2008), indicating *that IDA*, *HAE* and *HSL2* could be involved in the same pathway. A further indication that these genes are in the same pathway is that the over-expression phenotype of *IDA* is lost in the *haehsl2* background (Stenvik *et al.*, 2008).

IDL1 and the IDL1 EPIP, expressed by *IDA's* promoter, have been shown to rescue the *ida* phenotype (Stenvik *et al.*, 2008), probably by interacting with the receptors of IDA. It is therefore possible that the receptors of IDL1 are closely related to the receptors of IDA..The

closest relative of *HAE* is *HSL1*. Based on a previous microarray experiment (Birnbaum *et al.*, 2003) and publicly available expression data, it is hypothesized that HSL1 could be the receptor of IDL1 in the root cap. In addition to HSL1 and HSL2 other relatives of HAE (figure 1.3) might also be involved in cell separation pathways, such as IKU2L2. In this thesis IKU2L2 was investigated for a possible function.

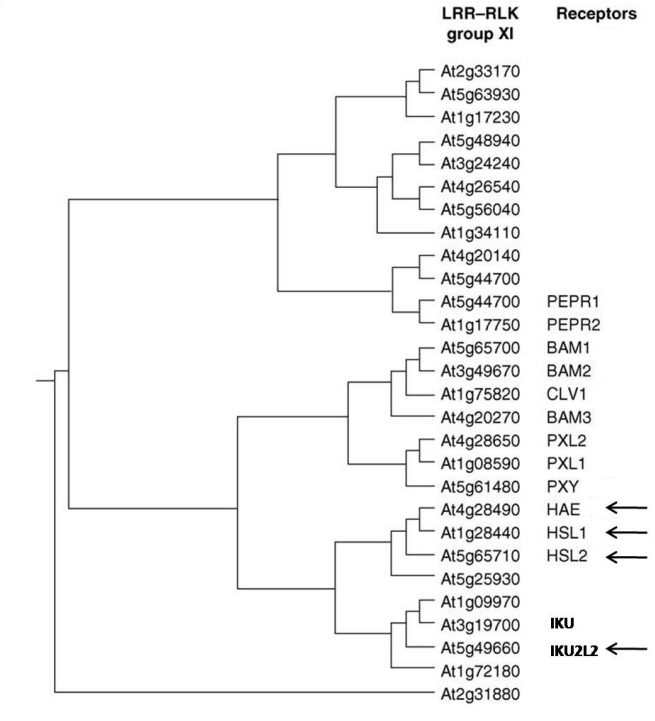


**Figure 1.3 The LRR-RLK group XI of proteins identified in *Arabidopsis.*** The figure shows the alignment of the full length amino acid sequence of the 28-member subfamily of LRR-RLKs XI proteins. Names are given for receptors with known biological function (except IKU2L2). Arrows indicate LRR-RLKs of most relevance to this thesis. (Butenko *et al.*, 2009)

### 1.4.3 Aim of Study

This study is a part of a larger project, where the goal is to characterize the five *IDL* genes and proteins and their putative target receptors of the LRR-RLK group XI, including HAE and its close relatives. The aim of this study was to investigate potential overlapping expression patterns of the LRR-RLKs *HSL1* and *IKU2L2* with the putative peptide ligands of the *IDA* and *IDL* family. Furthermore, since IDL1 rescues *the* ida mutant it was of interest to investigate the putative physical interaction between IDL1 and IDA's receptors HAE/HSL2. In addition, the physical interactin between IDL1 and HSL1, a proposed receptor candidate

for IDL1, was investigated. The interaction between the IDL proteins and HAE/HSL2 in the floral organ abscission zone was investigated using a genetic approach. A subsidiary goal of this thesis was to nvestigate the roles of the putative ligand-receptor pairs in different cell-separation processes. By studying possible root phenotypes in plants over-expressing *IDL1*, both in wild type background and *haehsl2* background, and a SALK line for *HSL1*, the role of IDL1, HAE, HSL1 and HSL2 in root development was investigated.

# 2 MATERIALS AND METHODS

## 2.1 Plant studies

### 2.1.1 Surface sterilization and growth conditions

Seeds were surface sterilized using 70 % ethanol for 5 min, bleached in 20 % chlorine in 0.1% Tween20 for 5 min and then washed in 0.001 % Tween20 for 5 min. 0.1 % agar was added to the seeds before plating on MS medium (Murashige, 1962), supplemented with 2 % sucrose (MS-2). For segregation analysis, selections of transformed lines, and genetically modified lines, either kanamycin (Km) (50 mg/l) or hygromycin (Hyg) (25 µg/ml), dependent on the construct used, was added to the medium. Plated seeds were cold treated at 4 °C for 18-32 hours and then transferred to growth chambers and cultivated at 18 °C, 8h dark and 16h light. After two weeks the seedlings were transferred to soil and further cultivated under the same conditions.

For segregation analysis T2 seedlings were scored for antibiotic resistance or sensitivity two weeks after germination. Seedlings that did not develop past the dicotylouse stage were considered to be antibiotic sensitive.

### 2.1.2 Transformation of *Arabidopsis thaliana* by floral dipping

The floral dipping method is based on the ability of *A. tumefaciens* to randomly integrate T-DNA, from its pTi plasmid, into the *Arabidopsis* genome (Bechtold *et al.*, 1993). The method is modified by Clough and Bent (Clough and Bent, 1998).

#### 2.1.2.1 Plant growth

*Arabidopsis* ecotypes Col and C24 were grown to flowering stage. To obtain more flowering buds, inflorescences were clipped; this encourages proliferation of numerous secondary bolts. Plants were transformed four to six days after clipping.

#### 2.1.2.2 Culturing of *A. tumefaciens* and transforming of plants

A T-DNA vector with the gene of interest was transformed into the *A. tumefaciens* strain C58 pGV2260. Bacteria were grown in YEB-medium, containing the appropriate antibiotics for

selection, at 28 °C to the stationary phase ($OD_{600}$ ~1.2). Cells were harvested by centrifugation for 10 min at room temperature at 5000 rpm and then resuspended in 5% sucrose solution (made fresh) to the final $OD_{600}$ of 0.8. Before dipping, Silwet L-77 was added to a concentration of 0.005%. The above-ground parts of the plants were inverted in the *Agrobacterium* solution for 30 seconds. Then the plants were transferred to a tray with moist paper, and covered with transparent plastic to maintain humidity. The plants were placed in a dark room ON and returned to the growth chamber the next day. Plants were grown for 4-6 weeks before seeds were harvested 1-2 times.

For selection of transformants MS-2 plates with carbenicillin and rifampicin in addition to either kanamycin or spectinomycin, depending on the vector, was used.

### 2.1.3 Histochemical GUS analysis

Plant tissue was prefixed in 90% cold acetone for 15 minutes, rinsed in staining buffer (50 mM $NaPO_4$ (pH 7,2), 2 mM $K_4Fe(CN)_6$, 2 mM $K_3Fe(CN)_6$, 0.1% Triton X-100) for 10 minutes, and incubated in staining buffer with 2 mM X-gluc substrate at 37°C for 30 minutes-20 hours. When investigated the stained tissue was rinsed in a graded ethanol (EtOH) series (15%, 35%, 50% EtOH in 50 mM $NaPO_4$), 10 minutes each. Post fixation was done by incubating the samples 30 minutes on ice in a 10:7:2:1 solution of 96% EtOH, $dH_2O$, 100% acetic acid and 37% formaldehyde. The tissue was then rehydrated in the reverse graded EtOH series.

Tissue was stored at 4°C in 50 mM $NaPO_4$. The material was mounted on microscope slides in clearing solution (8:2:1 chloral hydrate:water:glycerol) (Grini *et al.*, 2002) and incubated for minimum one hour at 4°C before inspection. The samples were investigated using a Zeiss Axioplan 2 imaging microscope.

### 2.1.4 Subcellular localization of promoter::YFP constructs

The subcellular localization of the promoter::YFP constructs were investigated using a Nikon SMZ800 stereomicroscope and a Leica TCS SP5 confocal microscope.

## 2.2 Working with bacteria

### 2.2.1 Growth and storage of bacteria

For permanent storage of all cultures, 1 ml culture containing 8% glycerol was made and stored at -80 °C.

#### *2.2.1.1 E. coli*

*E. coli* cultures were grown in LB-medium (10g/l Bacto tryptone, 5g/l Bacto yeast extract, 0.17M NaCl) at 37 °C with shaking. *E. coli* cells were plated onto LA-plates (LB-medium containing 15g agar per liter) to obtain single colonies.

- One Shot ® TOP 10 chemically competent cells (Invitrogen)
  For Gateway and TOPO cloning TOP10 cells were used. This strain does not contain the F'-episome which contains the *ccdA* gene. The *ccdA* gene is an antidote to the *ccdB* gene toxicity, and will prevent negative selection by the *ccdB* gene in the Gateway system.
- BL21-SI$^{TM}$ (Invitrogen)
  For induction of IDL1 protein BL21-SI$^{TM}$ competent cells were used

#### *2.2.1.2 A. tumefaciens*

The *A. tumefaciens* strain C58 pGV2260 was used for transformation of both wild type (wt) *Arabidopsis* and the mutant line *haehsl2*. *Agrobacterium* cultures were grown in YEB-medium (5 g/l Bacto beef extract, 1 g/l Bacto yeast extract, 1 g/l Bacto peptone, 5 g/l sucrose, pH 4.7, added 2 ml 1M $MgSO_4$ per liter) at 28 °C with shaking. *Agrobacterium* cells were plated onto YEB-plates (YEB-medium containing 15 g agar per liter) to obtain single colonies.

*Agrobacterium* cells containing pMDC 162 were selected on YEB-plates containing carbenicillin (100 µg/ml), rifampicin (100µg/ml) and kamamycin (50µg/ml) and cells containing pHGY and pH7WG2 were selected on YEB-plates containing carbenicillin (100 µg/ml), rifamicin (100µg/ml), and spectinomycin (100µg/ml).

### 2.2.2 Transformation of bacteria

#### 2.2.2.1 Transformation of *E. coli*

For TOP10 all transformations were done by heat-shock, as described by the manufacturer (Invitrogen). Cells were then plated onto LA-plates containing the appropriate antibiotic for selection, and incubated ON at 37 °C.

#### 2.2.2.2 Transformation of *A. tumefaciens*

All transformations of *A. tumefaciens* were done by electrotransformation. Electrocompetent C58 pGV2260 was thawed on ice and plasmid was added. Then the mixture was added to a cold electroporation cuvette (Bio Rad) and shocked at 25 µFD, 200 Ω and 1.3 V. SOC medium was added and the cells were incubated 1 h at 28 °C with shaking. For selection of transformants the cells were spread on YEB-plates with the appropriate antibiotic and incubated at 28 °C.

## 2.3 Working with yeast

### 2.3.1 Yeast Two-Hybrid

Yeast two-hybrid screening is a molecular tool used to identify putative protein-protein interactions or protein-DNA interactions by investigating the binding properties between two molecules. The key principle behind this lies in the ability of most eukaryotic transcription factors, such as the GAL4 transcription factor, to function properly without the covalent binding between their activating and binding domains. Even when the transcription factor is split in two it can still activate transcription if the two separated domains are indirectly connected.

The protein to be tested for putative binding partners is fused to the GAL4 DNA binding domain (BD), which will bind to several different upstream activating sequences (UAS) of downstream reporter genes. Prey proteins are made as fusion proteins to the GAL4 activating domain (AD), and if binding occurs between bait and prey, the GAL4 transcription factor is indirectly connected and the reporter genes are actively transcribed. Prey fusion proteins are usually constructed on the basis of a cDNA library with the preceding RNA isolated from a

given type of tissue, representing all the protein expressed in that specific tissue in an organism (or the proteome of that tissue).

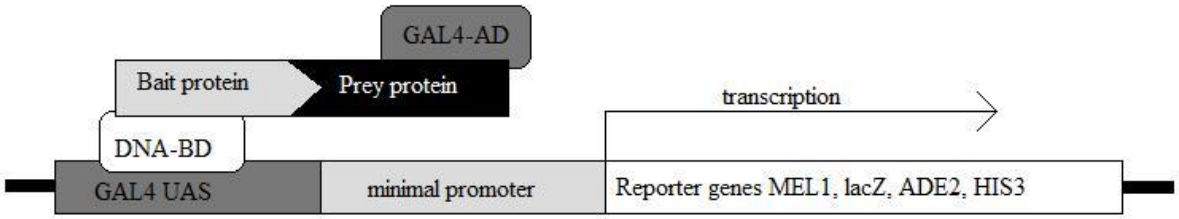


**Figure 2.1 The principle of yeast two-hybrid**

Interaction between the bait and prey protein indirectly connect the binding (DNA-BD) and GAL4 activating domain (GAL4 AD) with the BD binding the upstream activating sequences (UAS) of GAL4, leading to transcriptional activation of downstream reporter genes. The figure is modified from the MATCHMAKER Library Construction & Screening Kit User Manual (Clontech).

In the MATCHMAKER systems (Clontech) the downstream reporter genes included are MEL1, lacZ, ADE2 and HIS3. DNA-BD and GAL4-AD fusions are constructed by cloning cDNAs into the vectors pGBKT7 and pGADT7, respectively (figure 2.1).

### 2.3.2 Growth of yeast

The yeast strains were initially grown on YPDA-medium containing adenine and then selected on SD-medium (Synthetic Dropout) without either leucine (-L) or tryptophan (-T).

### 2.3.3 Transformation of yeast

The yeast cells were resuspended in 1X TE containing LiAc and herring testes carrier DNA and plasmid was added. PEG containing LiAc was added and the mix was incubated 30 min at 30 °C with shaking. After incubation DMSO was added and the cells were heat-shocked at 42 °C according to the protocol (Clontech Matchmaker Library Construction and Screening Kit)

### 2.3.4 Direct mating

One colony from each positive clone was resuspended in 2X YPDA, mixed with bait and control samples in a 96 well microtiter plate and incubated at 30 °C with shaking ON. The different matings were then plated on SD/-L/-T plates to attain diploid colonies and incubated at 30 C until colonies were observed. To find positive interactions three diploid colonies from each mating were then plated on both TDO-plates and QDO-plates containing X-α-gal and incubated at 30 °C for three days to look for positive interactions.

## 2.4 Standard DNA techniques

### 2.4.1 Agarose gel electrophoresis

Separation of DNA fragments according to size was done by agarose gel electrophoresis 1 % agarose (SeaKem®LE agarose, Cambrex Biosciences) gels with 1 µg/ml SyberSafe (Invitrogen) were run in a 1xTAE buffer (40mM Tris-acetate, 1mM EDTA) at 80-90 V for 30 min. To determine the size of DNA fragments GeneRuler™ 1 kb DNA ladder (Fermentas) was used.

### 2.4.2 Purification of DNA fragments

For purification of PCR fragments the Wizard SV gel and PCR Clean-Up System (Promega) was used. The procedure was followed according to the protocol supplied by the manufacturer.

### 2.4.3 Isolation of plasmids from *E. coli* cell cultures

#### 2.4.3.1 Miniprep (Promega)

This method is based on the fact that treatment with SDS (sodium dodecyl sulfate) and alkali leads to cell lysis and denaturation of proteins and genomic DNA, while the plasmids are released in the supernatant. Isolation of plasmids from 4 ml culture was done with Wizard Plus SV Miniprep DNA Purification System (Promega) according to the manual supplied with the kit.

### *2.4.4* Isolation of genomic DNA from *Arabidopsis*

#### 2.4.4.1 Miniprep (Omega)

Isolation of small amounts of genomic DNA from plant tissues was done using e.Z.N.A SP Plant DNA Mini Kit (Omega). DNA was extracted from $N_2$-frozen rosette leaves following the manual from the manufacturer.

#### 2.4.4.2 Extraction of DNA for genotyping

For plants that were to be genotyped, DNA was extracted using the ULTRAPrep® Genomic DNA Plant Kit (AHN Biotechnologie). The procedure was followed according to the protocol supplied by the manufacturer.

### 2.4.5 Quantification of DNA

Quantification of DNA samples was done using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technonlogies) as described by the manufacturer.

### 2.4.6 Using the Gateway® Technology (Invitrogen) to make constructs

The Gateway Method is a cloning method based on site specific recombination performed by the bacteriophage lambda (Landy, 1989). DNA segments that flanked by recombination sites (*att* sites) are exchanged between vectors.

Two recombination reactions constitute the basis of the Gateway technology. The BP reaction is catalyzed by the BP Clonase mix (Invitrogen), which recombines the *att*B sites of a PCR product or and *att*B expression clone and a donor vector containing *att*P sites. The BP reaction creates an entry clone bearing the insert of interest flanked by *att*L sites and a by-product flanked by *att*R sites. The LR reaction is a recombination reaction between an entry clone and a destination vector (with an *att*R substrate) to create an *att*B-containing expression clone and a by-product containing *att*P sites.

The Gateway technology has both positive and negative selection. The entry clone and the destination vector contain different antibiotic resistance genes for a positive selection of the entry or the expression clone. Both donor vectors, destination vectors, and the by-products of the BP and LR reactions contain the cytotoxic *ccdB* gene, which conveys a negative selection. Only plasmids with the appropriate antibiotic resistance and without the *ccdB* gene will yield colonies.

#### 2.4.6.1 YFP and GUS-constructs

The PCR products containing the promoter regions of *HSL1* and *At5g49660*, flanked by *att*B sites, were amplified using the primers attB1 SP2/*At1g28440*P, attB2 ASP2/*At1g28440*P, attB1 SP2/*At5g49660*P and attB2 ASP2/*At5g49660*P. The PCR products were recombined into the pDONR™/zeo. The entry clones were confirmed using PCR and sequenced using the same primers that were used amplifying attB flanked PCR products.

The destination vector pMDC 162 (Curtis and Grossniklaus, 2003), which contains the GUS marker, was used to obtain GUS-expression clones. The entry clones containing the promoter regions upstream of the coding sequence of *HSL1* and *IKU2L2* (2172 bp and 2485 bp respectively) were recombined with the destination vector pMDC162 in an LR reaction in front of the *gusA* gene (figure 2.2).


**Figure 2.2 The GUS construct.** The inserted promoter region is flanked by *attB* sites (25 bp) and followed by the coding sequence (CDS) of *gusA*, encoding the β-glucuronidase. RB: Right border, LB: Left border, *tNos*: Nos terminator, *Hygro*$^R$: Hygromycin resistance gene, *Km*$^R$: Kanamycin resistance gene (*NptII*). The figure is not to scale.

For the YFP expression clones the destination vector pHGY (RIKEN Plant Science Center) was used (figure 2.3). The entry clones containing the promoter sequences of *HSL1* and *IKU2L2* were recombined to the pHGY vector in an LR reaction in front of the *YFP* gene.


**Figure 2.3 The YFP construct.** The inserted promoter region is flanked by *attB* sites (25 bp) followed by the CDS of the *YFP* gene. *Sp*$^R$: Spectinomycin resistance gene. The figure is not to scale.

The expression clones for both *GUS* and *YFP* expression were analyzed by PCR using the primers attB1 SP2/*At1g28440*P, attB2 ASP2/*At1g28440*P, attB1 SP2/*At5g49660*P and attB2 ASP2/*At5g49660*P and by sequencing using the M13F and M13R primers in addition to the other four primers (see Appendix 1).

### 2.4.6.2 Constructs for direct mating

Prey- and bait constructs were also made using the Gateway® Cloning Technology. For the prey construct genomic DNA encoding the ECD of *HSL1* (1888 bp) was amplified using PCR. The ECD contains the leucine-rich repeat (LRR) sequence presumably needed for ligand binding. Amplification was done by using the primers *attB1* HSL1 cds SP and *attB2* HSL1 cds ASP, where the antisense primer contained a stop codon. The *att*B flanked PCR product was then recombined into the vector pDONR™/Zeo (Invitrogen) in a BP reaction. The entry clones were verified by PCR and sequencing using the ECD specific primers (Appendix 1). The entry clones were then recombined into the destination vector pADN by a LR reaction. This resulted in a prey vector with the GAL4 Activation Domain (AD) upstream of and in reading frame with the LRR region of *HSL1*. This was confirmed using the AD insert screening primers (5' and 3') (Appendix 1).

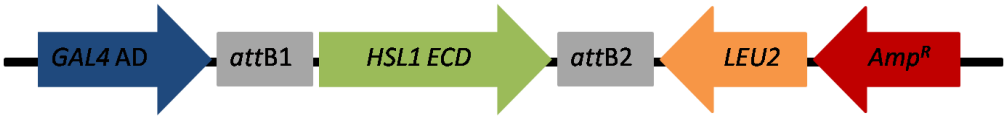


**Figure 2.4 The prey fusion construct.** The *GAL4* AD is positioned upstream of and in reading frame with the *HSL1* ECD. The *HSL1* ECD is flanked by *att*B sites (25 bp). *LEU2*: β-isopropylmalate dehydrogenase. $Amp^R$: Ampicillin resistance gene. The figure is not to scale.

