Cell-cell communication in early developing seeds of *Arabidopsis thaliana*

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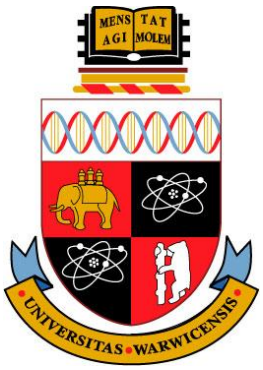

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“The important thing is not to stop questioning.

Curiosity has its own reason for existing.”

Albert Einstein

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Abbreviations:

ANOVA	Analysis of Variance
At	*Arabidopsis thaliana*
BLAST	Basic Local Alignment Search Tool
CaMV	*Cauliflower mosaic virus*
cDNA	copy DNA
CLE8	*CLAVATA3-ESR LIKE 8*
Col-0	Columbia
DAP	days after pollination
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphophates
EDTA	ethylenediaminetetraacetic acid
EMS1	*EXCESS MICROSPOROCYTES*
EXS1	*EXTRA SPOROGENOUS CELL*
ER	*ERECTA*
GFP	green fluorescent protein
GSH	glutathione
IPT	*ISOPENTHYL TRANSFERASE*
IKU2	*HAIKU2*
IKL	*IKU2 LIKE*
LB	Luria Broth
L*er*	Landsberg *erecta*
l.s.d.	least significant difference
LRR	leucine rich repeat
miRNA	microRNA

NaCl	sodium chloride
NaOH	sodium hydroxide
NASC	Nottingham *Arabidopsis* Seed Centre
NCBI	National Centre for Bioinformatic Information
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RNAi	RNA interference
RLK	receptor like kinase
RLP	receptor like protein
SD	short day(s)
SEM	standard error of the mean
SERK1	*SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE 1*
SERK2	*SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE 2*
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
Tris	Tris/Tris HCl 2-Amino-2 (hydroxymethyl) – 1-3 propanediol
WT	wild type
UTR	untranslated region

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Abstract

Sexual reproduction and the formation of seeds are key events in the life cycle of flowering plants. The development of the seed is achieved through the coordinated growth of its three components: embryo, endosperm and maternal integuments. Recent genetic studies have revealed that the tight co-ordination of the growth of the three tissues is mediated by cell-cell communication, most likely involving transmembrane receptors and secreted peptides that act as ligands. However, precisely which proteins are involved and how their activities are regulated remains poorly understood. The aim of my thesis is to investigate the putative roles of receptor like kinases (RLKs) in the coordination of growth during early seed development. Although there are 600 RLKs in the Arabidopsis genome, only a few of them have been assigned with a biological function. Mutation in one leucine-rich-repeat RLK (*IKU2*) gene confers a small seed phenotype. To decipher the role of *IKU2* during seed development and reveal its interaction with other receptor kinases known to play a key role during plant reproduction and development, genetic analyses were performed with *SERK1/SERK2, EMS1/EXS, ERECTA* and the newly identified *IKU2-LIKE*. The results from these analyses, suggest that both *IKU2* and *ERECTA* genetically interact to regulate seed size. Interestingly, this work has also revealed that an *IKU2* homologue, named *IKU2-LIKE* (*IKL*), as well as *EMS1* also genetically interact with *IKU2* and play a role in seed development. To better understand the function of the IKU2 receptor kinase a biochemical analysis was conducted, which revealed that the kinase domain is strictly required for IKU2 function. Together these findings support a pivotal role for *IKU2* and associated receptor like kinases, specifically *ERECTA*, in regulating the co-ordinated growth and development of the Arabidopsis seed.

Declaration:

The present thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Plant and Environmental Sciences. The present work was entirely performed by the student and has not been submitted for any additional degree at any other University.

Chapter 1

General Introduction

The development of multicellular organisms requires correct spatial and temporal expression of genes; a process tightly coordinated by defined signalling pathways (Lindsey, Casson and Chilley 2002). Plants, unlike animals, are sessile organisms that must constantly respond to external and internal stimuli (pathogens, fungi, insects, light and temperature) to regulate their development and physiological processes. Also, plant cells are surrounded by a cell wall and the channels known as plasmodesmata are of fundamental importance for intercellular communication (Cilia and Jackson 2004). To date, a wide range of signalling molecules involved in communication have been identified and consist of phytohormones (Santner, Calderon-Villalobos and Estelle 2009), mobile transcription factors (Schlereth et al. 2010), noncoding RNAs (Carlsbecker et al. 2010) as well as small signalling peptides implicated in short range cell-cell communication (Busch and Benfey 2010, Wu and Gallagher 2011).

1.1 Formation of the seed requires cell-cell communication

Plant sexual reproduction culminates in seed formation which requires the input of various signalling molecules, such as phytohormones, receptors and ligands to coordinate intercellular communication between its three genetically distinct components: embryo, endosperm and maternally derived integuments. Therefore seeds are an excellent model for studying cell-cell communication. Moreover, crop seeds are agronomically important therefore understanding the mechanisms governing aspects of seed development such as regulation of seed size is of great importance. Seeds arise within the female gametophyte from the fusion of female and male gametes during the haploid phase of the plant cycle (Fig. 1.2) (Dresselhaus 2006).

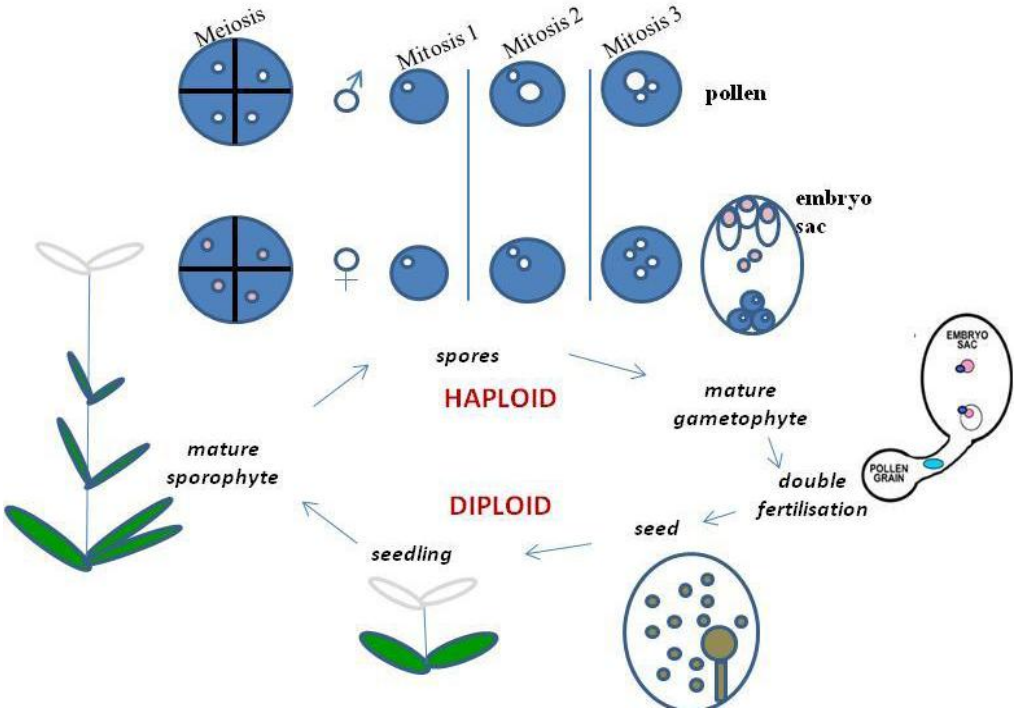

Figure 1. 1 **Schematic diagram of a life cycle of *Arabidopsis thaliana* plants adapted from Berger, Grini and Schnittger 2006**

In flowering plants, the predominant life form is the sporophyte (the vegetative plant). In the flowers, the sporophyte asexually generates female and male spores. The male spore undergoes two rounds of cell divisions. The mature pollen comprises of a large vegetative cell and embedded in this, two sperm cells. Female gametogenesis includes three rounds of nuclear divisions followed by cellularisation, resulting in a seven-celled, eight nucleate embryo sac. The embryo sac contains an egg cell flanked by two synergid cells and the central cell. During the double fertilisation process, the sperm cells are delivered by the pollen tube to the embryo sac. One sperm cell fuses with the egg cell and generates the diploid zygote, whereas the second sperm cell fuses with the central cell giving rise to the triploid endosperm. The endosperm supports the developing embryo of the seed.