For the bait vector the coding sequence of *IDL1* (244bp) without the sequence encoding the SP (*IDL1ΔSP*) was cloned into the pDONR™/Zeo (Invitrogen) in a PB reaction. The primers used for amplifying the truncated *IDL1* transcript were *att*B1 IDL1 cds SP and *att*B2 IDL1 cds ASP (Appendix 1). After sequencing, the entry clone was recombined into the destination vector pGBKT7 (Clontech) by an LR reaction. The resulting bait vector contained the *GAL4* Binding Domain (BD) upstream of and in reading frame with the coding sequence of *IDL1*, in order to make a translational fusion protein (figure 2.5). This was confirmed by sequencing using the BD insert screening primers (5' and 3') (Appendix1).


**Figure 2.5 The bait fusion construct.** The *GAL4* BD is positioned upstream of and in reading frame with *IDL1ΔSP*. *IDL1ΔSP* is flanked by *att*B sites (25 bp). *Trp1*: Phosphoribosylanthranilate isomerise. The figure is not to scale.

All BP and LR reactions were performed as recommended by the manufacturer.

### 2.4.7 TOPO TA cloning (Invitrogen)

The TOPO® Technology (Invitrogen) is a fast and efficient way of cloning PCR products into a vector. A key to the technology is the enzyme DNA topoisomerase, which has both restriction and ligase activity. Topoisomerase I from Vaccinia virus recognizes specific DNA sequences and cleaves the phosphodiester backbone of one strand. The energy from the reaction is conserved in the formation of a covalent bond between the 3' thymidine phosphate in the cleaved strand and a tyrosine residue in the topoisomerase. The plasmid vector used, pCR®2.1 II TOPO®, is supplied linearized with the topoisomerase covalently bound to the 3'

end of each strand. The 5' hydroxyl of the PCR product will then attack the phosphotyrosine bond between the 3' thymidine phosphate and the topoisomerase, so that the first reaction is reversed, the enzyme is released, and the PCR product is recombined into the TOPO vector. Topo cloning reactions were performed according to the manufacturer's recommendations.

#### 2.4.7.1 Constructs for identification of T-DNA insertion sites

The PCR products containing the flanking left border (LB) of the T-DNA insertion in Salk line SALK_108127 were amplified using the primers LBb1 and SALK_108127 RP. The PCR products were ligated into the pCR®2.1 II TOPO® (Invitrogen) vector (figure 2.6). The clones were confirmed using PCR and sequenced using the same primers that were used in amplifying the PCR products.


**Figure 2.6 The Topo construct.** The PCR fragment is cloned into the *LacZα* gene, encoding α-galactosidase, thus disrupting the function of *LacZ*. $P_{lac}$: *LacZα* promoter, f1 ori: f1 origin of replication, $Km^R$: Kanamycin resistance gene, $Amp^R$: Ampicillin resistance gene. The figure is not to scale.

## 2.5 Genotyping

In order to indentify the genotype of plants PCR genotyping was performed

### *2.5.1* Genotyping of *35S:IDL* genes, *hae* and *hsl2*

Transgenic plants containing the *35S:IDL* over-expression constructs were identified using the primers 35S L, *att*B2 IDL1 stop, *att*B2 IDL2 stop and *att*B2 IDL3 stop (figure 2.7). As these are dominant alleles, there was no need to distinguish between hemizygous (HZ) and homozygous (HM) plants. To determine whether plants were wt, HZ or HM for T-DNA insertions in the *HAE* or *HSL2* alleles, the primers LBb1, HAE RP, HAE LP, HSL2 RP and HSL2 LP were used. The expected fragment lengths were (Table 2.1):

| Primers | Band | Length |
|---|---|---|
| 35S L + *att*B2 IDL1 stop | 35S promoter-genomic flanking region | 538 bp |
| 35S L + *att*B2 IDL2 stop | 35S promoter-genomic flanking region | 563 bp |
| 35S L + *att*B2 IDL3 stop | 35S promoter-genomic flanking region | 576 bp |
| HAE RP + LBb1 | T-DNA-genomic flanking region | 526 bp |
| HAE RP + HAE LP | wt | 1042 bp |
| HSL2 RP + LBb1 | T-DNA-genomic flanking region | 561 bp |
| HSL2 RP + HSL2 LP | wt | 1055 bp |

**Table 2.1 Expected fragment lengths**

For the *HAE* and *HSL2* T-DNA insertions, a wt plant would give a 1042/1055 bp band on an agarose gel, a HM plant would give a 526/561 bp band and a HZ plant would give both bands (figure 2.8). All three primers (LP, RP and LBb1) were run in the same PCR reactions, and all PCRs were run at standard conditions.

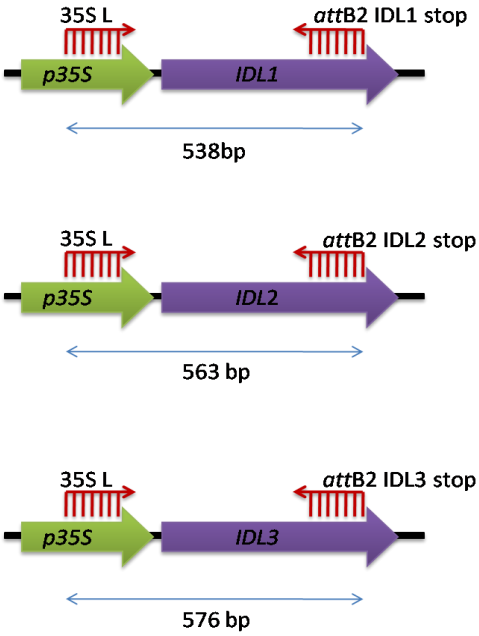


**Figure 2.7 Genotyping for the *35S:IDL* constructs.** The expected fragment lengths were 538 bp, 563 bp and 576 bp, as indicated by the blue arrow. *P35S*: Constitutive 35S promoter.

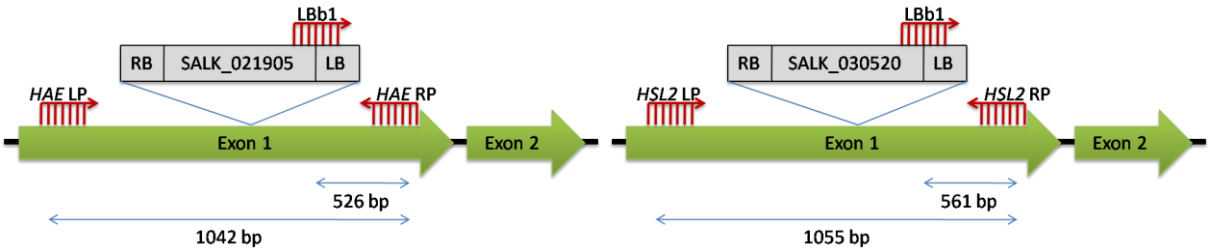


**Figure 2.8 Genotyping for *HAE* and *HSL2* T-DNA insertions.** A T-DNA insertion results in a PCR product of 526 bp (*HAE*) or 561 bp (*HSL2*), whereas no insertion results in a PCR product of 1042 bp (*HAE*) or 1055 bp (*HSL2*), as indicated by the blue arrows.

### *2.5.2* Genotyping of SALK and SAIL lines for *HSL1* and *IKU2L2*

SALK and SAIL lines supplied from the Salk Institute were genotyped to look for a line homozygous for the T-DNA insertion. The primers used were LBb1, LB1, SALK_104365 RP/LP, SALK_108126 RP/LP, SALK_108127 RP/LP and SAIL_268_H07 RP/LP (Appendix 1)

## 2.6 Polymerase chain reaction (PCR)

PCR was used for amplification of desired DNA fragments for cloning, screening for positive bacteria colonies and genotyping of T-DNA mutants. Standard setup for one reaction was 1X reaction buffer, 200 µM dNTP (deoxyribonucleotide triphosphate), 0.2 µM primers and 0.5-1 U DNA polymerase (*Taq* DNA Polymerase (New England BioLabs) or Phusion™ High-Fidelity DNA Polymerase (Finnzymes)). A positive control was included when possible and a negative control was always included.

*Taq* DNA Polymerase is a thermostable polymerase for standard PCR. Phusion™ High-Fidelity Polymerase is a thermostable polymerase with 3'→5' exonuclease activity that gives accurate amplification of DNA, thus making it suitable for cloning. *Taq* Polymerase generates a 3' A-overhang which facilitates ligation into a TOPO vector, whereas Phusion™ High-Fidelity Polymerase generates blunt ends.

All programs used were variations of the general program: 94 °C 5 min, 94 °C 30 sec, 52-68 °C 15-30 sec, 72 °C 3 min, 72 °C 7 min, and 4 °C ∞.

## 2.7 Sequencing

Sequencing was performed with an Applied Biosystems 3730 DNA analyzer using the ABI BigDye Terminator sequencing buffer and v3.1 Cycle Sequencing kit provided by the sequencing facility at CEES, Department of Biology and Molecular Biosciences.

## 2.8 Protein methods

### 2.8.1 Induction of proteins from pGEX-AB-GAW expression clones

BL21-SI™ cells (Invitrogen), optimal for expression of protein, were used for expression of recombinant GST-fusion proteins. The coding sequences of these proteins are incorporated in isopropyl-β-D-thiogalactopyranoside (IPTG) inducible pGEX-AB-GAW expression clones. All expression clones used were constructed by Grethe-Elisabeth Stenvik (figure 2.9).


**Figure 2.9 The expression clone for GST-IDL1ΔSP.** The expression clone was construcyed by Grethe-Elisabeth Stenvik.

One colony of cells was diluted in 10 ml LB containing 100 µg/ml ampicillin and cultured over night at 37°C with shaking. At $OD_{600}$ = 0.9 the cultures were split in two and IPTG was added to a concentration of 150 µM to one of the two cultures. Protein expression was induced at 150 rpm for 1 and 4 hours at 30°C.

To investigate induction of GST-fusion proteins, 18 µl of induced and not induced cell culture were run on the same SDS-PAGE gel for comparison of band strength.

### 2.8.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used for detection of proteins, where proteins are separated according to their electrophoretic mobility. SDS is an anionic detergent that denatures the secondary and the tertiary structures of proteins, in addition to coating the proteins in a uniform layer of negative charge, almost proportional to the mass of the protein. Linearized, net negatively charged proteins can thus be separated solely by their molecular weight when they migrate towards the anode in an electrical field. 2-mercaptoethanol is added to the loading buffer, in addition to SDS, do reduce intrinsic disulfide bonds. Estimation of the weight of the polypeptides was done by running a standard with known molecular weight in a separate lane.

Polyacrylamide gels are separated in two layers: the stacking gel (5% 30% acrylamide/Bis solution (BioRad), 200 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulfate (APS), 0.1% N, N, N', N'-Tetramethylenediamine (TEMED)) and the separating gel which can have various concentrations of acrylamide (10/12/15% 30% acrylamide/Bis solution, 390 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS, 0.04% TEMED). The stacking gel concentrates or "stacks" the proteins in thin bands before the polypeptides, with equal starting times, are subjected to separation in the separating gel. Proteins samples were mixed with the appropriate amount of 4X SDS loading buffer, boiled at 95°C for 5 min, and centrifuged at max speed for 6 sec. Denatured proteins were loaded onto the gel and run in running buffer (50mM Tris pH 8.3, 196 mM glycine, 0.1% SDS) at 45-55 mA for ~1 hour. PageRuler$^{TM}$ Prestained Protein Ladder (Fermentas) was used as a size marker.

Staining of the gel was done in Coomassie Brilliant Blue dye (0.25 g/l Coomassie Brilliant Blue in 10% acetic acid, 45% methanol) for approximately 30 min before the gel was de-stained by boiling for 5 min.

### 2.8.3 Western blot analysis

Western blotting, or immunoblotting, is used to detect a specific protein by exploiting the specificity of antigen-antibody recognition. Proteins are separated by SDS-PAGE and transferred, by electroblotting, to a PVDF membrane with non-specific affinity for amino

acids, so that the proteins are immobilized on the membrane and readily accessible for analysis. Proteins are then probed with antibodies specific to the target protein.

Equal protein samples were run on three identical SDS-polyacrylamide gels, where one gel was subjected to Coomassie-staining, while the other two were blotted onto PVDF membranes.

#### 2.8.3.1 Blotting

Two Scotch-brite™ pads (3M Company) and two pieces of Whatman 3MM papers (Whatman®) were soaked in blotting buffer (25 mM TRIS, 192 mM glycine, 20% methanol), while the Immobilon™ PVDF membrane (Millipore) was soaked in methanol for 3 sec, as a hydrophilic treatment, saturated in $dH_2O$ and soaked in blotting buffer before all layers were sandwiched together with the protein gel next to the membrane. The "sandwich" was then placed in an electrophoresis blotting chamber and run in cold blotting buffer at 100 V for 1 hour at 4°C. To reduce the heating caused by the electric current a cooling element and a magnetic stirrer was included in the setup.

#### 2.8.3.2 Immunolabeling

To reduce unspecific binding of antibody, the membrane was first left in blocking buffer (1X PBS, 2.5% skimmed milk, 0.1% Tween-20) for 1 hour with shaking. Blocking buffer was changed, the primary antibody was added in a 1000X dilution (goat anti-GST (Amersham Biosciences) and rabbit anti-IDL1) and incubated for 1 hour at room temperature. The membrane was then washed in wash buffer (1X PBS, 0.1% Tween-20) for 15 min with shaking, and then it was washed again for 3 x 5 min with fresh changes of wash buffer, this to remove any unbound antibody. Secondary antibody was then diluted 10000X (rabbit anti-goat (Sigma) and goat anti-rabbit (Thermo Scientific)) in blocking buffer and the membrane was soaked in this for 1 hour with shaking.

SuperSignal® West Pico Stable Peroxide Solution and Enhancer Solution (PIERCE) was mixed in a 1:1 ration, transferred to the membrane and incubated for 5 min. The membrane was then drained for excess liquid, wrapped in plastic foil and placed in a Hypercassette™ (Amersham pharmacia biotec).

#### 2.8.3.3 Detection

Shortly after incubation with working solution, a sensitive High Performance Chemiluminescence Film (GE Healthcare) was exposed to the protein membrane for various

amounts of time, depending on the expected signal strength. The secondary antibody is coupled to horseradish peroxidase (HRP), that will react with the working solution and emit a light signal that leaves a band on the processed x-ray film. The films were developed in the Optimax® X-ray Film Processor (PROTEC).

## 2.9 Bioinformatics

### 2.9.1 Sequence alignment and primer design

The Invitrogen software tool for sequence alignment and data management, Vector NTI Advance™, was used to analyze and align nucleotide sequences in addition to designing primers.

## 2.10 Statistical analysis

### 2.10.1 Standard deviation (SD)

Standard deviation (SD) was calculated using the formula;

$$SD = \sqrt{\sum (\frac{(X - M)^2}{n - 1})}$$

Where *X* is the individual data points, *M* refers to the mean and n refers to the number of data points. ∑ (sigma) means that all $(X\text{-}M)^2$ are added to find the sum for all *n* data points.

### 2.10.2 The chi-square test

The chi-square test is performed to see if the observed and the expected frequency of results are of significant value supplied by the statistic $\chi^2$ (chi-square), given by the formula;

$$\chi^2 = \sum \frac{(O - E)^2}{E}$$

Where *O* is the observed value and *E* is the expected value. Expected values are computed on the basis of our hypothesis. A 0.05% confidence and 1 degree of freedom was used in this test, and for $\chi^2 < 3.84$ the hypothesis holds with 95% accuracy and is not rejected.

### 2.10.3 Two-sample T-test

In the two-sample T-test the null hypothesis is defined so that there is no difference between the population means. The T-value is given by the formula;

$$T = \frac{|\overline{Y_1} - \overline{Y_2}|}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

Where $n_1$ and $n_2$ are the number of samples in group 1 and group 2, respectively, $\overline{Y_1}$ and $\overline{Y_2}$ are the sample means, and $s_1^2$ and $s_2^2$ are the sample variances (variance = $\sigma^2$ where $\sigma$ is the standard deviation). In an unpaired T-test there are $n_1 + n_2 - 2$ degrees of freedom. A 0.05 significance value and 1degree of freedom was used in the test, and for $|T| > 2.02$ the $H_0$ is rejected, meaning that the population means are different with 95% accuracy.

# 3 RESULTS

A possible physical interaction between IDL1 the native receptors of IDA, HAESA and HSL2, in addition to the closely related receptor HSL1 (Shiu and Bleecker, 2001a), was investigated using the yeast two-hybrid system. To see whether the IDL proteins were signaling through the native receptors of IDA in the floral organ AZ of plants over-expressing the IDL proteins, a genetic approach was also applied by investigating the phenotype of *haehsl2* plants over-expressing *IDL1, IDL2* and *IDL3*.

For *IDL1,* which is normally expressed in the columella root cap, over-expression has been shown to result in shorter roots (Nora Tandstad, unpublished results). Due to the early abscission phenotype observed for over-expression of all the *IDL* genes, it was therefore of interest to investigate if this over-expression phenotype was also found when over-expressing IDA, IDL2 and IDL3.

The expression pattern of the two genes *At1g28440* (*HSL1*) and *At5g49660* (*IKU2L2)* was examined by promoter::reporter gene analysis in order to compare the expression pattern to the observed expression pattern of the *IDL* genes. According to publicly available microarray data *HSL1* and to a lesser extent *IKU2L2* could be the native receptor(s) of IDL1 in roots, as they both seem to be expressed in the root. Therefore were three SALK lines for *HSL1* and one SAIL line for *IKU2L2* investigated in order to look for mutant phenotypes to investigate if any of these receptors could be the receptor of IDL1.

## 3.1 Can IDL1 interact with IDA's receptors?

IDL1 is as efficient as IDA in the floral organ abscission zone (AZ), rescuing the abscission defect of *ida,* probably by interacting with IDAs receptors (Stenvik *et al.*, 2008). We therefore wanted to test whether we could detect a direct interaction between IDL1 and IDA's, receptors HAE and HSL2. It was also of interest to look for an interaction between IDL1 and its potential receptor HSL1.

The direct interaction of IDL1 and HAE, HSL2 and HSL1 is expected to occur between the exported part of IDL1, the SP was therefore not included in the construct, and the extracellular domain (ECD) of the receptors containing the LRR domain. The constructs for

HSL1 ECD and IDL1ΔSP were made according to Materials and Methods, section 2.4.6.3. Yeast cell strain AH109 (MATa) was transformed with the prey vector containing the sequence encoding the ECD of HAE, HSL2 and HSL1 in frame with the GAL4 AD. The constructs containing HAE and HSL2 were made by Even S. Riiser (Riiser, 2009). Cells of the strain Y187 (MATα) were transformed with the bait vector containing IDL1ΔSP in frame with GAL4 BD. After growth on appropriate selective medium (SD/-Leu and SD/-Trp, respectively), the two strains were mated and plated on SD/-Leu/-Trp. The SD/-Leu/-Trp medium allows for selection of diploid yeast containing both the bait and prey vector.

To test the interaction between IDL1 and the receptors the diploid yeast cells were streaked onto selective medium. As negative controls, Y187 cells containing only the bait vector pGBKT7 (BD) and pGBKT7-Lam (*Human Lamin* C::*GAL4 BD*) (LAM) were mated to AH109 containing the respective prey vectors. As a positive control, diploid yeast cells expressing two proteins with a known interaction, ASHR3 and AMS (Thorstensen *et al.*, 2008) were used.

Growth of diploid yeast colonies were in all cases observed on the SD/-Leu/-Trp medium (figure 3.1, left column), indicating that the mating reactions were successful. For both the TDO and QDOX selective medium, growth was seen for the positive control (ASHR3/AMS), as expected (figure 3.1 middle and right column). The colonies with a positive interaction were coloured blue on QDOX medium. This colour is due to the cleavage of X-α-gal into a blue end-product by the α-galactorsidase encoded by the reporter gene *Mel1*. No growth was observed for the negative controls, showing that neither GAL4 BD alone nor GAL4::Human Lamin C interacts with any of the prey constructs. For the *IDL1ΔSP::GAL4BD* bait and the ECD of *HAE::/HSL2::/HSL1::GAL4AD* fusion proteins no growth was observed. This result concludes that no direct interaction between IDL1ΔSP and the ECD of HAE, HSL1 or HSL2 could be detected using yeast two-hybrid.