In animals, the germ cells are specified during early embryogenesis and remain as separate stem cells. However, flowering plants contain populations of undifferentiated stem cells that continuously produce vegetative tissues and organs in the sporophytic phase, which then later switch to the production of reproductive organs containing diploid sporogenous cells. The developmental signals that activate the fate of the cells to undergo meiotic division and produce megaspore and microspore mother cells of the female and male gametophytes, respectively, are

unknown. During microsporogenesis, the sporogenous cells develop within the anther. The microspore mother cell undergoes meiotic division to produce four haploid gametes collectively known as the tetrad. These tetrads separate and undergo two rounds of mitotic division, generating the mature pollen grain that comprises one vegetative cell and two haploid sperm cells (Fig.1.1) (Drews and Yadegari 2002).

Female gametophyte development occurs within the ovary of the flower. The megaspore mother cell undergoes meiotic division producing four haploid megaspores (Lohe and Chaudhury 2002). Three of these meiocytes degenerate and the remaining one undergoes three rounds of mitotic division to generate the seven celled/ eight – nucleate mature female gametophyte that is common to the majority of angiosperms (Friedman and Diggle 2011). In the maturing embryo sac three nuclei group at the micropylar pole and differentiate into one haploid egg cell flanked by two synergids, another three nuclei are located at the opposite pole and differentiate into the antipodal cells (Fig. 1.1).

A large number of female gametophyte mutants were identified and help to understand this process. In the *female gametophyte* (*fem1*) mutant the female gametophyte disintegrates soon after cellularisation (Drews, Lee and Christensen 1998). Mutations may also affect nuclear division, polar nuclei migration, polar nuclei fusion, cellularisation, and antipodal cell death or proliferation. For instance *hadad* (*hdd), indeterminate gametophyte* (*ig),* and *prolifera* (*prl)* mutants affect nuclear division (Springer et al. 1995, Huang and Sheridan 1996, Moore et al. 1997). The mutations in *SLOW WALKER* genes affect a mitotic cell cycle and thus result in a sterile female gametophyte (Liu et al. 2010, Li et al. 2009). There have been a few genes regulating RNA biosynthesis identified to regulate female gametophyte development. Amongst them genes involved in pre-mRNA spilicing, such as

LACHESIS (*LIS*), *GAMETOPHYTIC FACTOR1* (*GFA1*)/*CLOTHO* (*CLO*) and *ATROPUS* (*ATO*) (Gross-Hardt et al. 2007, Moll et al. 2008, Liu et al. 2009), Mutations in *GAMETOPHYTE DEFECTIVE1* (*GAF1*) encoding a predicted *Arabidopsis* RNase P/MRP protein subunit RPP30 involved in RNA biogenesis resulted in disrupted female gametophytic development (Wang et al. 2012b). Formation of the female gametophyte has also been proved to be regulated by small RNA silencing pathways, since a semi-dominant mutation (*ARGONAUTE5* (*ago5-4))* resulted in defects in the initiation of megagametogenesis (Tucker et al. 2012). Also MYB transcription factors, such as FOUR LIPS (FLP) and its paralogue MYB88, were shown to regulate megasporogenesis via regulation of cell cycle genes (Makkena et al. 2012). There are also signalling molecules involved in megagametogenesis, such as an egg cell specific secreted peptide, *ZmEAL1*, that act as a signal preventing antipodal cells adopting central cell fate (Krohn et al. 2012) and wall-associated RLK *OsDEES1* (Wang et al. 2012a).

The process of double fertilisation initiates when mature pollen grains which carrying two haploid sperm cells land on the receptive female stigma. The directional growth of the pollen tube is controlled by complex interactions with the female sporophyte and gametophyte (Hamamura, Nagahara and Higashiyama 2012). The two synergid cells which flank the egg cell were proved to be the source of gematophytic diffusible signals that attract pollen tubes. Attractant molecules, cysteine rich polypeptides LURE1 and LURE2 were found to be specifically expressed in the synergid cells (Okuda et al. 2009). In *Zea mays* an additional signalling molecule that attracts the pollen tube is EGG APPARATUS 1 (ZmEA1) a small membrane protein necessary for micropylar guidance (Marton et al. 2005). Also, a putative glycosyl phosphatidylinositol (GPI)-anchored protein was identified

to be implicated in reception of the pollen tube by the female gametophyte (Tsukamoto et al. 2010). Small regulatory RNAs have also been shown to be implicated in double fertilisation. Male gametophytic *kokopelli* (*kpl)* mutants encoding *cis*-nat-siRNAs caused a single-fertilisation event, in which only the egg or the central cell was fertilised resulting in seed abortion (Ron et al. 2010). Once the pollen tube reaches the micropyle, the receptive synergid cell starts to degenerate and releases the signal to the pollen tube to stop growth and initiate rupture. As a result the two sperm cells are released, one fertilises the egg cell to produce a diploid zygote that will give rise to an embryo, and the second sperm cell fuses with the homodiploid central cell nucleus to generate the triploid endosperm of the seed. It has been shown that fusion of the central cell with the sperm cell requires the function of BAHD acyl-transferase (Leshem et al. 2012). The fusion of the gametes prevents the attraction of multiple pollen tubes and ensures reproductive success (Beale, Leydon and Johnson 2012).

1.1.1The seed endosperm

Endosperm is a triploid product of a fertilisation of a homodiploid central cell and haploid sperm. The endosperm encapsulates the embryo within the seed and therefore it acts to support and regulate embryonic growth (Costa, Gutierrez-Marcos and Dickinson 2004). Other roles for the endosperm are maintaining high osmotic potential around the embryo and providing mechanical support during early embryo growth as well as storage reserves and nutrients during germination (Chaudhury et al. 2001).

Endosperm development is characterized by four phases: syncytial, cellularisation, differentiation and death. The early phase of endosperm development is marked by nuclear proliferation to generate a large multinucleate cell. Recently, a

cell wall invertase, *GhCWIN1* identified in cotton revealed the important role of CWINs in generating glucose as a signal stimulating nuclear division of the syncytial endosperm (Wang and Ruan 2012). This free-nuclear structure (syncytium or coenocyte) later undergoes cellularisation, a process of partitioning nuclei into individual cells (Olsen 2004, Brown and Lemmon 2001). In addition, cellularisation of the endosperm is patterned along the anterior-posterior axis that runs from the micropylar, embryo surrounding region (ESR) to the chalazal pole, where the maternal vascular tissue adjoins the seed integuments (Berger 2003). This process is however impaired in the Arabidopsis mutant *spatzle* (Sorensen et al. 2002).

Following cellularisation, cell differentiation occurs, which leads to the formation of a variety of tissues including a well-defined outer layer and a central endosperm in Arabidopsis (Berger 1999). Also a polar axis is set up at this stage. In maize and Arabidopsis a micropylar domain/embryo surrounding region (ESR). The ESR consists of small cells with dense cytoplasm which implies high metabolic activity in these cells (Olsen 2004). In maize, the expression pattern of *Esr* genes is endosperm-specific and is restricted to this region and could be involved in either nutrient transfer to the embryo or in signalling processes (Bonello et al. 2000, Bonello et al. 2002, Opsahl-Ferstad et al. 1997). Identification of genes exclusively expressed in the ESR, such as *ZHOUPI(ZOU)/RETARDED GROWTH OF EMBRYO1(RGE1)* (Kondou et al. 2008) and subtilisin-like serine protease *ALE1* (Tanaka et al. 2001) led to the conclusion that the endosperm controls embryo development due to mutations in these genes affecting cuticle formation (Yang et al. 2008, Tanaka et al. 2001).

In maize, the region adjacent to the pedicel (referred to as chalazal in Arabidopsis (Costa et al. 2004)) is characterized by the presence of transfer cells

which are involved in uptake of maternal nutrients (mainly sucrose) into the endosperm (Berger et al. 2006). Two genes have been shown to promote cell fate and differentiation of this tissue type, namely the MYB transcription factor ZmMRP1 (Barrero et al. 2009) and small cysteine rich peptide encoding imprinted gene *Meg1* (Costa et al. 2012, Gutie?rrez-Marcos et al. 2004).

The outermost epidermal aleurone cell layer, and the central starchy endosperm are areas where enzymes involved in starch synthesis are located. During germination, aleurone layer is responsible forf the synthesis of enzymes which mobilise reserves from internal layers. Aleurone is also the site of anthocyanin synthesis (Lopes and Larkins 1993). In the maize mutant, *crinkly4*, the aleurone layer does not differentiate correctly. In rice, similarly to Arabidopsis and maize (Gifford et al. 2005, Becraft, Kang and Suh 2001), *CRINKLY4* (*OsCR4*) RLK was found to promote epidermal cell differentiation in organs forming an interlocking mechanism that protects floral organs and the developing kernel (Pu et al. 2012).