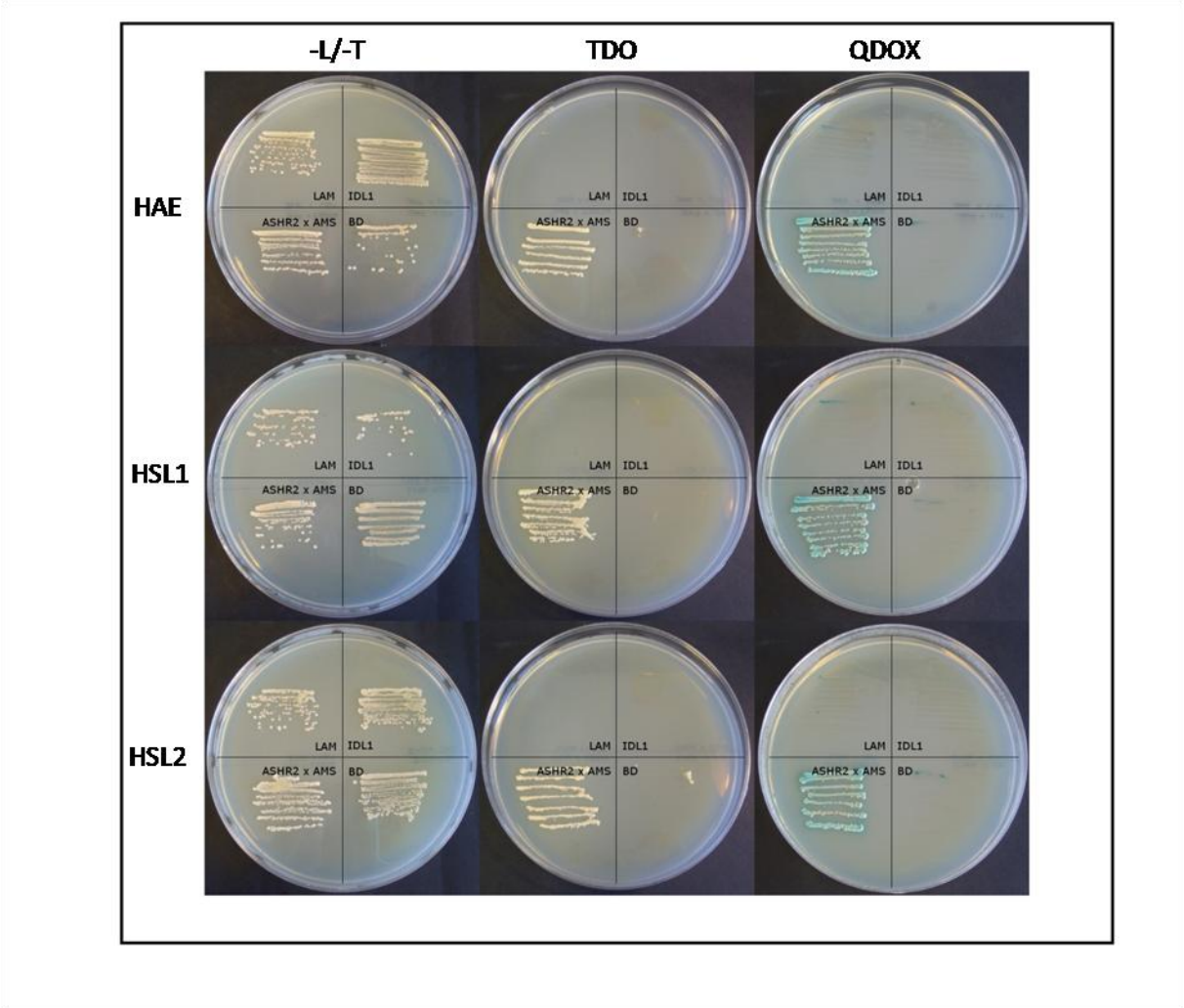


**Figure 3.1 Direct interaction assay.** Growth was observed in all sectors of the –L/-T plates (left column), confirming the successful mating reactions. On TDO medium (middle column) and QDOX (right column) growth was observed for the positive control (lower left sector), while no growth was seen for the negative controls (upper left and lower right sector). Furthermore, no growth was observed for the cells containing both bait and prey fusion proteins was observed (upper right sector).

## *3.2* Production of recombinant IDL1 protein in *E. coli*

The lack of interaction in the Y2H assay could be due to lack of processing of IDL1 within yeast cells. To delineate the active peptide we plan to use Cauliflower meristem extract which has been shown to process IDA (Stenvik *et al.*, 2008). In order to be able to produce protein to

be used in the processing assay, it is necessary to be able to express the protein in *E. coli*. To express the protein an expression clone was therefore constructed.

A recombinant IDL1 protein was created by cloning *IDL1ΔSP* in front of the gene encoding a GST (glutathione S-transferase)-tag in the vector pGEX-AB GAW (Stenvik *et al.*, 2006) and then transformed into *E. coli* BL21-SI$^{TM}$ cells (materials and methods section 2.8.1) (performed by Grethe-Elisabeth Stenvik).

GST-IDL1ΔSP was expressed in *E. coli* (figure 3.2) as seen in figure 4.2. The recombinant GST-IDL1ΔSP protein was visible as a strong band at ca. 30 kD, corresponding to the GST tagged IDL1ΔSP peptide of approximately 279 aa (ca. 30.7 kD). To verify that this was the fusion protein Western blots were probed with antibodies against the variable region of IDL1 and GST. The antibody against the variable region of IDL1 resulted in much background noise, and could not be used to detect the recombinant IDL1 protein. The antibody against GST however gave one strong band of approximately 30 kD and three weaker bands at approx. 28 kD, 27.5 kD and 24 kD. The 30 kD band corresponds to the recombinant protein and possibly degradation products of this recombinant protein.

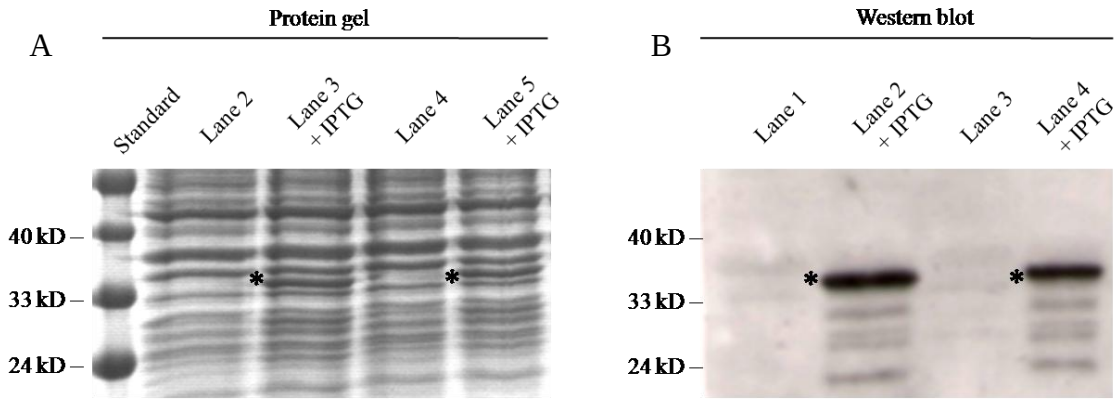


**Figure 3.2 GST-IDL1ΔSP.** (A) Expression of the recombinant protein has been induced in lane 3 and 5 and a clearly visible band is seen at approximately 30 kD, corresponding to the 279 aa recombinant protein GST-IDL1ΔSP (asterisk). (B) Western blot of the induced GST-IDL1ΔSP. In lanes 2 and 4, where protein expression has been induced, a strong band of approximately 30 kD is visible (asterisk). This corresponds to the GST-IDL1ΔSP recombinant protein. The three bands of approximately 28 kD, 27.5 kD and 24 kD are possibly the degradation products of the recombinant protein.

## 3.3 Functional redundancy of IDA and IDL proteins in roots

Since the direct interaction approach did not function, we went back to genetic and *in planta* experiments. It is known that the *35S:IDL* genes exhibit a phenotype in the floral abscission zone (AZ) similar to that of *35S:IDA*, namely early abscission of floral organs and secretion of arabinogalactan in the AZ (Stenvik *et al.*, 2006; Stenvik *et al.*, 2008). An interesting question to be answered is; can these proteins be functionally redundant also in other tissues than the AZ? Preliminary experiments performed by Nora Tandstad have indicated that *35S:IDL1* plants have a specific short root phenotype (Tandstad, 2005) and this was the background for setting up a root experiment. The purpose of the experiment was to see if only *35S:IDL1* plants had the short root phenotype, or if other *35S:IDL* genes also could exhibit the same short root phenotype.

Plants harbouring *35S:IDA* and *35S:IDL* gene constructs in Col background were investigated for short root phenotypes. The plants were grown vertically on MS plates for 17 days (see materials and methods). The results show that the plants over-expressing IDA and IDL1 have significantly ($P < 0.001$) shorter roots than the wt Col plants from day 5 and onwards (figure 3.3). The plants over expressing IDL2, IDL3 and IDL5 were also shown to have significantly shorter roots, although at a lower level of significance ($P < 0.01$ and $P < 0.05$). The plants over-expressing IDL4 however, did not show any significant difference in root length compared to the wt control (figure 3.3).

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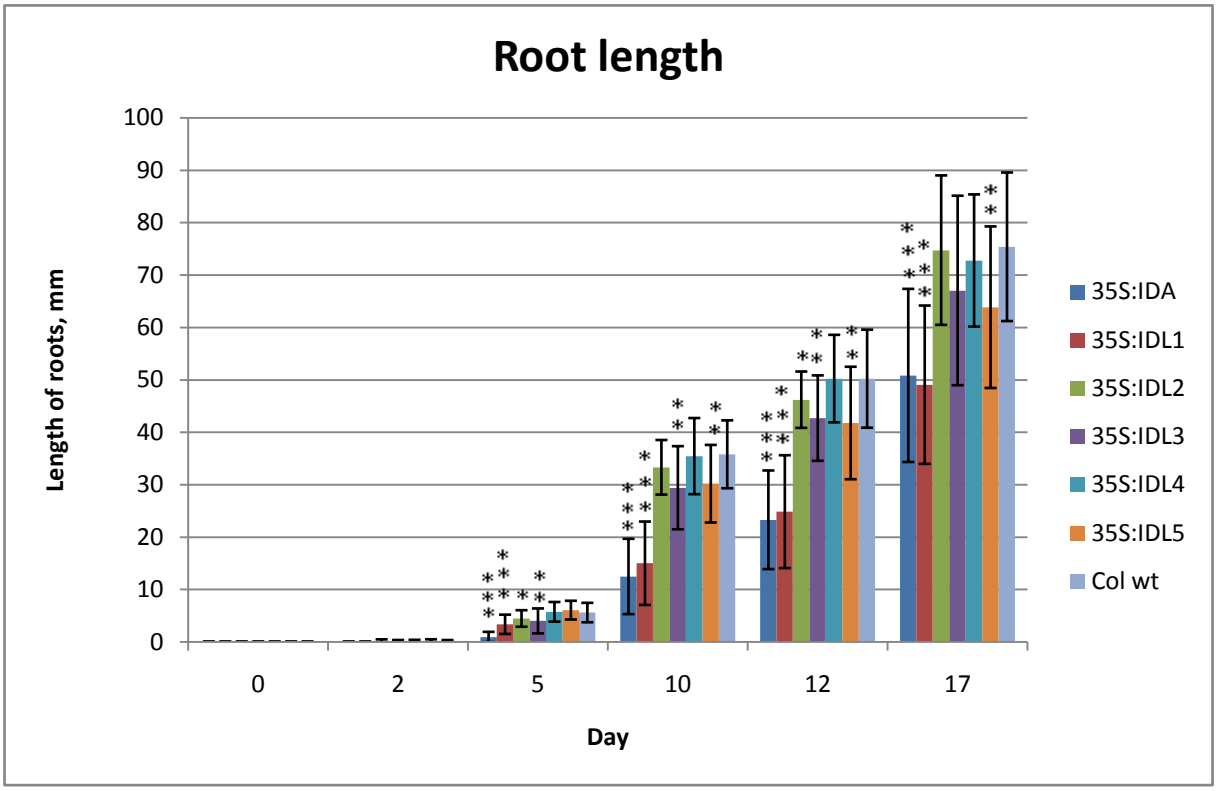


**Figure 3.3 Bar diagram showing length of roots (mm) relative to days after exposure to light.** The bars show that over-expression lines of IDA, IDL1, IDl2, IDL3 and IDL5 have shorter roots relative to the Col wt control line. *35S:IDL4* plants did not at any time exhibit a difference in root length from the wt control. *** P-value < 0.001, ** P-value < 0.01, * P-value < 0.05. N = 23.

After observing these results it was decided to also measure the total above-ground length of the plants to see whether the *35S:IDA* and the *35S:IDL1* plants have a specific short root phenotype, or if they are generally shorter than the wt Col. The data showed that the above-ground length of plants harbouring the *35S:IDL* constructs were significantly shorter than the Col wt (figure 3.4).

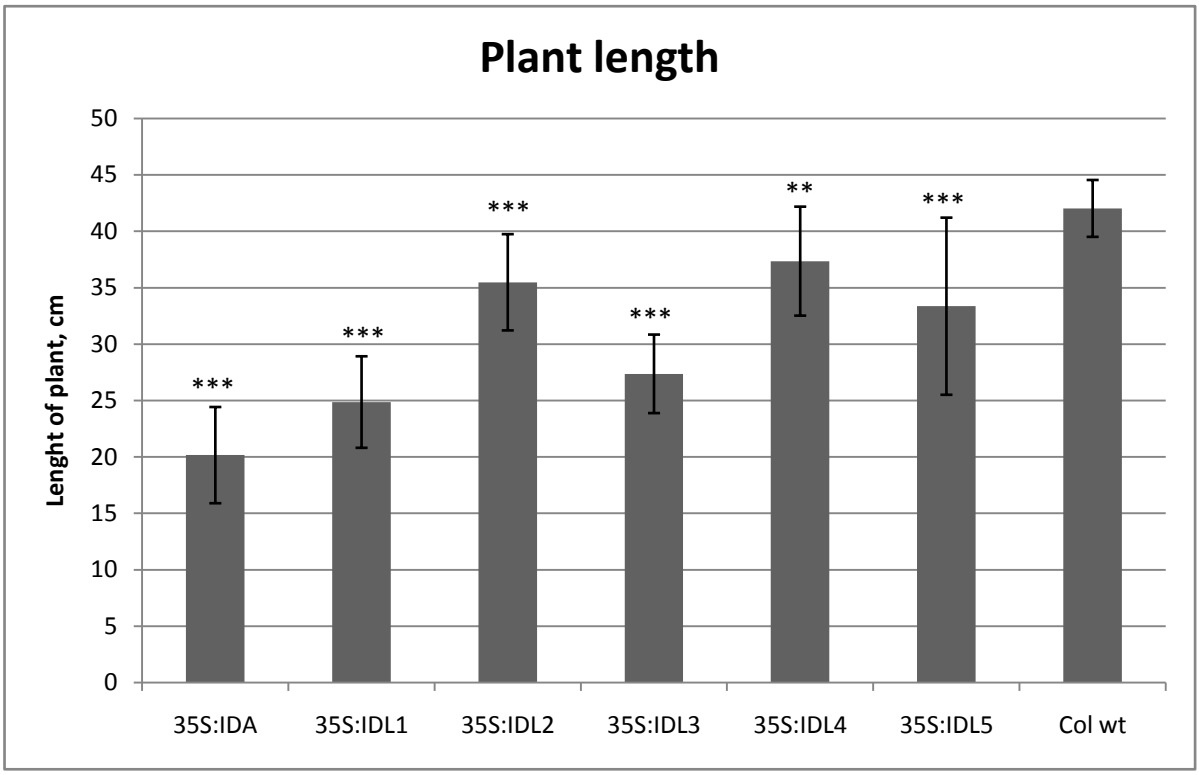


**Figure 3.4 Bar diagram showing length of plant (cm) relative to plant.** All plants are significantly smaller than the Col wt plants. *** $p < 0.001$, ** $p < 0.01$. N = 14

In order to be able to say if *35S:IDA* and *35S:IDL1* plants have a shorter root phenotype, the length of the root had to be seen relative to the length of the above-ground plant. This showed that the *35S:IDL1* plants, when looking at the ratio between plant length and root length clearly have significantly shorter roots ($p < 0.05$), whereas the *35S:IDA* plants do not have a specific shorter root phenotype (Table 3.1 and figure 3.5). It is known that IDA signals through HAE and HSL2 in the AZ (Cho *et al.*, 2008; Stenvik *et al.*, 2008), so it would be expected that *35S:IDA* and *35S:IDL1* plants would have the same root phenotype if they signal through the same receptors. Since *35S:IDA* does not exhibit a shorter root phenotype, it is plausible to think that IDA and IDL1 signals through different receptors in the root.

| Plant | Ratio plant length:root length | Standard deviation | p-value |
|---|---|---|---|
| *35S:IDA* | 14,6 | 8,845 | 0,187 |
| *35S:IDL1* | 22,2 | 19,902 | 0,050 |
| *35S:IDL2* | 8,7 | 4,583 | 0,123 |
| *35S:IDL3* | 8,9 | 4,360 | 0,131 |
| *35S:IDL4* | 12,6 | 9,528 | 0,583 |
| *35S:IDL5* | 9,2 | 6,984 | 0,359 |
| *Col wt* | 11,1 | 3,267 | 1,000 |

**Table 3.1 Ratio between plant length and root length**

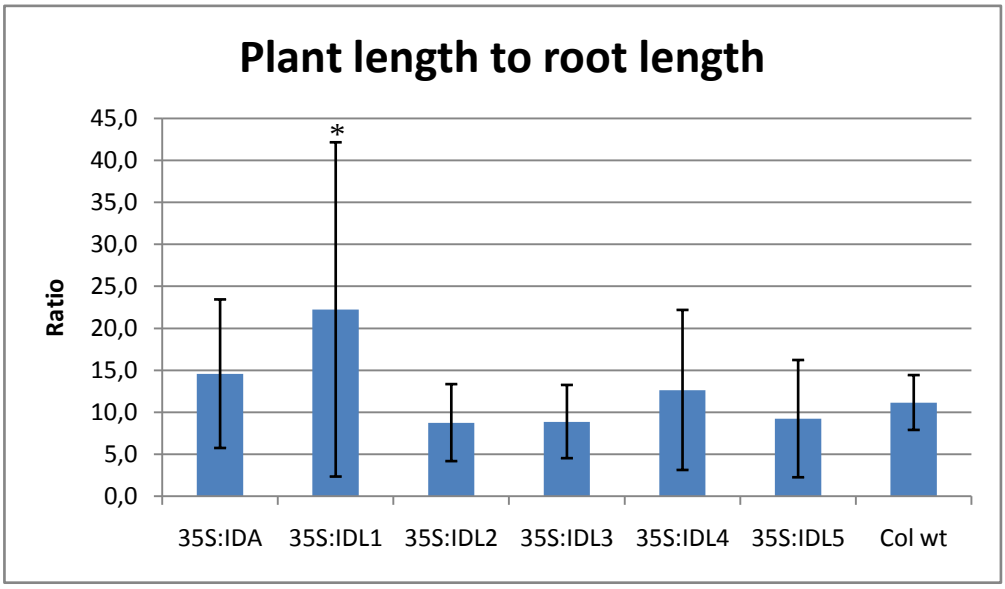


**Figure 3.5 Bar diagram showing the average ratio between plant length and root length.** The *35S:IDL1* plants have significantly ($p < 0.05$) shorter roots in comparison with the wt Col background. * $p < 0.05$. N = 14

## 3.4 Over-expression of IDL1, IDL2 and IDL3 in *haehsl2* background

Since over-expression of the IDL proteins all show early abscission, we wanted to test whether the early abscission observed is dependent on HAE/HSL2 signaling, or if another receptor could be involved. *35S:IDL1*, *35S:IDL2* and *35S:IDL3* constructs (Stenvik *et al.*, 2008) were transformed into *haehsl2* plants and investigated for abscission phenotypes, and any additional phenotypes. After selection of T1 and T2 plants, the transformants was

genotyped to make sure that they contained the over-expressing constructs for the *IDL* genes and were homozygous for the T-DNA insertions in the *HAE* and *HSL2* genes (figure 3.6).