In contrast to maize, the Arabidopsis endosperm is rather ephemeral, as only a single outermost layer of endosperm cells known as the aleurone layer remains in the mature seed. The endosperm in monocots is by contrast persistent and comprises similar cellular domains, but the enlarged cells of central starchy endosperm and aleurone remain until germination (Costa et al. 2004). As a result, the cereal endosperm encapsulates and provides nutrients to the developing embryo (Lohe and Chaudhury 2002). In all plant species the endosperm ceases to exist after germination as the central portion of the endosperm becomes consumed by the embryo. Studies in maize indicate the presence of programmed cell death in the endosperm (Young, Gallie and DeMason 1997). In the *shrunken2* mutant, increase of ethylene production coincides with precocious and more extensive cell death than in

the wild type. It is not clear in seeds with non-persistent endosperm whether endosperm death is autonomous, or linked to signals provided by other parts of the seed (Berger 1999, Olsen 2001).

1.1.2 Embryogenesis

Embryogenesis begins when one of the two sperm cells fuses with the egg cell to form the zygote. The zygote then divides asymmetrically to produce an apical cell that develops into an embryo proper and a basal cell that generates the hypophysis and the suspensor (Fig.1.3). Embryogenesis is characterised by seven main stages: two-celled, four-celled, octant, dermatogens, globular, heart and torpedo. Mutations affecting the developmental stages have helped to characterise embryogenesis.

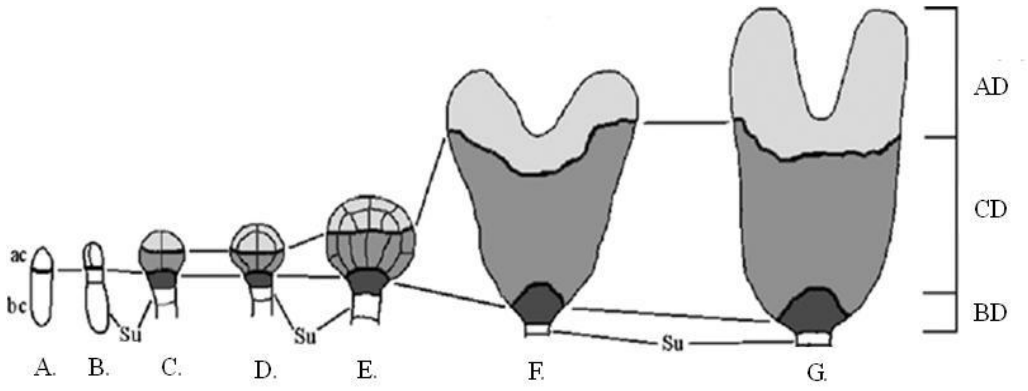

Figure 1. 2 **Schematic drawing showing representative stages of Arabidopsis embryo development**,
(AD) apical , (CD) central and (BD) basal embryonic domains. The developmental stages depicted in the figure involve embryo at (A) two-celled, (B) four-celled, (C) octant-stage, (D) dermatogen-stage, (E) globular-stage, (F) heart-stage, (g) torpedo-stage embryo. ac, apical cell; bc, basal cell; su, suspensor. Adapted from (Park and Harada 2008)

Mutations in the recently identified *LONO1 (LNO1)* gene abolishes first asymmetrical cell division of the zygote, affects early embryogenesis and eventually causes developmental embryo arrest (Braud, Zheng and Xiao 2012). The different fates of the two daughter cells are marked by differential gene expression.

ARABIDOPSIS THALIANA MERISTEM LAYER1 (*ATML1*), a class IV homeodomain-leucine zipper gene normally active later in development in the L1 layer of the shoot apex, is expressed in the apical but not basal cell (Lu et al. 1996). *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes are also differentially expressed in apical and basal cells (Haecker et al. 2004, Breuninger et al. 2008). *WOX2* and *WOX8* are expressed in the egg cell and in the zygote, but their expression becomes restricted to the apical cell and basal cell, respectively, after the first division (Haecker et al. 2004). It was demonstrated that *WOX2* initiates expression of auxin transport protein PIN FORMED1 (PIN1) (Breuninger et al. 2008) and *WOX9/STIMPY* are involved in maintenance and growth of shoot meristem (Skylar et al. 2010). The activation/expression of *WOX8* requires zinc-finger transcription factor *WRKY2* for early Arabidopsis embryo patterning (Ueda, Zhang and Laux 2011). Auxin responsive gene, *PIN7*, is higher in apical than basal cells (Friml et al. 2003). Auxin transport and the localization of auxin maxima are required for many aspects of embryogenesis and postembryonic development (Berleth, Krogan and Scarpella 2004, Barbez et al. 2012). The quadruple mutation of *pin1, pin3, pin4* and *pin7* auxin efflux facilitator genes causes significant defects in apical–basal organization (Friml et al. 2003). A MAP kinase signalling pathway is also implicated in the specification of basal cell lineage. Loss-of-function mutations in the *YODA* gene encoding a MAP kinase kinase cause defects in elongation of basal cells, whereas gain-of-function alleles result in a long suspensor phenotype and suppression of proper embryo development (Lukowitz, Mayer and Jurgens 1996, Lukowitz et al. 2004, Bayer et al. 2009, Wang et al. 2007).

In the octatant stage (Fig.1.2), the embryo can be divided into three domains: the top and bottom halves of the embryo proper corresponds to the apical and central

domains, respectively whereas the hypophysis constitutes the basal domain. It has been shown that Aux/IAA and ARF transcription factors are required for hypophysis specification and root meristem initiation (Rademacher et al. 2012). The apical domain is divided into three subdomains that generate the SAM, RAM and the cotyledons. The SAM consists of a population of small stem cells that will give rise to future plant organs (stem, leaves and flowers).

Studies have identified two classes of mutations affecting postembryonic shoot apical meristem formation. Mutations in *SHOOT MERISTEMLESS* (*STM*), *WUSCHEL* (*WUS*) and *CUP-SHAPED COTYLEDON* (*CUC*) result in seedlings with no or reduced shoot apical meristems (Laux et al. 1996, Aida et al. 2004, Byrne et al. 2000). *STM* is a member of the class I *KNOTTED LIKE HOMEOBOX (KNOX)* gene family and its phenotype in SAM was found to be affected by the expression of the MYB domain protein ASYMMETRIC LEAVES1 (AS1) (Byrne et al. 2000). The *clavata1* (*clv1*), *clv2* and *clv3* mutants have enlarged SAM (Clark, Running and Meyerowitz 1993, Kayes and Clark 1998). The central domain gives rise to the hypocotyls, radicle and part of the cotyledons. There are a few genes that play a role in the organogenesis of a central domain. Among these are *FACKEL* which encodes a sterol C-14 reductase involved in sterol biosynthesis (Schrick et al. 2000), *MONOPTEROS* (*mp*) that encodes an auxin response factor (*ARF5*), and a transcription factor that regulates auxin-responsive genes (Hardtke and Berleth 1998). BDL (IAA12) is an Aux-IAA protein that is thought to bind with MP and inhibit its transcriptional activation (Hamann et al. 2002, Hardtke et al. 2004, Weijers et al. 2005).

The root apical meristem derives from both the central and basal domains of the embryo and gives rise to below ground plant organs. At the globular stage the

hypophysis divides asymmetrically and this is correlated with a shift in auxin concentration maxima within the embryo. Auxin releases from inhibition the *MP/ARF5* transcription factor gene which in turn activates *TARGET OF MP 5* (*TMO5*) and *TMO7* required for root meristem initiation (Schlereth et al. 2010). *OBERON1* (*OBE1*) encoding a plant PHD-finger protein (Saiga et al. 2008) was found to be involved in the mediation of *TMO5* and *TMO7* transcription (Saiga et al. 2012). Auxin influx is mediated by the PIN1, PIN4 and PIN7 facilitators (Friml et al. 2003) and MP was also found to control this mechanism via PIN1 (Weijers et al. 2006). A Rho GTPase is also involved in the trafficking of PIN and thus pattern formation in roots (Lin et al. 2012a) Response to the auxin signal may be mediated at least in part by the *PLETHORA* (*PLT*) genes, *PLT1* and *PLT2* (Aida et al. 2004). *PLT* genes encode putative APETALA2-domain transcription factors whose expression is dependent on auxin and auxin responsive transcription factors, including MP (Galinha et al. 2007).