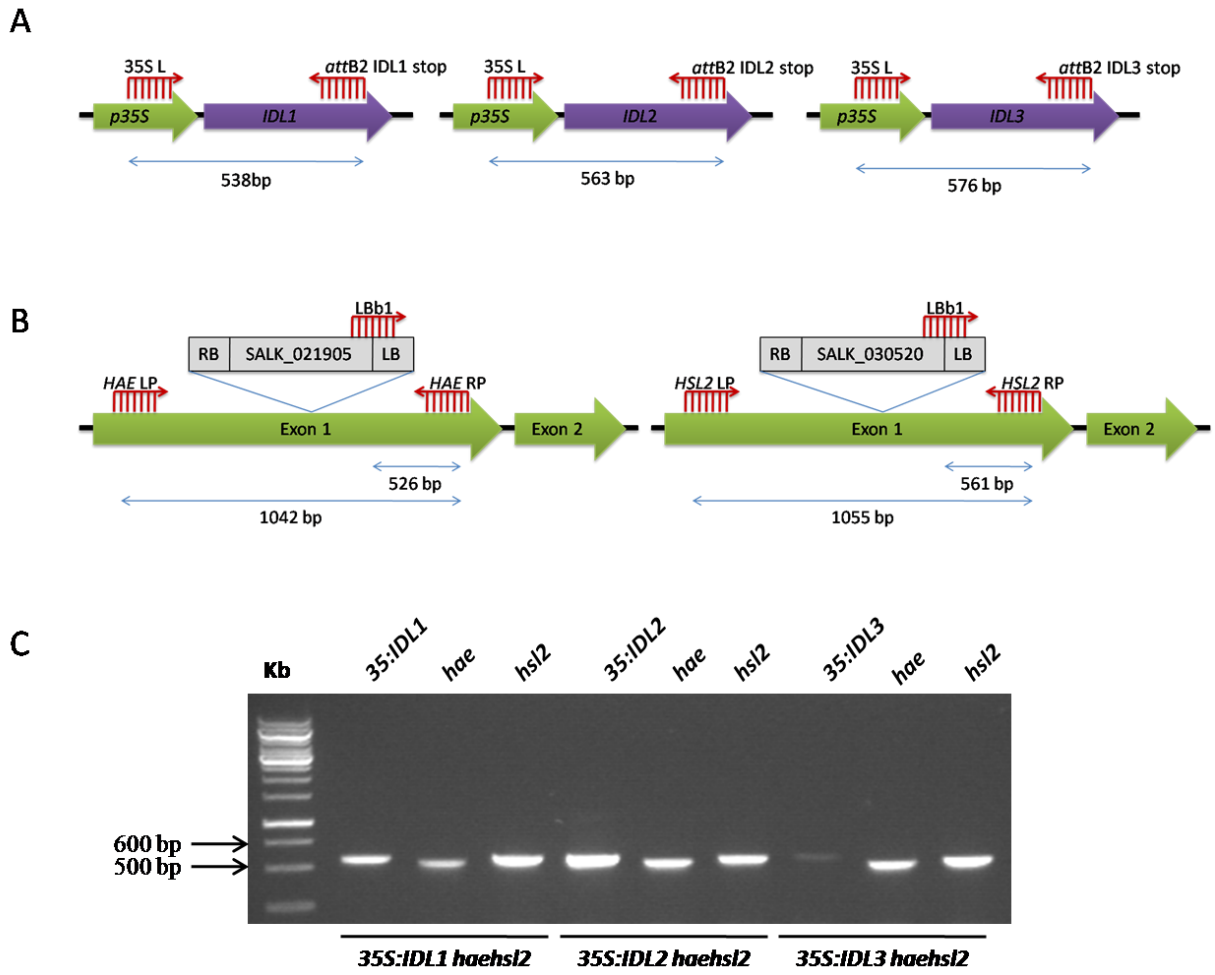


**Figure 3.6 Genotyping of transformants.** The investigated transformants did all contain the *35S:IDL* of choice and was homozygous for the T-DNA insertions in the *HAE* and *HSL2* genes, making them *35S:IDL haehsl2* plants. (A) The primers used to genotype for the *35S:IDL* constructs and the expected fragment lengths. (B) The primers and expected lengths of fragments for the T-DNA insertions in *HAE* and *HSL2*, respectively. (C) The gel showing the genotyping. For the T-DNA insertions all three primers were used in the same reactions.

### 3.4.1 Over-expression of *IDL* genes in *haehsl2* background retains the *haehsl2* phenotype

When examining the plants in both T1 and T2 generation it became clear that they exhibited the *haehsl2* phenotype, with no abscission of floral organs, masking the early abscission

*35S:IDL* phenotype (figure 3.7). The phenotypes of the plants were further confirmed when looking closer at the siliques of the plants. They all retained their floral organs even after silique development, just like the *haehsl2* mutant (figure 3.8). Since over-expression of IDL1, IDL2 or IDL3 did not rescue the *haehsl2* phenotype this indicates that signalling of the IDL proteins in the floral organ abscission zone is dependent on HAE and HSL2.

When the receptors are present and the IDL proteins are over-expressed, the signal is strengthened and the plants display early abscission (Stenvik *et al.*, 2006; Stenvik *et al.*, 2008). Removing the receptor on the other hand stops the IDA signal from being relayed and the plants are unable to abscise their floral organs (Cho *et al.*, 2008; Stenvik *et al.*, 2008). The fact that the *haehsl2* phenotype is retained also in the IDL over-expression lines is a clear indication that all signaling is dependent on HAE and HSL2 in the floral AZ.

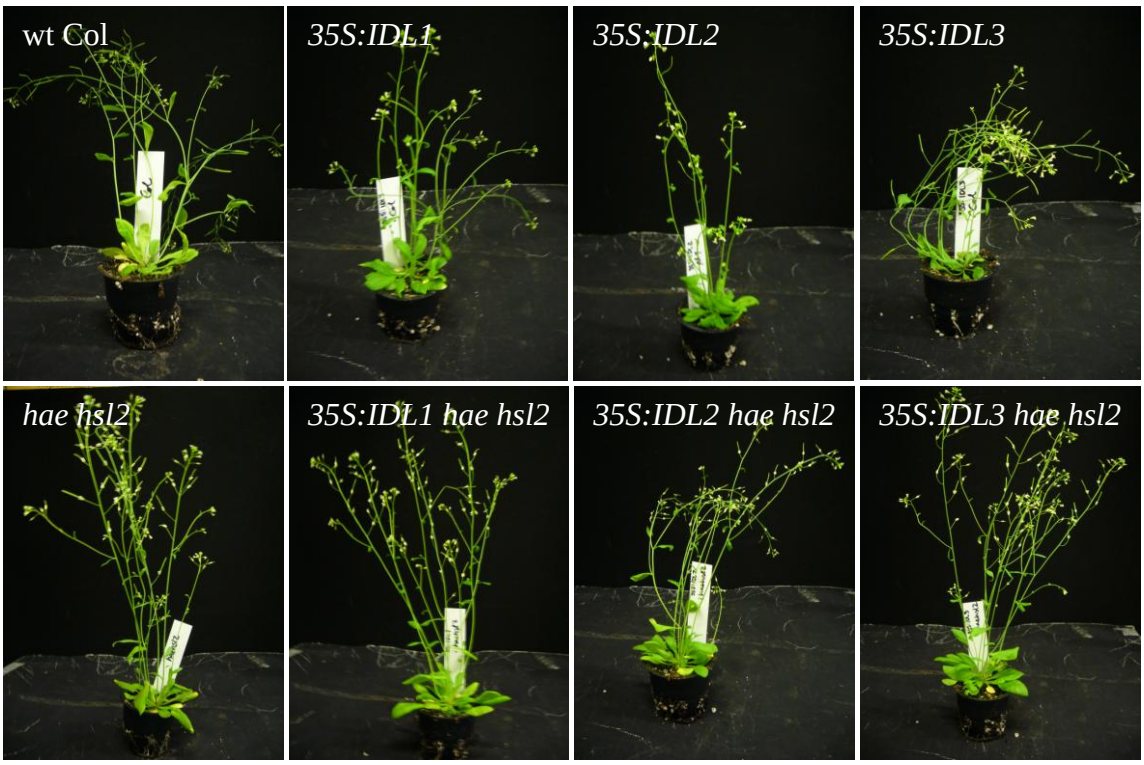


**Figure 3.7 Phenotypes of the transformants.** The *35S:IDL haehsl2* plants retain the *haehsl2* phenotype, masking the *35S:IDL* phenotype.

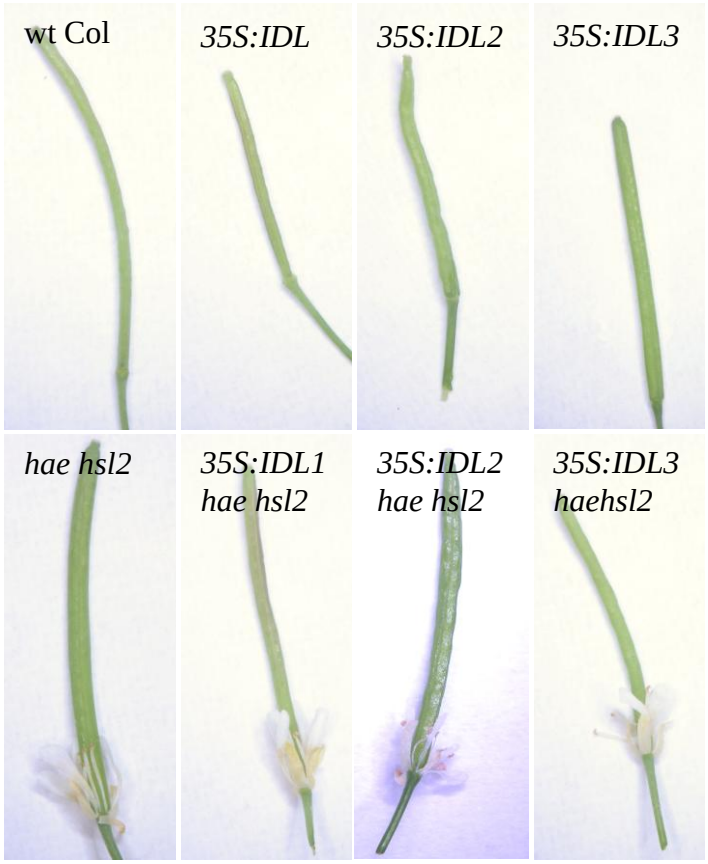


**Figure 3.8 Floral organ phenotypes.** Siliques from position 10. Over-expression of *IDL1*, *IDL2* and *IDL3* in *haehsl2* background does not rescue the *haehsl2* phenotype, indicating that signaling of IDL proteins in the floral organ AZ is dependent on HAE and HSL2

### 3.4.2 *35S:IDL1* plants in *haehsl2* background have long roots

*35S:IDL1* plants, but not *35S:IDA* plants, have been shown to have a specific short root phenotype in wt Col background ( see section 3.3) and these results indicate that HAE/HSL2 is not the receptor pair of IDL1 in the root although IDL1 is able to interact with HAE and HSL2 in AZs. On this background it was interesting to investigate the *35S:IDL1* root phenotype in a *haehsl2* background, and also because *HSL2* is expressed in the root cap of the main root (Riiser, 2009), and *haehsl2* have long roots (Riiser, 2009).

T1 plants of *35S:IDL1*, *35S:IDL2* and *35S:IDL3* in *haehsl2* background were selected on vertical MS/hygromycin plates, and the root length was investigated. Since wt Col and

*haehsl2* plants cannot grow on this medium, the experiment was performed without control plants. The primary results however, did indicate that the roots of the *35S:IDL1* plants in *haehsl2* background were significantly ($p < 0.001$) longer than that of the *35S:IDL2* and *35S:IDL3* plants in *haehsl2* background (figure 3.9). In the wt background the roots of *35S:IDL1* plants are shorter than the roots of *35S:IDL2* and *35S:IDL3* plants (figure 3.3). If one assumes that all the IDL proteins are signaling through the same receptor(s), then one would expect that they would all have the same root phenotype in the *haehsl2* background. The length of the *35S:IDL* roots are however clearly different from the *35S:IDL2* and *35S:IDL3* roots in the *haehsl2* background, indicating that they do not signal through the same receptor(s) in the root.

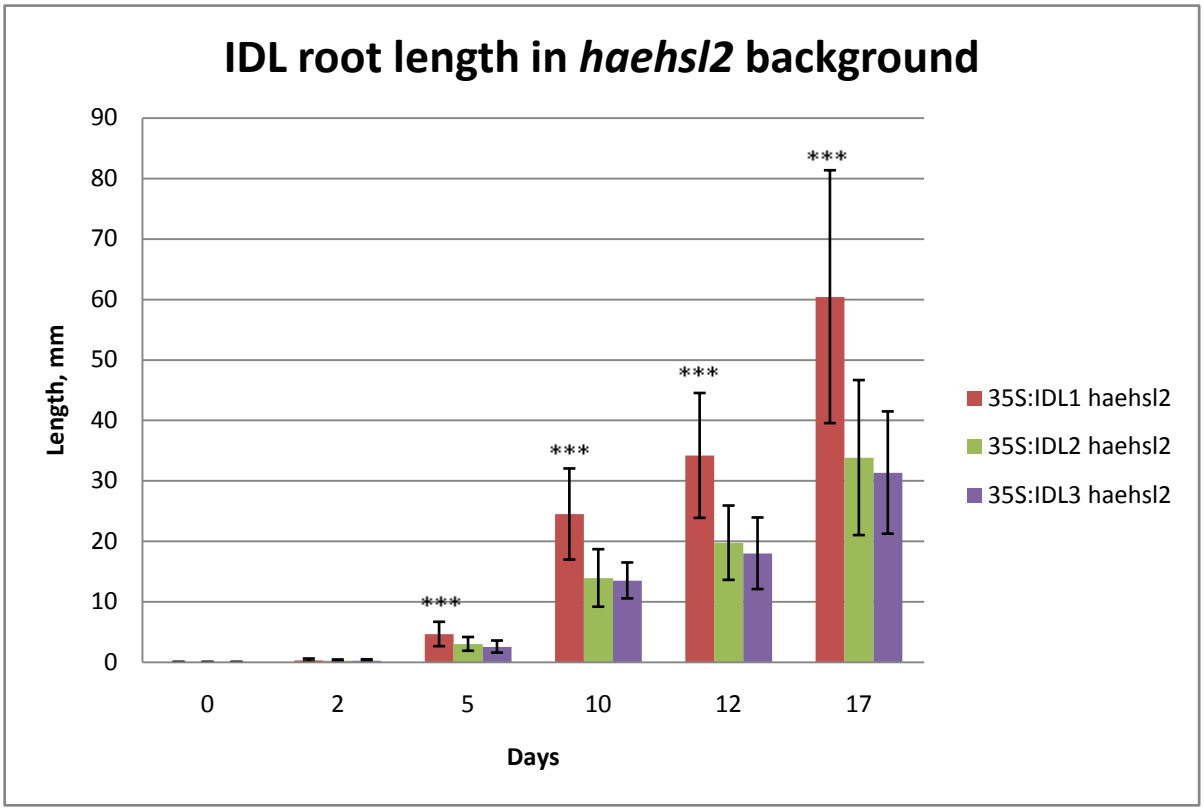


**Figure 3.9 Bar diagram showing length of roots (mm) relative to days after exposure to light.** From day 5 and onwards show that *35S:IDL1hae hsl2* has significantly ($p < 0.001$) longer roots than *35S:IDL2 hae hsl2* and *35S:IDL3 hae hsl2* roots, indicating that HAE/HSL2 could be the receptor of IDL1 in the root. N = 21

Only measuring the root length is however not enough to determine whether *35S:IDL1* in *haehsl2* background have a shorter root phenotype or not. It was also necessary to measure the plant length above-ground as well (figure 3.10).

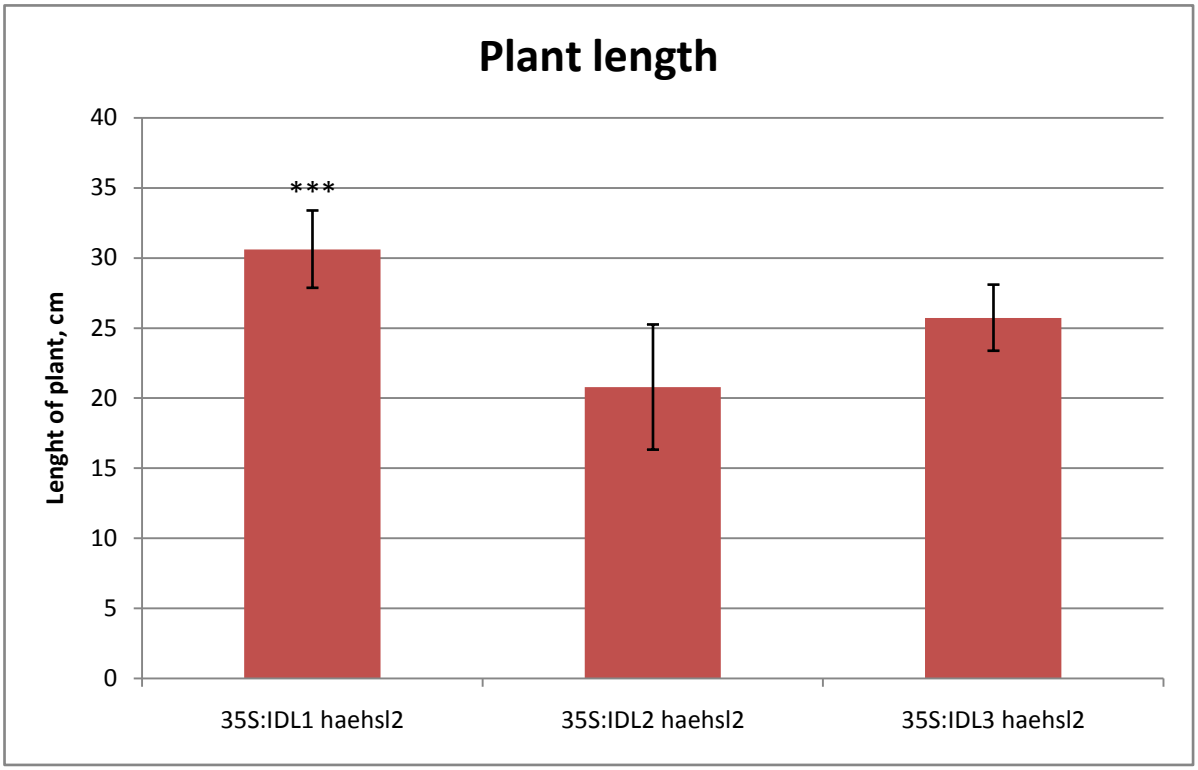


**Figure 3.10 Bar diagram showing length of plants (cm).** The plants of *35S:IDL1* in *haehsl2* were significantly ($p < 0.001$) longer than the *35S:IDL2* and *35S:IDL3* plants in *haehsl2* background. *** $p < 0.001$. N = 10

Measuring the above-ground plant length showed that the *35S:IDL1* plants also were higher above-ground than the *35S:IDL2* and *35S:IDL3* plants. The ratio between plant length and root length showed that the roots of the *35S:IDL1* plants in *haehsl2* background are significantly ($p < 0.05$) longer than the roots of the *35S:IDL2* and *35S:IDL3* plants relative to the above-ground plant length (Table 3.2 and figure 3.11).

| Plant | Ratio plant length:root length | Standard deviation | p-value |
|---|---|---|---|
| *35S:IDL1* in *haehsl2* | 9,4 | 5,049 | 0,01665 |
| *35S:IDL2* in *haehsl2* | 15,4 | 11,379 | 0,53126 |
| *35S:IDL3* in *haehsl2* | 18,4 | 9,491 | 1,00000 |

**Table 3.2 Ratio between plant length and root length**

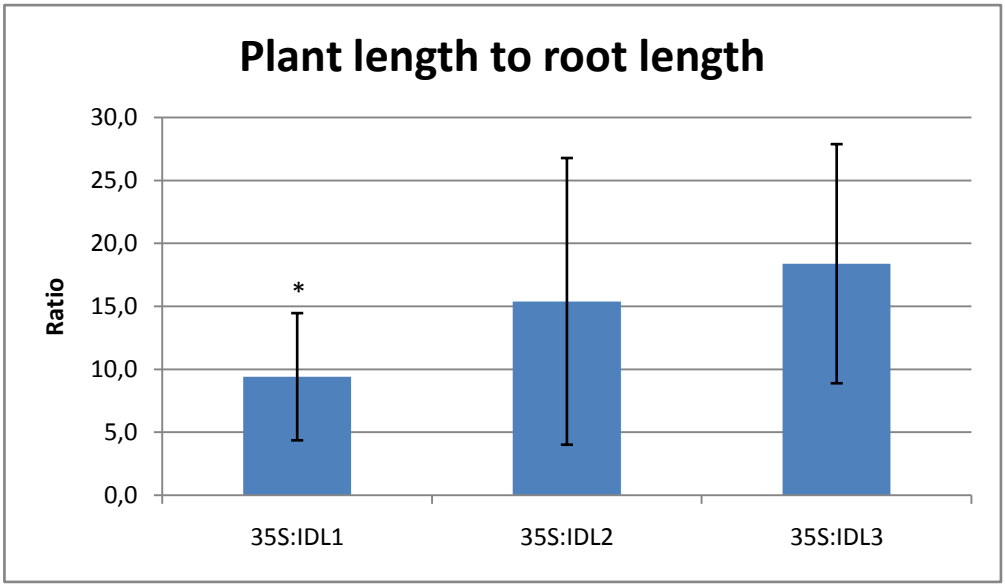


**Figure 3.11 Bar diagram showing ratio between plant length and root length.** The *35S:IDL1* plants in *hae hsl2* background have significantly ($p < 0.05$) longer roots than the *35S:IDL2* and *35S:IDL3* plants in the same background, compared to the above-ground organs. * $p < 0.05$. N = 10

The results from the root experiment, showing that *35S:IDL1* plants in *haehsl2* background have longer roots than *35S:IDL2* and *35S:IDL3* in *haehsl2* background, indicate that IDL1, but not IDL2 and IDL3 signal through HAE and/or HSL2 in the root.

## 3.5 Looking for new partners

When the IDL genes are expressed using IDA's own promoter, only *IDL1* could rescue the *ida* mutant phenotype, while *IDL2*, *3* and *4* gave partial rescue, and *IDL5* no rescue (Stenvik *et al.*, 2008). Thus these genes are only partially redundant with IDA and all the *35S:IDL* phenotypes may not rely on signaling through the HAE/HSL2 receptors. This is the reason for why we are looking for other related receptors that could be partners for the IDL proteins.

*HSL1* and *IKU2L2* are two leucine-rich repeat receptor-like kinases (LRR-RLKs) closely related to the members of the HAESA family (Shiu and Bleecker, 2001a). We were interested in knowing the expression pattern of these two genes in order to see if they could fit as the receptor for some of the IDL proteins. The promoter sequences of the genes were cloned in

front of the *GUS* gene in the vector pMDC 162 and transformed into Col wt plants via *A. tumefaciens*.

The same promoter sequences were also cloned in front of the *YFP* gene in the pHGY vector and transformed into Col wt plants.

### 3.5.1 *pHSL1::GUS* expression

Four primary transformants and six secondary transformants (from the same mother plant) harboring the promoter::reporter gene construct for *HSL1* were investigated for GUS expression. Tissue from seedlings, rosette leaves, cauline leaves, flowers and siliques was incubated in X-gluc mixture at 37 °C for 1 hour (see materials and methods section 2.1.3).

Examination of GUS expression in 14 day old T2 seedlings showed GUS to be expressed in the hydathodes and root tips of seedlings (figure 3.12). Examination of flower buds, flowers, cauline leaves and rosette leaves in adult T2 plants showed GUS to be expressed in stomata and hydathodes. Expression in root tip was not examined in the adult plants. The same expression was observed in three of the primary transformants and all of the six secondary transformants carrying the *pHSL1::GUS* construct.

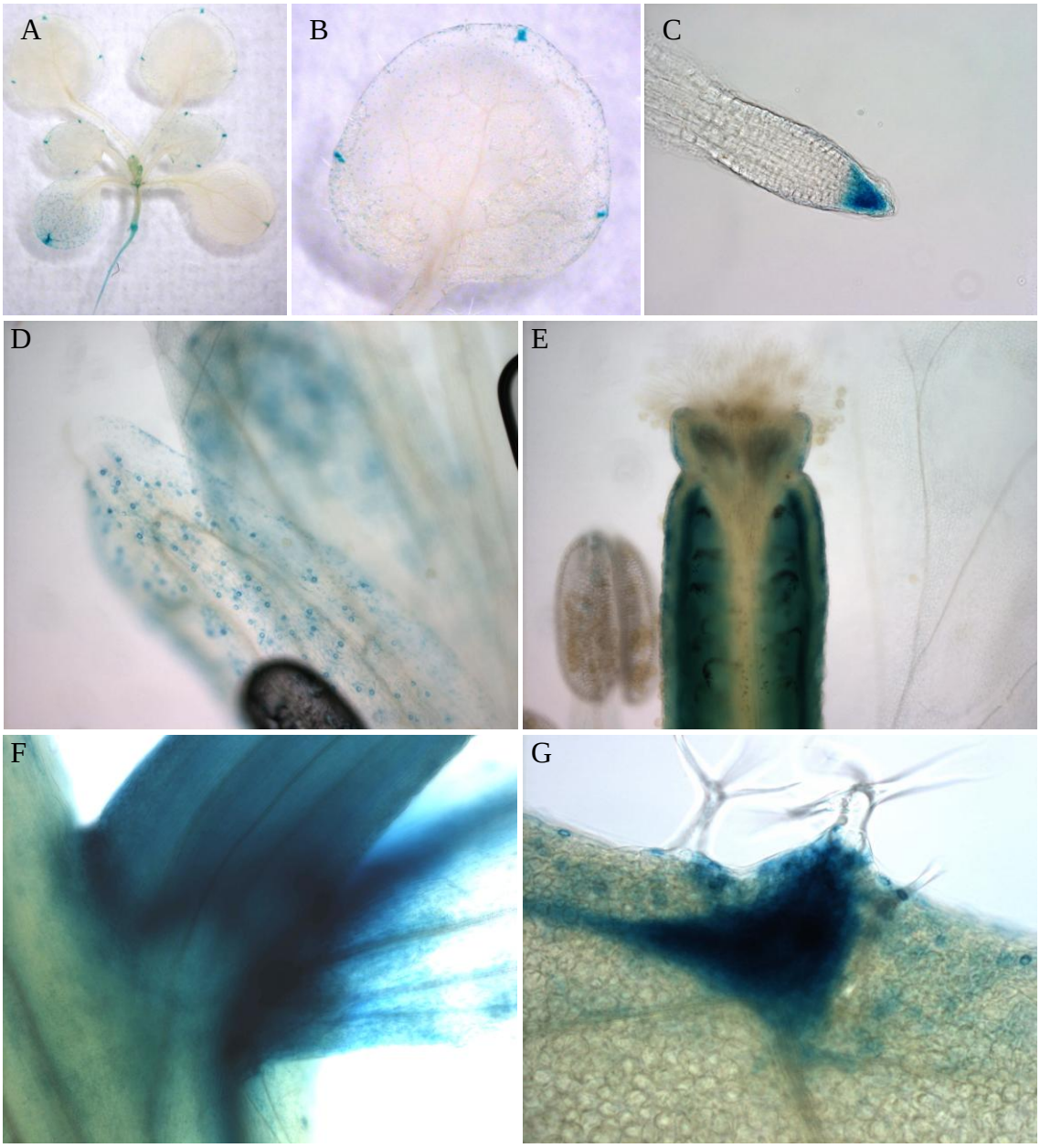


**Figure 3.12 *pHSL1::GUS* expression in T2 plants.** Expression of GUS seen in the entire seedling (A), leaf (B) and root tip (C). Expression of GUS in the guard cells in the petals of the flower (D), carpel (E) branching point (F) and hydathodes of the rosette leaves (G).

### 3.5.1.1 *HSL1* is expressed in the root tip

The GUS expression in the root tip was of special interest as *IDL1* is also expressed in the root cap (Tandstad, 2005). From a timeline set up for *IDL1* it was shown that *IDL1* expression starts in the primary root already at 36 hours after germination (Tandstad, 2005). It was therefore interesting to investigate when the expression of *HSL1* starts in the root tip and a

timeline was set up (figure 3.13). The results show that *HSL1* expression starts at day 4 after germination and continues throughout root development. It is worth noting that *HSL1* is not expressed in the first layer of columella cells as *IDL1* is (Tandstad, 2005), but rather in the second and third layer of columella cells (fig 3.13 D-J).

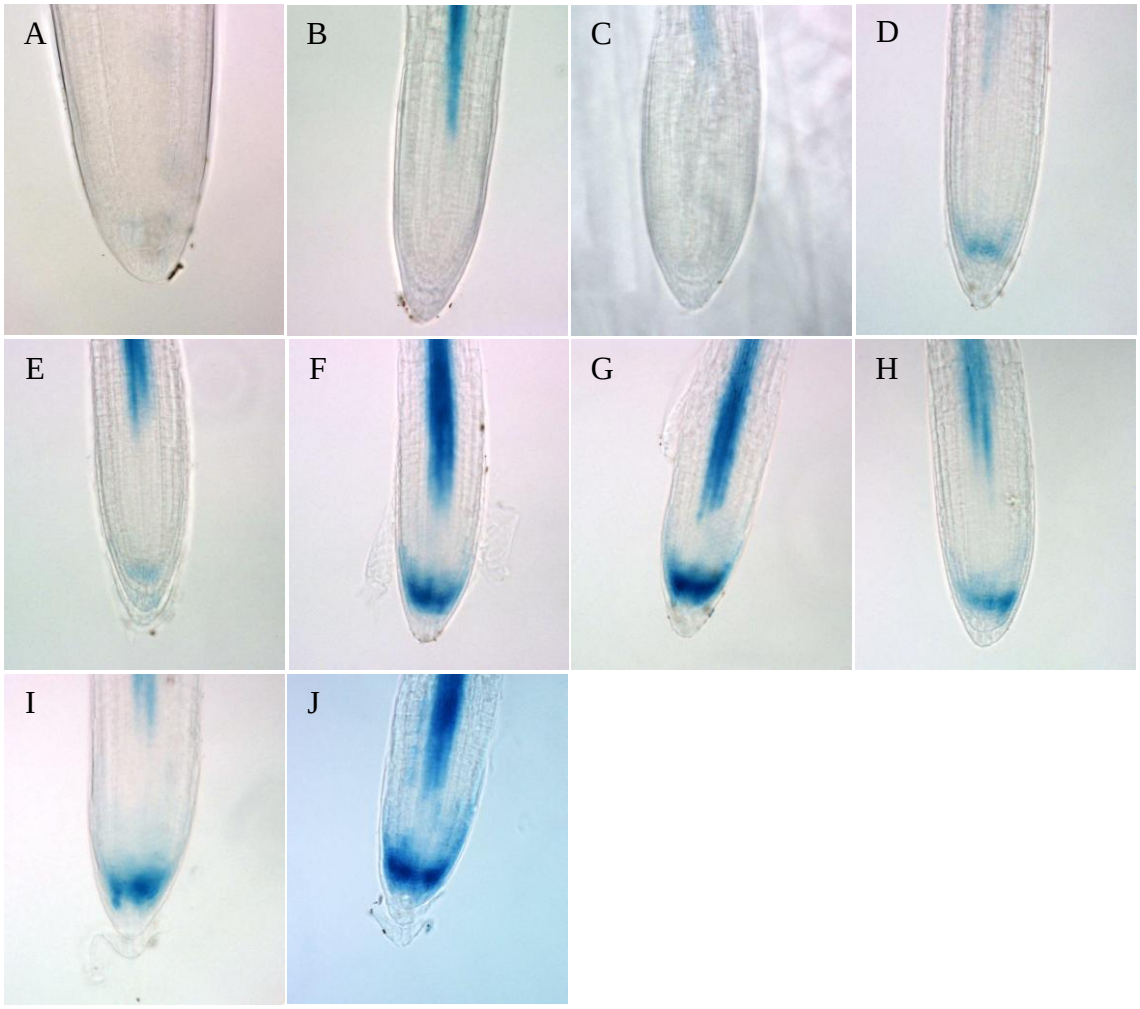


**Figure 3.13 Timeline of the GUS expression of pHSL1::GUS in 1 to 10 day seedlings.** (A to E) Day 1 to day10. GUS expression is first observed at day 4 (D). Also notice the recently shed root cap in E, I and J.

### 3.5.2 *pHSL1::YFP* expression

To confirm the GUS results primary transformants harbouring the promoter::YFP construct were investigated for YFP expression. This was done by Even S. Riiser and postgraduate student Ane Kjersti Vie at NTNU. Three primary transformants harboring the promoter::reporter gene construct for *HSL1* were investigated for YFP expression.

Examination of 14 day old T1 seedlings showed expression of YFP in stomata (figure 3.14). No YFP could be detected in the hydathodes or in the root tips. The same expression was observed for all three lines, confirming the GUS results for stomata expression.

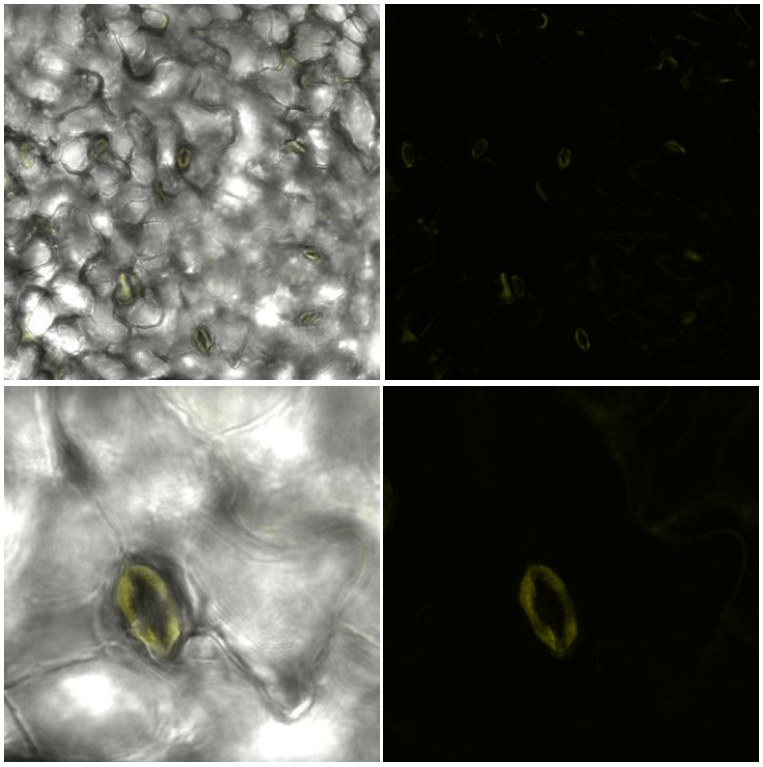

**Figure 3.14 *pHSL1::YFP* expression in stomata.** Examination of 14 day old T1 seedlings harbouring the *pHSL1::YFP* construct showed expression of YFP in stomata.

### *3.5.3* Identifying a SALK line for *HSL1*

Due to the interesting expression pattern of the promoter of *HSL1*, it was decided to try to identify an *hsl1* mutant to look for possible phenotypes. A search for SALK lines with T-DNA insertions within the promoter- or coding regions of *HSL1* was done in the SIGnAL database (http://signal.edu/cgi-bin/tdnaexpress).

Three SALK lines were identified using T-DNA Express, SALK_104365, SALK_108126 and SALK_108127. These were genotyped according to section 2.5.2 to look for a homozygous

T-DNA insertion. Only in line SALK_108127 were homozygous plants found for the T-DNA insertion (figure 3.15).

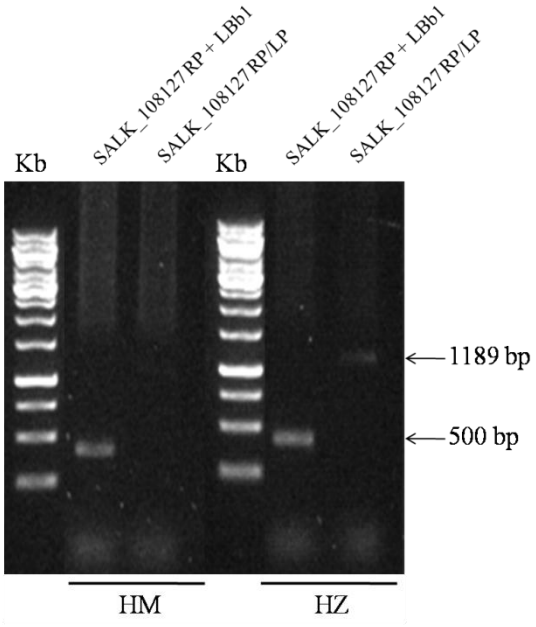


**Figure 3.15 Genotyping of *HSL1* SALK T-DNA insertion line.** Homozygous plants for the T-DNA insertion were only found in the Salk line SALK_108172. For the homozygous (HM) plants for the T-DNA insertion a band of ca. 500 bp was observed using the primers SALK_108127 RP and LBb1, and no band was observed using the two genomic primers (SALK_108127 RP and SALK_108127 LP). Heterozygous (HZ) plants gave two bands, one using the genomic primer and the T-DNA primer of ca. 500 bp and one using the two genomic primers of 1189 bp.

The Salk database predicted the T-DNA insertion to be in exon 1, +1857 bp relative to the start codon. The identification of insertion sites done by the Salk Institute are however high throughput sequencing reactions, and the insertion site could be from 0 to 300 bp from the predicted site. Identification of the correct T-DNA insertion was done by sequencing the flanking LB of the T-DNA and aligned with the genomic sequence of *HSL1*. After sequencing and alignment it was showed that the T-DNA was inserted at +1636 bp relative to the start codon, in the region encoding the LRRs (figure 3.16). An insert in this region would probably lead to a knock-out of the *HSL1* gene due to the fact that the T-DNA would disrupt the open reading frame, producing a dysfunctional protein.

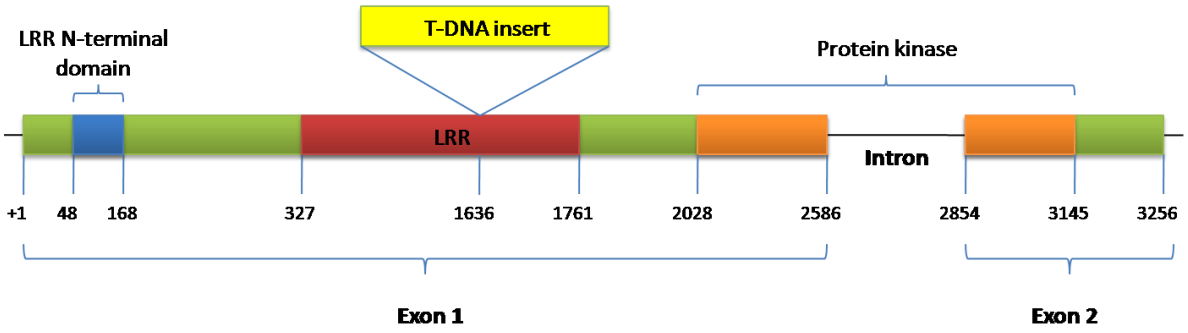


**Figure 3.16 The T-DNA insert in SALK_108127.** The T-DNA insert in SALK_108127 is inserted in the region of the gene that encodes the LRRs, at +1636 bp from the start codon. The figure also shows the LRR N-terminal domain and the protein kinase domain. The figure is not to scale.

### 3.5.3.1 Investigation of the phenotypes of SALK_108127

The homozygous line SALK_108127 with known T-DNA insertion site was investigated for phenotypes. No phenotype in the above-ground organs deviating from the wt phenotype was observed (figure 3.17)


**Figure 3.17 Phenotypes of the homozygous T-DNA insertion line.** Six week old plants. No phenotype in the above ground organs differing from the wt was observed.

As *HSL1* is expressed in the roots it was natural to investigate the roots of SALK_108127. A root experiment comparing the SALK line to wt Col and *35S:IDL1* was set up. The results show that the *35S:IDL1* plants have significantly ($p < 0.001$, except at day 17 where $p < 0.05$) shorter roots than the wt Col plants. SALK_108127 showed no significant difference in root length from the Col plants until day 17, where the roots were significantly ($p < 0.01$) longer than the Col plants (figure 3.18). Opposite phenotypes may indicate that HSL1 could be the receptor of IDL1.

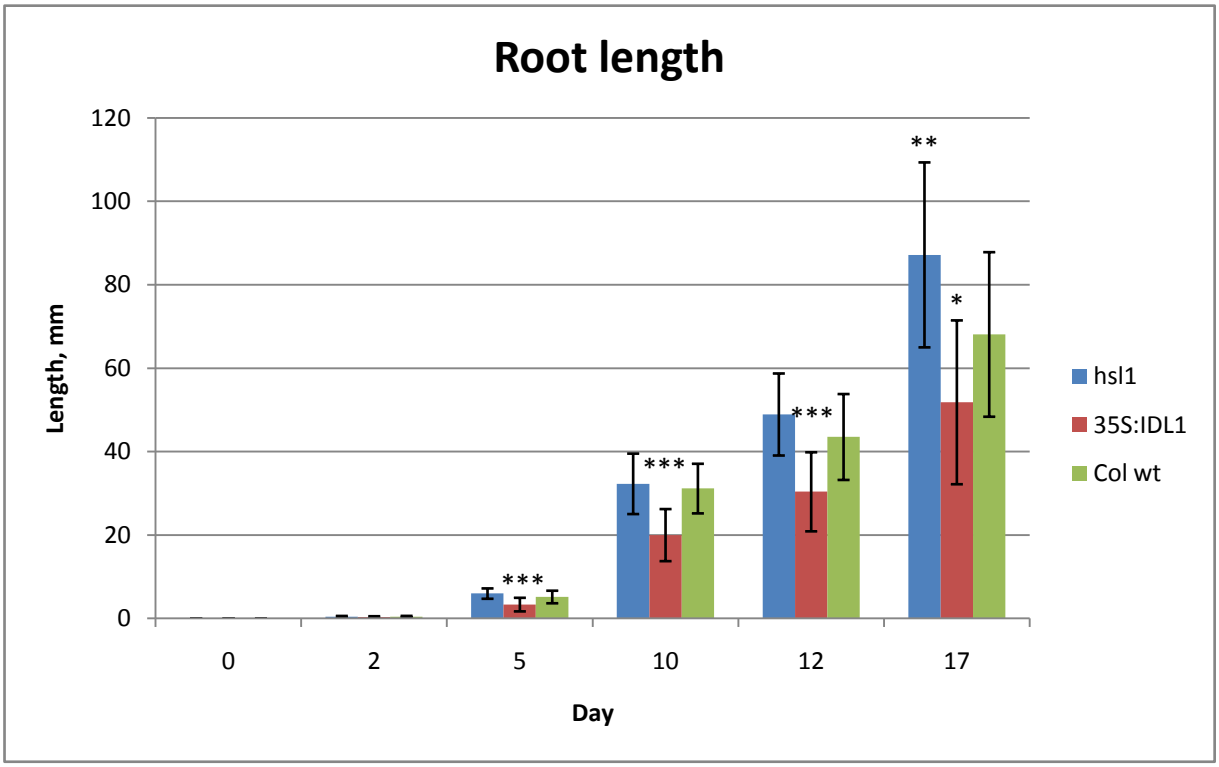


**Figure 3.18 Bar diagram showing length of roots (mm) relative to days after exposure to light.** The SALK line does not show a difference in root length relative to the Col plants until day 17, where the SALK line seems to have significantly ($p < 0.01$) longer roots. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

### 3.5.4 *pIKU2L2*::GUS expression

For the second receptor investigated in this thesis, seven primary transformants and four secondary transformants, from the same mother plant, harbouring the *pIKU2L2::GUS* construct were investigated for GUS expression.

When 14 day-old seedlings were stained, GUS activity was observed in the vascular tissue of the seedlings (figure 3.19 A-C). Examination of the flower bud, flower, rosette leaves, cauline leaves and stalk revealed expression of *IKU2L2* in the vascular tissue of all organs (figure 3.19 D-G). GUS expression was detected in the vascular tissue in six of the primary transformants and all of the secondary transformants.

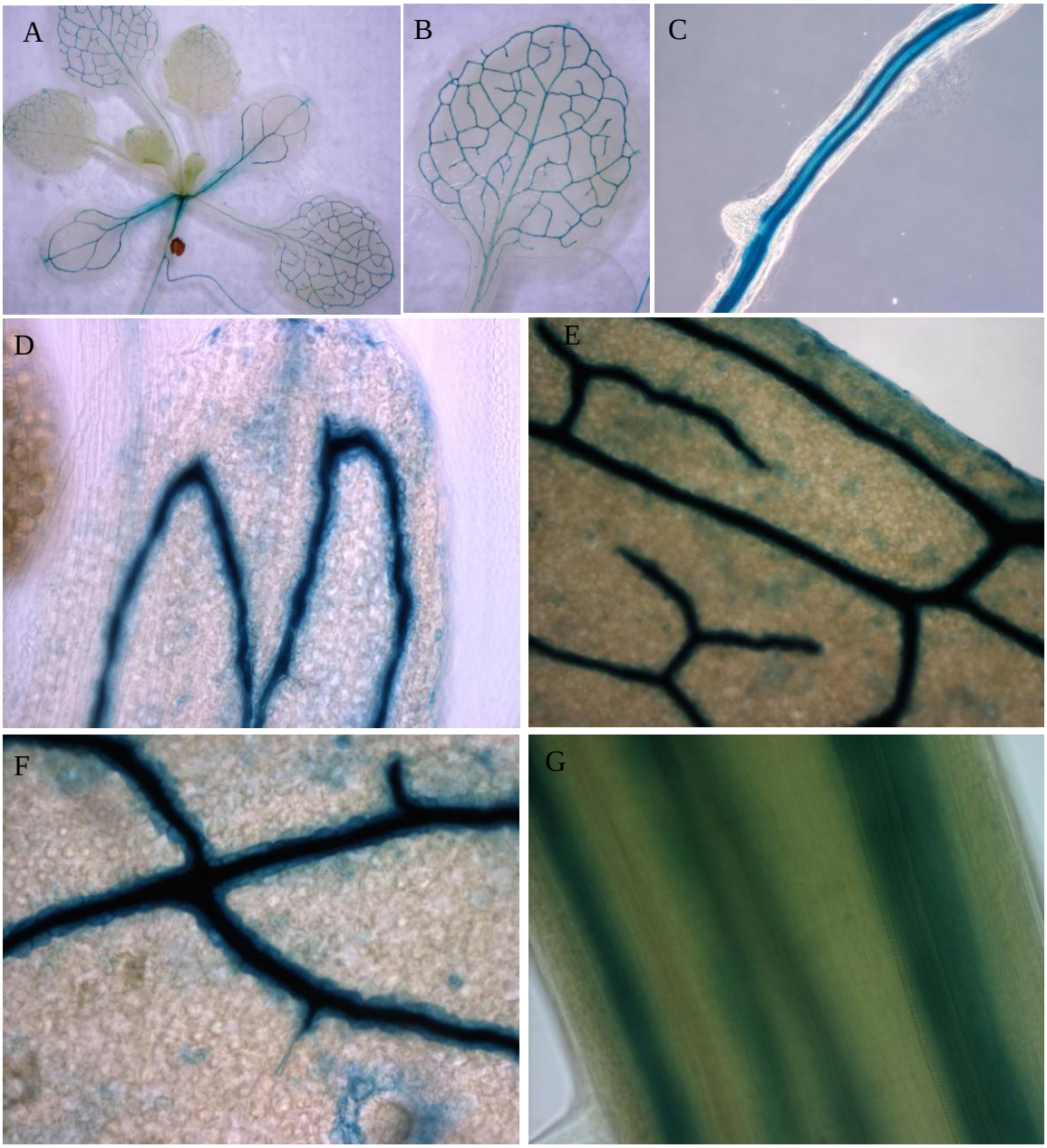


**Figure 3.19 expression of *pIKU2L2::GUS*.** Expression as shown in the entire seedling (A). Leaf (B). Root (C). Vascular tissue in the petal (D). Vascular tissue of rosette leaf (E). Vascular tissue of cauline leaf (F). Vascular tissue of the stalk (G).

### 3.5.4.1 The vascular tissue expression of *IKU2L2*

As *pIKU2L2::GUS* was expressed in the vascular tissue of the seedling it was of interest to see when this expression started. Vascular tissue development is a cell separation event starting after seed germination and during primary growth of the stem (Baucher *et al.*, 2007).

*IKU2L2* could be involved in this cell separation event and a timeline from day 1 to 10 after germination was set up. This showed that GUS was expressed already at day1 in the vascular tissue (figure 3.20 A and B).

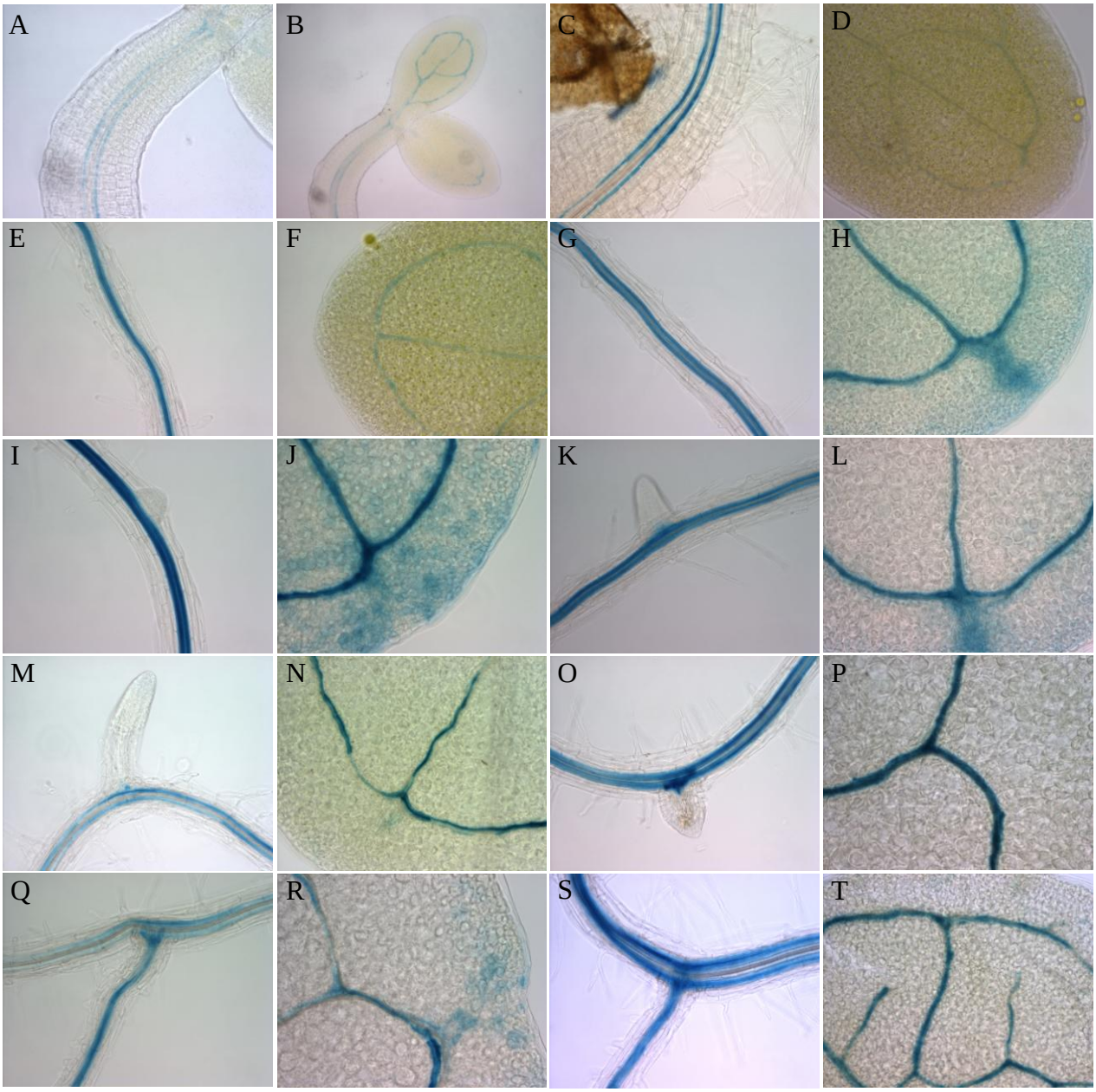


**Figure 3.20 Timeline of the GUS expression of *pIKU2L2::*GUS in 1 to 10 day seedlings.** Day 1 (A, B), day 2 (C, D) day 3 (E, F), day 4 (G, H), day 5 (I, J), day 6 (K, L), day 7 (M, N), day 8 (O, P), day 9 (Q, R) and day 10 (S, T). GUS expression started already at day 1 (A and B).

### 3.5.5 *pIKU2L2::YFP* expression

Three primary transformants harbouring the *pIKU2L2::YFP* construct were investigated for YFP expression. This was done by Even S. Riiser and postgraduate student Ane-Kjersti Vie at NTNU.

Examination of 14 day old T1 seedlings showed YFP to be expressed in the vascular tissue of the seedlings (figure 3.21). The same expression was observed for all three lines, confirming the GUS expression seen in section 4.5.4.

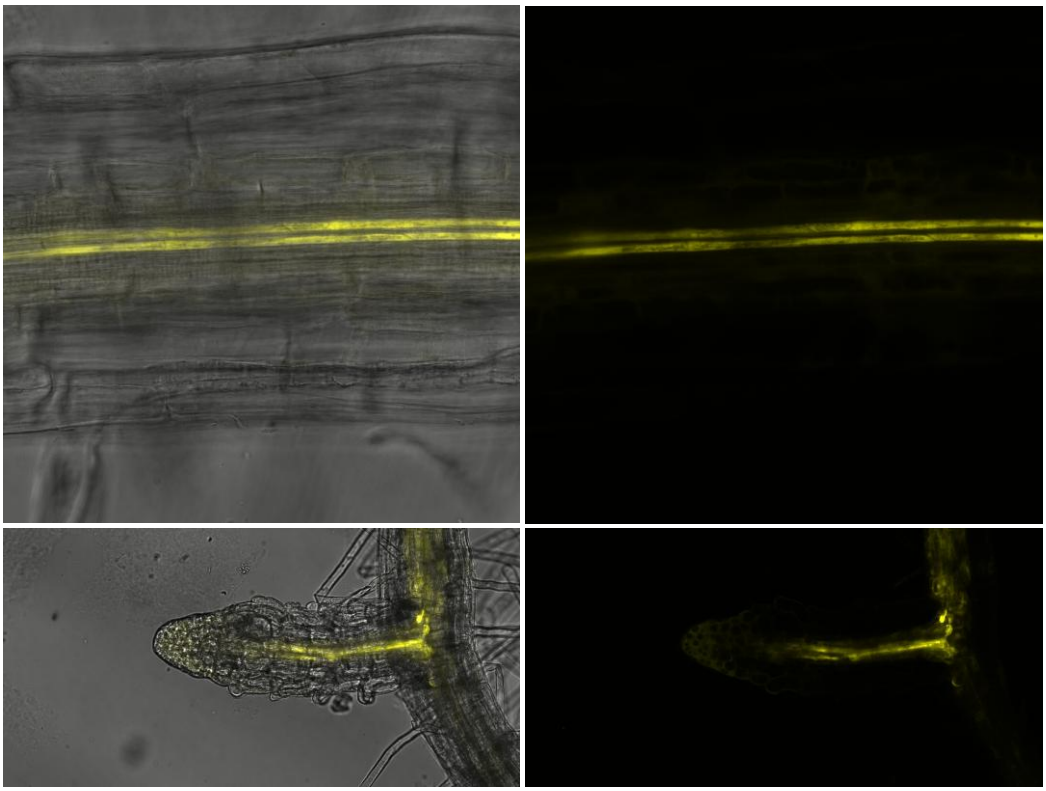

**Figure 3.21 *pIKU2L2::YFP* expression in vascular tissue.** Examination of 14 day old T1 seedlings harbouring the *pIKU2L2::YFP* construct showed expression of YFP in the vascular tissue of the root.

### *3.5.6* Finding a T-DNA insertion line for *IKU2L2*

With the results from the GUS and YFP experiments it was decided to look for an *iku2l2* mutant, in order to study a possible phenotype. The SIGnAL database was searched for a T-DNA insertion line with T-DNA inserted within the promoter- or coding sequence of *IKU2L2*.

One SAIL line was found using the T-DNA Express tool, SAIL_268_H07. Plants from this line were genotyped, using PCR, in order to find a homozygous line. No homozygous or heterozygous plants for the T-DNA insertion were found and, consequently no *iku2l2* mutant was identified.

# 4 DISCUSSION

The IDL proteins are thought to signal through members of the HAE and HSL family of LRR-RLKs (Butenko *et al.*, 2009). By matching the expression of an *IDL* to the expression of an *HSL* we are hoping to identify possible ligand-receptor pairs.

In this thesis the expression pattern of the two genes, *HSL1*and *IKU2L2*, has been investigated using GUS and YFP analysis. Comparison to the expression pattern of the *IDL* genes can be used to propose a number of new ligand-receptor pairs.

Because the *IDL* genes have a similar over-expression phenotype, genetic crosses were used to investigate if the IDL proteins could signal through IDA's receptor in the floral organ AZ, HAE and HSL2 (Cho *et al.*, 2008; Butenko *et al.*, 2009), and furthermore whether the short plant and root phenotypes of the over-expressing lines are likely to signal through the same receptor(s) (Tandstad, 2005).

## 4.1 Matching ligands and receptors based on expression patterns

The *IDL* genes are differentially expressed on sites where cell separation/ degradation of the cell wall take place (figure 4.1) (Stenvik *et al.*, 2008). It is therefore believed that the IDL proteins could be involved in cell separation/degradation processes at different times and places. In a recent paper (Stenvik *et al.*, 2008) it is found that *IDL1* is expressed in the two outermost layers of the columella root cap, *IDL2*, *IDL3* and *IDL4* are, like *IDA*, expressed in the pedicel and in floral organ AZ but with peaks of expression at different positions. *IDL2* and *IDL4* are also detected in the funicle AZ. Expression of *IDL2*, *IDL3*, *IDL4* and *IDL5* is also observed in the vascular tissue (Stenvik *et al.*, 2008).

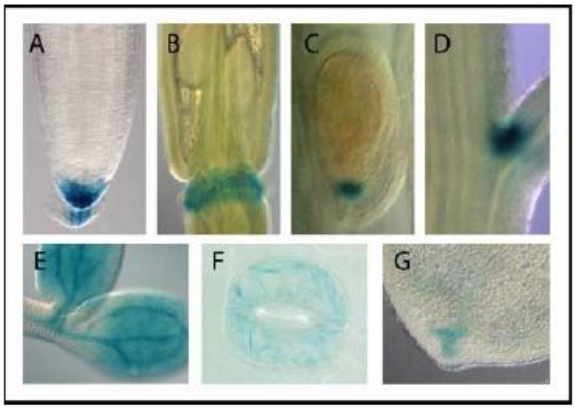


**Figure 4.1 GUS expression under the control of the *IDL* promoters.** (A) *IDL1:GUS* expression in the columella root cap and in cells that are shed from the root. (B) to (D) Expression at the base of pedicel, in the floral organ AZ and the funicle AZ, represented by *IDL2:GUS*. (E) *IDL3:GUS* expression in vascular tissue of a young seedling. Expression in vascular tissue was also observed for *IDL2:GUS, IDL4:GUS* and *IDL5:GUS*. (F) *IDL4:GUS* expression in guard cells. (G) GUS activity was seen in the hydathodes both for *IDL4:GUS* and *IDL5:GUS*, here represented by *IDL5:GUS*. The figure is taken from Stenvik *et al.* 2008.

One approach to match receptors to the different IDL peptides is to find overlapping expression patterns. Due to the common over-expression phenotypes of IDA and IDL proteins we propose that these proteins, if ligands, through their common EPIP motif can interact with the same or similar receptors. Receptor candidates for the five IDL ligands are likely to be found among members of the LRR-RLK receptor family. The phenotypic similarities resulting from over-expression of *IDA* and *IDL* genes suggest that one should start looking among those molecules most closely related to HAE and HSL2. So far only the expression of *HAE* and *HSL2* has been published (Jinn *et al.*, 2000; Cho *et al.*, 2008). By *promoter::GUS* analysis and in situ RNA hybridization *HAE* is shown to be expressed at the base of petioles and pedicels as well as the floral organ AZ (Jinn *et al.*, 2000; Cho *et al.*, 2008). *Promoter:GUS* analysis in the Aalen lab shows that *HAE* and *HSL2* are expressed in the floral organ AZ, base of pedicel and, and in addition at the base of cauline leaf (Riiser, 2009). *HSL2*, but not *HAE,* is also expressed in the columella root cap of the main root (Riiser, 2009).

When examining two close relatives of *HAE*, *HSL1* and *IKU2L2* (Shiu and Bleecker, 2001a; Butenko *et al.*, 2009), we have identified two new putative receptors for the IDL proteins. They both show overlapping expression with several of the *IDL* genes.

### 4.1.1 HSL1

When 14 day old seedlings, buds, flowers, cauline leaves and rosette leaves of plants harbouring the *pHSL1::GUS* construct were examined, GUS was found to be expressed in hydathodes, guard cells and in the columella root cap. From previous GUS experiments it is known that IDL4 and IDL5 are expressed in the hydathodes, IDL4 is expressed in guard cells and IDL1 is expressed in the columella root cap (Stenvik *et al.*, 2008). This not only strengthens the hypothesis that HSL1 could be the receptor of IDL1, but it also introduces the hypothesis of HSL1 being the receptor of IDL4 and IDL5.

From the timeline experiment it became clear that HSL1 is expressed in the root cap first at four days after germination (figure 3.13 D). Also, the expression seems to be confined to the second and third columella layer of the root cap. This is slightly different from IDL1, which is found to be expressed in the first and second columella layer (Tandstad, 2005). This does however not exclude an interaction, as they do in fact overlap in expression in the second columella layer. IDL proteins are also assumed to be exported out of the cell where they are expressed, so that their receptors may not be expressed in the same cell layer.

### 4.1.2 IKU2L2

When 14 day old seedlings, buds, flowers, cauline leaves, rosette leaves and stalks of plants harbouring the *pIKU2L2::GUS* construct are examined, GUS is found to be expressed in the vascular tissue. YFP is also expressed in the vascular tissue, when investigating plants harbouring the *pIKU2L3::YFP* construct, confirming the GUS result. From previous GUS experiments it is known that IDL2, IDL3, IDL4 and IDL5 are expressed in the vascular tissue (Stenvik *et al.*, 2008), indicating that IKU2L2 could be the receptor of any of these putative ligands.

After the timeline experiment it became clear that IKU2L2 was expressed in vascular tissue already at day 1 after germination (figure 3.20 A and B). It would be interesting to repeat this experiment on seeds and siliques to see if IKU2L2 is expressed at an even earlier stage of development. So far no mature or differentiating vascular elements is identified in the *Arabidopsis* embryo, but a continuous network of procambial cells distributed along the

hypocotyl-root axis and the cotyledons make up the embryonic vascular system (Busse and Evert, 1999). Not until germination and during primary growth of the stem does the procambium produce xylem centripetally and phloem centrifugally, leading to the formation of vascular bundles (Baucher *et al.*, 2007). It would therefore be interesting to investigate if *IKU2L2* could be involved in the differentiation and/or formation of vascular bundles.

All of the seeds for the T-DNA insertion line SAIL_268_H07, supplied from NASC, were genotyped to look for a plant homozygous for the T-DNA insertion. Because none of the plants were found to contain the T-DNA insertion an *iku2l2* mutant could not be identified. It would be interesting to order new seeds from the supplier, as the batch of seeds could have been bad, to look for vascular defects in an *iku2l2* mutant, as GUS and YFP analysis confirmed *IKU2L2* to be expressed in the vascular tissue.

## 4.2 The IDL proteins signals through HAE and HSL2 in the floral abscission zone

IDA signals through the receptors HAE and HSL2 in the floral organ AZ (Stenvik *et al.*, 2008). When a single locus *35S:IDA* line was crossed to *haehsl2* the offspring exhibited the *haehsl2* phenotype and none of the *35S:IDA* early abscission of floral organs (Stenvik *et al.*, 2008).

When the *IDL* genes are over-expressed they exhibit a phenotype similar to that of *35S:IDA*, with early abscission and secretion of arabinogalactan from the AZs (Stenvik *et al.*, 2006; Stenvik *et al.*, 2008). This is an indication that *IDA* and the *IDL* genes may act redundantly. On the basis of these observations it is hypothesized that the IDL proteins may act through IDA's native receptor in the floral organ AZ. To investigate this, the interaction between *IDL1* and *HAE* and *HSL2* was first investigated using a yeast two-hybrid assay. The interaction between *IDL1* and its proposed native receptor *HSL1* was also investigated. As the Y2H assay suggested no interaction between IDL1 and HAE, HSL1 or HSL2 it was decided to create a GST (glutathione S-transferase) tagged IDL1 protein. This will be used in further studies to investigate a possible proteolytic processing of IDL1. It was also decided to go back to genetic and *in planta* experiments to further investigate the possibility that the IDL proteins might signal through HAE and HSL1 when expressed in the floral organ AZ. *haehsl2* plants

were transformed with constructs for *35S:IDL1*, *35S:IDL2* and *35S:IDL3* and the transformants were investigated for phenotypes.

### 4.2.1 The yeast two-hybrid suggests no interaction between IDL1 and HAE, HSL1 or HSL2

As IDL1 rescues the *ida* mutant when expressed by *IDA's* promoter and is the IDL protein most similar to IDA it was suggested that IDL1 could interact with the proposed receptors of IDA, HAE and HSL2(Stenvik *et al.*, 2008). A direct mating experiment was performed in order to look for a direct interaction between IDL1 and HAE and HSL2. As both IDL1 and HSL1 and HSL2 are expressed in the root, and HSL1 and HSL2 are suggested to be the native receptors of IDL1, the interaction between IDL1 and HSL1 and HSL2 was also investigated using the direct mating approach.

No growth was observed for the mated cells containing both the BD-IDL1ΔSP fusion protein and the AD-HAE/HSL1/HSL2 ECD fusion protein. These results indicate that there is no interaction between the putative peptide ligand IDL1 and IDAs proposed receptors HAE and HSL2, or between IDL1 and its proposed receptor HSL1. This does neither support the hypothesis that IDL1 can signal through the receptors of IDA, nor does it support the hypothesis that HSL1 could be the native receptor of IDL1. There are however, several weaknesses in using the yeast two hybrid/ direct interaction method, which forces us to interpret the results with caution. First of all is the lack of contextual specificity, as the interaction between bait and prey is confined to the nucleus of the yeast cell (Bao *et al.*, 2009). The interaction of IDL1 and its receptor(s) *in planta* is thought to happen in the extracellular space, between the secreted, extracellular ligand and the LRR-domain of the plasma membrane-embedded RLK. These two compartments have different pH, and proteins are known to be dependent of proper pH for proper folding. If either the peptide ligand or the receptor is wrongly folded in the nucleus there will be no interaction. Second, the recognition of the receptor by the ligand might depend on posttranslational modifications, such as glycosylation, of the LRR region of the RLK (Schaller and Bleecker, 1993). These modifications might not be correctly performed in the yeast. Third, and last, the fusion proteins might not fold and interact as wt proteins or even be transcribed or translated in the

yeast cell. The transcription level or the presence of the fusion proteins was not examined in this experiment.

The nature of the putative ligand-receptor interaction between IDL1 and HAE, HSL1 or HSL2 is not ideal when utilizing the Y2H system. However, it is possible to take certain measures if the experiment is to be repeated. Results from similar studies show that using only fragments of the LRR-domain might yield sufficient results, as in the case of the small protein TPD1 and the LRR-RLK EMS1 (Jia *et al.*, 2008). When investigating the interaction between the TPD1 and EMS1 the researchers created a series of prey vectors constructed by shorter cDNAs for truncated EMS1 LRRs. One of these fragments was found to interact with TPD1. This might also be done for the interaction between IDL1 and HAE, HSL1 or HSL2.

Other promising methods have also been developed for investigating the direct interaction of membrane bound proteins. The membrane yeast two-hybrid (MY2H) system is used to identify interactions between membrane embedded proteins with either membrane bound or cytosolic proteins (Stagljar *et al.*, 1998; Suter *et al.*, 2008b). MY2H is an adaptation of the split-ubiquitin assay (Johnsson and Varshavsky, 1994) where integral or peripheral membrane proteins are fused to the c-terminal half of ubiquitin, followed by a transcription factor. Preys (membrane or cytosolic proteins) are expressed as fusions with the N-terminal half of ubiquitin. Bait-prey interaction reconstitutes native ubiquitin, which is then cleaved by an endogenous ubiquitin specific protease. The transcription factor then enters the nucleus and activates reporter gene expression (Iyer *et al.*, 2005). This approach then avoids the lack of contextual specificity of the ordinary Y2H, as the interaction is allowed to happen outside of the nucleus. However, IDL1, like IDA (Butenko *et al.*, 2003), is thought to act extracellularly, thus making the MY2H assay partly unsuitable for the hypothetical interaction between IDL1 and HAE, HSL1 and HSL2. The group behind the MY2H assay is currently working on developing novel approaches to address potential ligand-receptor bindings (Suter *et al.*, 2008a), and this is of great interest and could be highly relevant in the work to reveal the biochemical interaction between IDL1, HAE, HSL1 and HSL2.

### 4.2.2 An active IDL1 peptide might be delineated using cauliflower meristem extract

CLV3 and the CLE proteins are a family of proteins in *Arabidopsis* that are subject to processing *in planta* (Fiers *et al.*, 2006). The CLE domain of these proteins is the functional

peptide released from a precursor protein (Kondo *et al.*, 2006; Ni and Clark, 2006). A similar mechanism is thought occur in the IDA and IDL proteins, with EPIP, the C-terminally conserved motif of IDA and the IDL proteins, as the active peptide (Stenvik *et al.*, 2008; Butenko *et al.*, 2009). IDL1 might be processed *in planta* and this might explain the lack of interaction in the Y2H assay. IDA has been shown to be processed by cauliflower meristem extracts (Stenvik *et al.*, 2008), and it is of interest to see if IDL1 possess the same ability. Several peptide ligands, such as phytosulfokine, systemin and CLV3 (Pearce *et al.*, 2001; Yang *et al.*, 2001; Matsubayashi, 2003; Fiers *et al.*, 2006; Kondo *et al.*, 2006) are processed *in planta* in order to release functional peptides.

We created a GST-IDL1ΔSP fusion protein, and successfully expressed it in *E.coli*. To confirm the expression Western blots were probed with antibodies against the variable region of IDL1 and GST. The antibody against the variable region of IDL1 resulted in a lot of background noise, making it impossible to draw any conclusions. Anti-GST however, yielded a strong band of approximately 30 kD, corresponding to the 279 aa fusion protein.

When a GST-IDAΔSP fusion protein was incubated in cauliflower meristem extracts it was shown to be proteolytically processed. It would be interesting to see if GST-IDL1ΔSP could be subject to the same processing. It has been shown that IDL1 synthetic EPIP rescues the *ida* mutant, which is an indication that the functional domain of IDA and the IDL proteinss might be the EPIP domain (Stenvik *et al.*, 2008). If IDL1 also is processed, then we would be one step closer to delineate the shortest peptide necessary for biological function and the potential release of this from an IDL proprotein. By incubating the purified GST-IDL1ΔSP fusion protein in cauliflower extracts, re-purification by GST affinity followed by separation on an SDS-PAGE and subsequent detection with an antibody, e.g. anti-GST (Ni and Clark, 2006), one will be able to see if IDL1 is subject to processing. The exact site of processing is then determined by mass spectrometry (Ni and Clark, 2006).

### 4.2.3 Plants over-expressing *IDL1, IDL2* and *IDL3* in a *haehsl2* background retain the *haehsl2* phenotype

Since a biochemical approach could not reveal an interaction between IDL1 and HAE and HSL2 it was decided to look for genetic evidence of an interaction between the IDL proteins and HAE and HSL2 in the floral organ AZ. In wt plants interaction between IDA and

receptors leads to abscission (figure 4.2), when IDA is absent no abscission occur (Butenko *et al.*, 2003), and when IDA is over-expressed the plant exhibits early abscission (Stenvik *et al.*, 2006). When the receptors are knocked-out, as in the case of the *haehsl2* mutant, both the plants normally expressing IDA and the plants over-expressing IDA exhibit no abscission (Cho *et al.*, 2008; Stenvik *et al.*, 2008). As the plants over-expressing *IDL* genes exhibit the same phenotype as the plants over-expressing *IDA*, it was therefore proposed that they signal through the same receptor as IDA.

To test this hypothesis genetically the over-expressing constructs were introduced into a *haehsl2* background. When investigating the above-ground organs of plants over-expressing IDL1, IDL2 and IDL3 they all retained the *haehsl2* phenotype, i.e. no abscission of the floral organs. The *35S:IDL* abscission zone features could not be seen, although they were confirmed to harbour the *35S:IDL* constructs. This is an indication that all the IDL proteins signal through the HAE/HSL2 receptor complex in the floral organ AZ (figure 4.2).

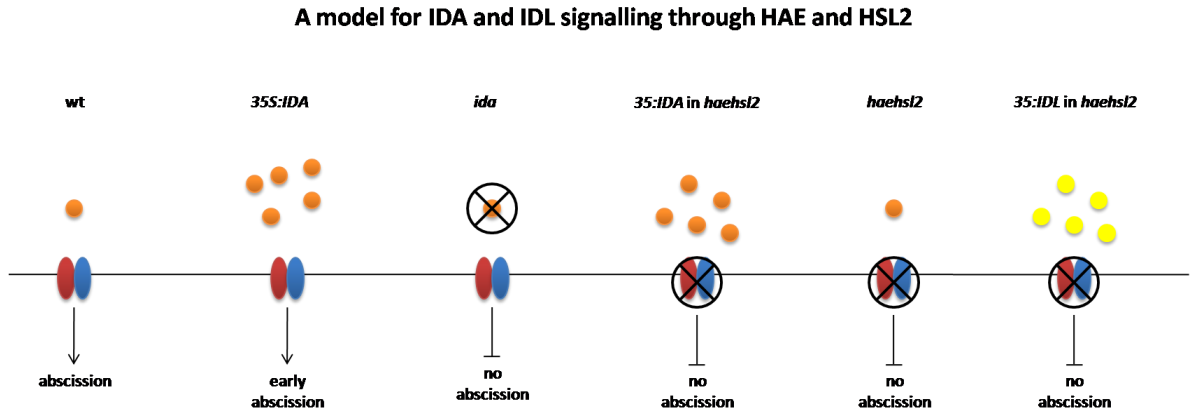


**Figure 4.2 A model for signalling of IDA through HAE and HSL2.** The interaction between IDA and receptors leads to abscission, when IDA is absent no abscission occur, and when IDA is over-expressed the plant exhibits early abscission. When the receptors are knocked-out, as in the case of the *haehsl2* mutant, both the plants normally expressing IDA, and the plants over-expressing IDA, exhibit no abscission. Key: orange: IDA, red: HAE, blue: HSL2

## 4.3 Short roots and putative receptors

Another approach for matching ligands and receptors could be to compare mutant phenotypes. The phenotypes of plants that either over-express a gene or has a mutated gene might tell us something about the function of the gene. The *ida* mutant for instance exhibits no abscission

(Butenko *et al.*, 2003), whereas plants over-expressing *IDA* exhibit early abscission (Stenvik *et al.*, 2006), indicating that *IDA* is involved in the floral organ abscission process. The *haehsl2* mutant does also exhibit a no abscission phenotype (Cho *et al.*, 2008) very similar to that of *ida*. *The haehsl2* mutant does also have a long root phenotype (Riiser, 2009). Preliminary results for an artificial micro RNA (amiRNA) line for IDL1 suggests that down regulation of IDL1 results in a longer meristematic zone, and an amiRNA line for IDL2 exhibits early seed abortion, impaired dehiscence and shedding of seeds (Robert Kumpf and Chun-Lin Shi, unpublished results). The mutation in IDL4 results in fewer lateral roots and a possible problem with the emergence of the lateral roots (Robert Kumpf and Chun-Lin Shi, unpublished results). RNAi lines for IDL1 also showed longer roots (Tandstad, 2005). Mutant lines for *IDL3* and *IDL5* have not yet been investigated. Plants over-expressing the *IDL* genes, however, have a phenotype similar to that of the plants over-expressing *IDA* (Stenvik *et al.*, 2006; Stenvik *et al.*, 2008). Plants over-expressing *IDL1* has also been reported to have shorter roots (Tandstad, 2005).

*IDL1* is expressed in the outermost layers of the columella root cap (Tandstad, 2005; Stenvik *et al.*, 2008) and it is, as the rest of the members of the *IDA* and *IDL* gene family, thought to interact with RLKs (Stenvik *et al.*, 2008). The EPIP domain of IDL1 rescues the *ida* mutant indicating that IDL1 is capable of interacting with the receptors of IDA. Over-expression of *IDL1* in a *haehsl2* background leads to retention of the *haehsl2* phenotype, indicating that IDL1, when expressed in the floral organ AZ interacts with the receptors of IDA, HAE and HSL2. Since IDL1 clearly is capable of signalling through HAE and HSL2 it was of interest to investigate if either HAE or HSL2 or both could be the native receptor of IDL1 in the root. Another proposed receptor is HSL1. Based on microarray experiments and work done in this thesis HSL1 has been shown to be expressed in the root cap of the main root, similarly to IDL1.

### 4.3.1 Over-expression of *IDL1* results in a short root phenotype

In order to see if the IDL proteins can act redundantly, also in other tissues than the AZ of the floral organs, a root experiment was set up. Root length was measured on 2, 5, 10, 12 and 17 day old *35S:IDL* plants grown under normal growth conditions. Compared to the control plants the roots of *35S:IDA*, *35S:IDL1*, *35S:IDL2*, *35S:IDL3* and *35S:IDL5* were shorter. To

answer the question if the shorter root phenotype was due to a specific effect on the root or if it was caused by generally stunted growth it was decided to measure the above-ground plant length and compare the plant lengths and root lengths. Plants with a specific short root phenotype were expected have a plant length to root length ratio larger than the ratio for the control plants, and plants with a stunted growth would have a similar ratio to the control plants. A significant larger ratio was observed for the *35S:IDL1* plants, indicating that plants over-expressing IDL1 have a short root phenotype. None of the other lines were shown to have this phenotype when comparing above-ground plant length to root length. In order to confirm these results they will have to be repeated and preferably the expression level of the *IDL* genes should be measured by qPCR in beforehand, as the *35S* phenotype is dose dependent.

Also, as mentioned earlier, if IDA and IDL1 signal through the same receptors, the plants over-expressing *IDA* should exhibit the same short root phenotype as plants over-expressing *IDL1*. Although *35S:IDA* plants are smaller than wt plants they do however not exhibit a specific shorter root phenotype, which may seem to contradict the hypothesis that HAE and HSL2 could be the receptors of IDL1 in the root.

### 4.3.2 Over-expression of IDL1, IDL2 and IDL3 in *haehsl2* background reveal a long root phenotype

In *haehsl2* background, the plants over-expressing IDL1 was found to have longer roots than plants over-expressing IDL2 and IDL3, the opposite of what was found for *35S:IDL1* plants in Col background. This could be an indication that IDL1 signals through HAE or HSL2 or both in the root. The *haehsl2* mutant has been shown to have long roots, just as *35S:IDL1* plants in a *haehsl2* background. These results are summed up in figure 4.3. Also plants over-expressing *IDL2* and *IDL3* retain their *35S* phenotypes, seemingly unaffected by the *haehsl2* mutation, indicating that they do not signal through the HAE/HSL2 receptor in the root.

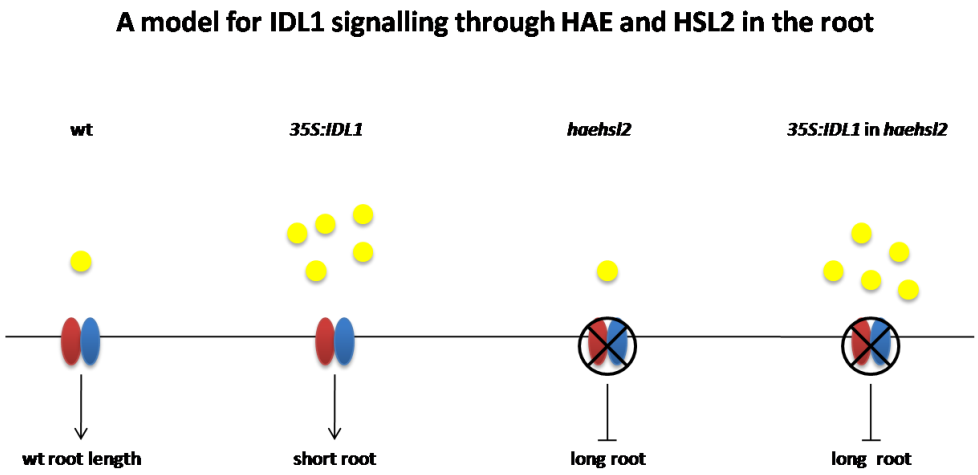


**Figure 4.3 A model for IDL1 signalling through HAE and HSL2.** The normal interaction between IDL1 and receptors leads to wt root length, but when IDL1 is over-expressed the plant exhibits short roots. When the receptors are knocked-out, as in the case of the *haehsl2* mutant, both the plants normally expressing IDL1 and the plants over-expressing IDL1, exhibit longer roots. Key: yellow: IDL1, red: HAE, blue: HSL2.

Based on the results in section 3.4.2 and GUS analysis (Riiser, 2009), a possible interacting partner for HSL1 could be HSL2. Earlier results have shown that IDL2 and IDL3 are capable of signaling through HAE and HSL2 in the AZ. If IDL1 signals through HAE and HSL2 in the root cap, one would also expect IDL2 and IDL3 to be able to interact with HAE and HSL2 in the root cap. One would expect to observe the same long root phenotype in plants over-expressing IDL1 in the *haehsl2* background as well as plants over-expressing *IDL2* and *IDL3* in a *haehsl2* background. However, plants over-expressing *IDL2* and *IDL3* in the *haehsl2*background did not have longer roots and neither does the expression pattern of HAE fit the expression pattern of IDL1, as it is not expressed in the main root cap (Riiser, 2009). These results indicate that HAE7HSL2 might not be the receptors for IDL1 in the root cap. However, HSL2 is expressed in the main root cap (Riiser, 2009), thus IDL1 could signal through HSL2 and maybe another receptor expressed in the root cap, assuming that IDL1 has a higher affinity for the other receptor than IDL2 and IDL3.

The root experiment was performed in T1 generation without a control samples, i.e. wt and *haehsl2* plants as well as *35S:IDL* plants, due to shortage of time. If the experiment is to be repeated it is advised to measure the expression level of the *IDL* genes in addition to having control samples. The controls were left out as that the plants had to grow on a medium containing hygromycin, which is toxic to wt plants, it is therefore also advised to do the experiment in the T2 generation of transformants. Hygromycin was used in the medium in

order to select for *haehsl2* plants harbouring the *35S:IDL* constructs, as this construct contains a hygromycin resistance gene. The long roots observed for the *35S:IDL1 haehsl2* plants could also be the result of low expression of *IDL1* and therefore it would be necessary to investigate the expression level of the *IDL* genes using qPCR.

### 4.3.3 The *hsl1* mutant has long roots

When the Salk line SALK_108127 was examined no differences in the above-ground organs between wt Col plants and the Salk line could be observed. The roots did however prove to be significantly ($p < 0.01$) longer than the roots of the Col control 17 days after germination. This strengthens the hypothesis that HSL1 might be the native receptor of IDL1, as seen in the model proposed in figure 4.4. More studies must however be done to confirm this hypothesis. If a *35S:IDL1* crossed to SALK_108127 also exhibits longer roots, it is plausible that IDL1 signals through HSL1.

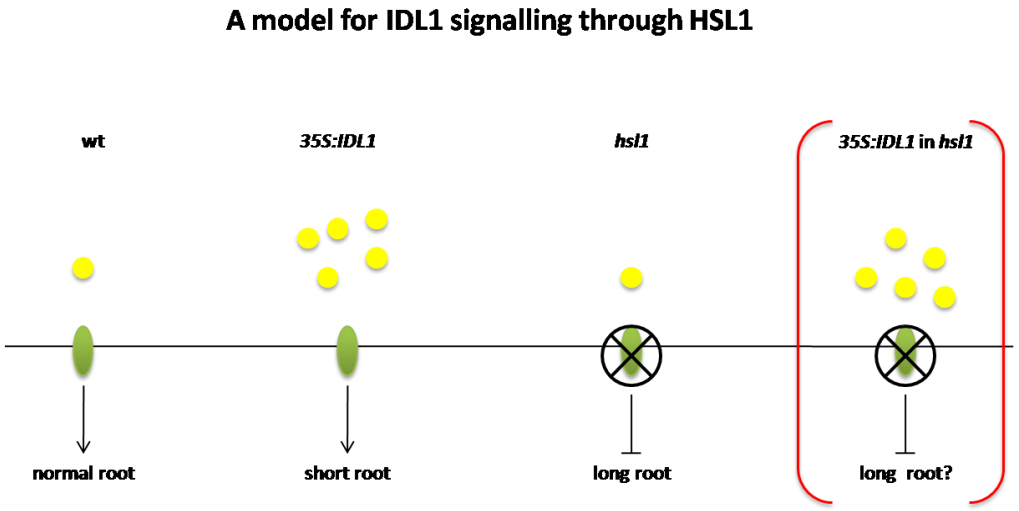


**Figure 4.4 A model for IDL1 signalling through HSL1.** The normal interaction between IDL1 and receptor leads to normal root length, but when IDL1 is over-expressed the plant exhibits short roots. When the receptor is knocked-out, as in the case of the Salk line SALK_108127, the plants normally expressing IDL1 exhibit longer roots. If the long root phenotype is also seen when *hsl1* plants are crossed or transformed to *35S:IDL1* plants (red brackets), the hypothesis is strengthened. Key: yellow: IDL1, green: HSL1.

In addition to HSL2 and HSL1, IKU2L3 is also found expressed in the root cap of the main root (Riiser, 2009), thus there are three candidates for receptors for IDL1. It is possible that IDL1 uses one set of receptors at one particular time of development and another set at another time of development, since HSL1 was not expressed until four days after germination. An interesting approach would be to look at the temporal expression of *HSL2* and *IKU2L3* and compare it to the temporal expression of *IDL1*, in order to see if any of them could be the receptors of IDL1 at an earlier stage of development.

More studies should be done on this and it would be interesting to measure the root lengths of the *hsl2* and *iku2l3* mutant. It would also be just as interesting to measure the root lengths of *35S:IDL1 hsl2* and *35S:IDL1 iku2l3* plants. If IDL1 signals through HSL2 or IKU2L3 one would expect *hsl2, iku2l3, 35S:IDL1 hsl2* and *35S:IDL1 iku2l3* plants to have long roots.

## 4.4 Summary and future perspectives

From the expression study, several novel ligand-receptor pairs can be postulated. The expression of HSL1 in the root cap makes it an excellent candidate receptor for the small signaling peptide IDL1. The HSL1 expression in the hydathodes and stomatal guard cells also makes it a candidate receptor for IDL4 and IDL5. *IKU2L2* was expressed in the vascular tissue and is proposed to be a candidate receptor for IDL2, IDL3, IDL4 and IDL5.

No biochemical interaction between IDL1 and HAE, HSL1 and HSL2 was detected. However, based on a genetic approach it is clear that IDL1, as well as IDL2 and IDL3, acts through the receptors if IDA, HAE and HSL2, when expressed in the floral organ AZ. A GST-tagged IDL1 protein was created in order to investigate possible processing of the ligand necessary for proper function.

Plants over-expressing IDL1 have a specific short root phenotype. When *IDL1* is over-expressed in the *haehsl2* background the plants have a long root phenotype, similar to that of the *haehsl2* mutant, indicating that IDL1 signals through either HAE or HSL2 or both in the root cap. The *hsl1* mutant also exhibits a long root phenotype, indicating that IDL1 signals through HSL1 in the root cap. HAE is not expressed in the columella root cap of the main root, but HSL2 is. It is therefore plausible to propose HSL2 and HSL1 as the native receptor pair of IDL1.

In order to increase our knowledge of peptide signaling in plants, and the mechanisms behind this form of cell communication, it is necessary to investigate putative ligand-receptor interactions between the IDL proteins and the HSL proteins. By doing more biochemical and genetic studies we will be able to identify more new ligand-receptor pairs and the downstream pathways that they regulate. As our knowledge increases, so does the development of new techniques to identify interactions.

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# ABBREVIATIONS

| | |
|---|---|
| 35S | CaMV 35S constitutive promoter |
| ABRC | Arabidopsis Biological Resource Center |
| AD | Activating domain |
| *ADE2* | *Phosphoribosylaminoimidazole carboxylase* |
| AG | Arabinogalactan |
| *Agrobacterium* | *Agrobacterium tumefaciens* |
| amiRNA | Artificial micro RNA |
| Amp | Ampicillin |
| $Amp^R$ | Ampicillin resistant |
| $Amp^S$ | Ampicillin sensitive |
| *AMS* | *ABORTED MICROSPORES* |
| *Arabidopsis* | *Arabidopsis thaliana* |
| *ASHR3* | *ASH1-RELATED 3* |
| *AUX1* | *Auxin influx carrier 1* |
| AZ | Abscission zone |
| BD | Binding domain |
| CaMV | Cauliflower mosaic virus |
| *ccdB* | *Controller of cell division or death B* |
| cDNA | Complementary DNA |
| CDS | Coding sequence |
| CFP | Cyan fluorescent protein |
| *CLE* | *CLAVATA3/ESR-RELATED* |
| *CLV* | *CLAVATA* |
| Col | Columbia (ecotype) |
| *CRN* | *CORYNE* |
| dNTP | deoxyribonucleotide triphoshate |
| *E. coli* | *Esherichia coli* |
| ECD | Extracellular domain |
| *EMS1* | *Excess microsporocytes 1* |
| EPIP | Extended PIP domain |
| EtOH | Ethanol |
| GAL4 | GAL4 transcription factor |

| | |
|---|---|
| GFP | Green fluorescent protein |
| GST | glutathione S-transferase |
| *GUS (gusA)* | *β-glucuronidase* |
| *HAE* | *HAESA* |
| *HIS3* | *Imidazoleglycerol-phosphate dehydratase* |
| HM | Homozygote |
| *HSL* | *HAESA-LIKE* |
| Hyg | Hygromycin |
| HZ | Hemi-/heterozygote |
| IAA | Auxin (indole-3-acetic acid) |
| *IDA* | *INFLORESCENCE DEFICIENT IN ABSCISSION* |
| *IDL* | *IDA-LIKE* |
| *IKU2* | *HAIKU2* |
| *IKU2L* | *HAIKU2-LIKE* |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| Km | Kanamycin |
| LA (medium) | Luria Broth medium w/ agar |
| *lacZ* | *β-galactosidase* in *lac*-operon |
| *Lam* | *Human Lamin C* |
| LB | Left border |
| LB (medium) | Luria Broth medium |
| Leu | Leucine |
| LP | Left primer |
| LRR | Leucine-rich repeat |
| MAPK | Mitogen-activated protein kinase |
| MAT | Mating type |
| *MEL1* | *α-galactosidase* |
| MY2H | Membrane yeast two-hybrid |
| NASC | Nottingham Arabidopsis Stock Center |
| *nptII* | *Neomycin phosphotransferase* |
| OD | Optical density |
| ON | Over night |
| PCR | Polymerase chain reaction |
| PEPR1 | *At*PEP1 receptor |

| | |
|---|---|
| PG | Polygalaturonase |
| pI | Isoelectric point |
| PSK1/PSKR1 | Phytosulfokine/PSK1-receptor |
| QDOX | Quadruple Dropout X-α-gal medium |
| RB | Right border |
| RLK | Receptor-like kinase |
| RP | Right primer |
| S.O.C | S.O.C cell growth medium |
| SAM | Shoot apical meristem |
| SRC/SRK | S-LOCUS CYSTEINE RICH/SRC receptor kinase |
| SDS | Sodium dodecyl sulphate |
| SIGnAL | Salk Institute Genomic Analysis Laboratory |
| SP | Signal peptide |
| Sp | Spectinomycin |
| T1 | First transformant generation |
| T-DNA | Transfer DNA |
| TDO | Triple Dropout Medium |
| *tNos* | *Nopaline synthase* terminator |
| TDP1 | Tapetum determinant 1 |
| Trp | Tryptophan |
| Wt | Wild type |
| X-α-gal | 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside |
| X-gluc | 5-bromo-4-chloro-3-indoyl-β-D-glucuronide |
| YEB-medium | Yeast extract broth |
| YFP | Yellow Fluorescent Protein |
| Zeo | Zeocin |

# APPENDIX 1 – Primer sequences

| Primer | Sequence |
|---|---|
| attB1 At1g28440P SP | 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACTTTGCTTATAACAATCTC-3’ |
| attB2 At1g28440P ASP | 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTATTCTTCGTCTTCCCCGGTATC-3’ |
| attB1 At5g49660P SP | 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATACATTCCAACTCGAAGTG-3’ |
| attB2 At5g49660P ASP | 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTATTCAGAGAAAGATCAAAAGTAACC-3’ |
| attB1 HSL1cds SP | 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTACCCACCGTCTTCTCTCTTAACCAAG-3’ |
| attB2 HSL1cds ASP | 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTACTATACATAGCCTCTCTTCTTAGCTTCA-3’ |
| attB1 IDL1cds SP | 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGCAAGGATCGGACCGATTAAGCTTTCTGA-3’ |
| attB2 IDL1cds ASP | 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGTGTTTGAGATTATTCACCACA-3’ |
| SAIL_268_H07 RP | 5’-GAATCTCCCTTTGGTCGAAAC-3’ |
| SAIL_268_H07 LP | 5’-TACCGGATTCAATCTGCAGTC-3’ |
| SALK_104365 LP | 5’-CTCGTTGATTTAGACCTTGCG-3’ |
| SALK_104365 RP | 5’-AATCCCTTGATATCCCCACAC-3’ |
| SALK_108126 LP | 5’-GCTCGTCAACAACTCGTTCTC-3’ |
| SALK_108126 RP | 5’-GTGAAGATACGAAAGCCCCTC-3’ |
| SALK_108127 LP | 5’-GATCTGTGTGCGAAAGGAGAG-3’ |
| SALK_108127 RP | 5’-CCAAGAGCTTGCAGTCTCTTG’3’ |
| pGEX3’ | 5’-CCGGGAGCTGCATGTGTCAGAGG-3’ |
| pGEX5’ | 5’-GGGCTGGCAAGCCACGTTTGGTG-3’ |
| M13 F | 5’-GTAAAACGACGGCCAG-3’ |
| M13 R | 5’-CAGGAAACAGCTATGAC-3’ |
| Insert screening primer 3’ | 5’-GTGAACTTGCGGGGTTTTTCAGTATCTACGATT-3’ |
| Insert screening primer 5’ | 5’-CTATTCGATGATGAAGATACCCCACCAAACCC-3’ |
| 35S L | 5’-CAACCACGTCTTCAAAGCAA-3’ |
| act2int2_antisense | 5’-CCGCAAGATCAAGACGAAGGATAGC-3’ |
| act2int2_sense | 5’-CCCTGAGGAGCACCCAGTTCTACTC-3’ |
| LBb1 | 5’-GCGTGGACCGCTTGCTGCAACT-3’ |
| LB1_SAIL | 5’-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3’ |
| LB2_SAIL | 5’-GCTTCCTATTATATCTTCCCAAATTACCAATACA-3’ |

| | |
|---|---|
| LB3_SAIL | 5’-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3’ |
| attB2 IDL1 stop | 5’-GGGGACCACTTTGTACAAGAAAGCTGGTTAGTGTTTGAGATTATTCACCACA-3’ |
| attB2 IDL2 stop | 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTACGAGCTATCCAAAAAATA-3’ |
| attB2 IDL3 stop | 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTATTAAGTCTTAGTACTACT-3’ |

# APPENDIX 2 – Statistical data

## Length of roots

| Dag | *35S:IDA* | σ | P-value | *35S:IDL1* | σ | P-value |
|---|---|---|---|---|---|---|
| 0 | 0,000 | - | - | 0,000 | - | - |
| 2 | 0,000 | - | - | 0,000 | - | - |
| 5 | 0,940 | 0,97833637 | 9,36507E-18 | 3,356 | 1,84093467 | 2,01845E-05 |
| 10 | 12,483 | 7,18598962 | 4,55289E-19 | 15,003 | 7,95181815 | 6,61854E-16 |
| 12 | 23,292 | 9,40920295 | 5,56529E-16 | 24,847 | 10,76336916 | 9,06295E-14 |
| 17 | 50,832 | 16,50920908 | 1,00842E-07 | 49,063 | 15,09730075 | 8,70579E-09 |

| Dag | *35S:IDL2* | σ | P-value | *35S:IDL3* | σ | P-value |
|---|---|---|---|---|---|---|
| 0 | 0,000 | - | - | 0,000 | - | - |
| 2 | 0,173 | 0,30962218 | 0,676188584 | 0,113 | 0,25022251 | 0,60927752 |
| 5 | 4,469 | 1,57728460 | 0,015873491 | 4,003 | 2,38006370 | 0,007069775 |
| 10 | 33,309 | 5,20647050 | 0,110258076 | 29,408 | 7,92881532 | 0,002005689 |
| 12 | 46,202 | 5,37655410 | 0,050316277 | 42,690 | 8,14689076 | 0,003707639 |
| 17 | 74,731 | 14,25810247 | 0,864358118 | 67,041 | 18,07240045 | 0,066138543 |

| Dag | *35S:IDL4* | σ | P-value | *35S:IDL5* | σ | P-value |
|---|---|---|---|---|---|---|
| 0 | 0,000 | - | - | 0,000 | - | - |
| 2 | 0,091 | 0,30761452 | 0,441363305 | 0,186 | 0,31283824 | 0,554265303 |
| 5 | 5,740 | 1,87526402 | 0,761578335 | 6,056 | 1,78496039 | 0,342727698 |
| 10 | 35,439 | 7,26442981 | 0,844018069 | 30,175 | 7,40106032 | 0,003521593 |
| 12 | 50,228 | 8,34733021 | 0,993981693 | 41,756 | 10,72613875 | 0,002459016 |
| 17 | 72,756 | 12,60739515 | 0,448416486 | 63,846 | 15,41070371 | 0,005192979 |

| Dag | Col wt | σ | P-value |
|---|---|---|---|
| 0 | 0,000 | - | - |
| 2 | 0,144 | 0,206284721 | 1 |
| 5 | 5,594 | 1,855029951 | 1 |
| 10 | 35,787 | 6,476649126 | 1 |
| 12 | 50,211 | 9,35746967 | 1 |
| 17 | 75,373 | 14,17186272 | 1 |

| |
|---|
| p < 0,001 |
| p < 0,01 |
| p < 0,05 |

## Plant length

| Plant | Mean length | σ | P-value |
|---|---|---|---|
| *35S:IDA* | 20,2 | 4,264271449 | 6,458370173126E-16 |
| *35S:IDL1* | 24,9 | 4,055625135 | 4,17043E-14 |
| *35S:IDL2* | 35,5 | 4,26517459 | 2,00817E-05 |
| *35S:IDL3* | 27,4 | 3,480079364 | 1,4832E-13 |
| *35S:IDL4* | 37,4 | 4,828763028 | 0,002489517 |
| *35S:IDL5* | 33,4 | 7,849640574 | 0,000346864 |
| Col wt | 42,0 | 2,520052909 | 1 |

**Root length in *haehsl2* background**

| Day | *35S:IDL1 haehsl2* | σ | P-value | *35S:IDL2 haehsl2* | σ | P-value | *35S:IDL3 haehsl2* | σ | P-value |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0,000 | - | - | 0,000 | - | - | 0,000 | - | - |
| 2 | 0,334 | 0,249 | 0,2325295 | 0,247 | 0,185 | 0,75735376 | 0,262 | 0,197 | 1,000 |
| 5 | 4,644 | 2,019 | 1,0847E-05 | 3,010 | 1,138 | 0,12364746 | 2,563 | 1,001 | 1,000 |
| 10 | 24,487 | 7,533 | 1,1659E-06 | 13,915 | 4,756 | 0,75026767 | 13,506 | 2,969 | 1,000 |
| 12 | 34,177 | 10,326 | 2,2477E-07 | 19,741 | 6,148 | 0,33442188 | 17,994 | 5,924 | 1,000 |
| 17 | 60,432 | 20,909 | 8,3595E-07 | 33,820 | 12,819 | 0,46432625 | 31,343 | 10,113 | 1,000 |

**Plant length in *haehsl2* background**

| Plant | Mean length | σ | P-value |
|---|---|---|---|
| *35S:IDL1 haehsl2* | 30,6 | 2,758340886 | 0,0005 |
| *35S:IDL2 haehsl2* | 20,8 | 4,466119369 | 0,0062 |
| *35S:IDL3 haehsl2* | 25,7 | 2,359872878 | 1,0000 |

**Root length SALK_108127**

| Day | *hst1* | σ | P-value | *35S:IDL1* | σ | P-value | Col wt | σ | P-value |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0,000 | - | - | 0,000 | - | - | 0,000 | - | - |
| 2 | 0,410 | 0,203 | 0,96803776 | 0,305 | 0,239 | 0,12737937 | 0,412 | 0,212 | 1,000 |
| 5 | 5,972 | 1,241 | 0,08754312 | 3,334 | 1,629 | 0,00076041 | 5,157 | 1,508 | 1,000 |
| 10 | 32,279 | 7,244 | 0,60054474 | 19,989 | 6,239 | 2,0758E-06 | 31,148 | 5,943 | 1,000 |
| 12 | 48,903 | 9,831 | 0,10302508 | 30,372 | 9,473 | 0,00019225 | 43,509 | 10,295 | 1,000 |
| 17 | 87,165 | 22,168 | 0,00724455 | 51,825 | 19,638 | 0,01398912 | 68,091 | 19,719 | 1,000 |