1.2 Co-ordinated growth and development of the seed

Gametogenesis and fertilisation takes place in an environment where sporophytic and gametophytic structures must interact. Recent genetic evidence supports the view that cell-cell communication exists between the three seed components: endosperm, embryo and maternal tissue (Fig. 1.4) (Harashima and Schnittger 2010) to coordinate seed growth and development. At very early stages, communication is thought to exist between the two female gametes to kick start seed development synchronously.

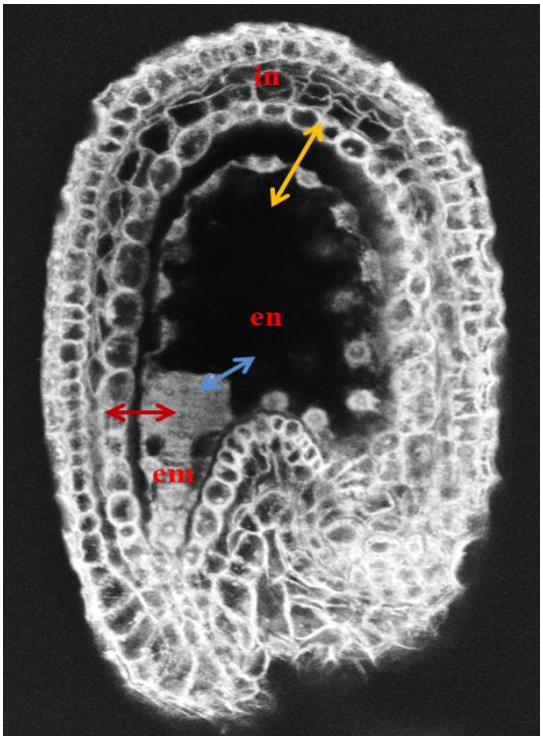

Figure 1. 3 **Diagram highlighting the different seed components and their hypothetical interactions (arrows)**
Cell-cell communication between three seed components: (in) integuments; (en) endosperm; (em) embryo that coordinate seed growth. Red arrow, communication between the embryo and integuments, blue arrow: communication between embryo and endosperm, yellow arrow: communication between endosperm and seed integuments.

Subsequently, the endosperm plays a central role in governing co-ordinated seed growth, as it resides at the interface between maternal and embryonic tissues. The endosperm is uniquely composed of 2:1 maternal:paternal genomes (Walbot and Evans 2003), with a paternal genome excess in the endosperm causing seed overgrowth, whereas a maternal genome excess reduces seed size (Scott et al. 1998). There is also strong genetic evidence that female sporophytic and gametophytic genes govern early endosperm gene expression and thus seed development (Luo et al. 2000).

1.2.1 Coordination of early seed development

Several studies indicate the existence of an interaction between the early embryo (zygote) and endosperm. In the Arabidopsis, *cdc2* mutant pollen carries a single sperm that in most cases preferentially fertilises only the egg cell. Interestingly, when *cdc2* pollen fertilises wild type females, the endosperm develops without fertilisation, thus suggesting that signals must exist between embryo and endosperm to coordinate seed development soon after fertilisation. These signals are unknown but involved in promoting proliferation of the central cell (Nowack et al. 2006) however seed development is not able to proceed past the globular stage. Similarly, in *glauce* mutants (*glc*) the central cell is impaired in fertilisation and embryo development does not proceed past the globular stage (Ngo et al. 2007). A good example of the intercellular interaction between the early embryo and endosperm comes from a study of ablating specific cells in the developing endosperm with a diphtheria toxin. Presence of the toxin in the endosperm resulted in cessation of seed growth (Weijers et al. 2003).

Identification of mutants in which seed development occurs in the absence of fertilisation has given a better insight into the molecular processes controlling the induction of early seed development. *mea/fis1, fis2,* and *fis3/fie* mutants undergo endosperm and seed coat development in the absence of fertilisation (Ohad et al. 1999, Ohad et al. 1996, Chaudhury et al. 1997). Genetic analysis shows that these three mutants segregate gametophytically expressed and maternally expressed in the endosperm post-fertilisation (Ohad et al. 1999, Ohad et al. 1996, Chaudhury et al. 1997). The phenotype of *fis* mutants show arrest of the embryo at the heart stage with the endosperm failing to cellularise (Chaudhury et al. 2001). *FIS1/MEDEA* and *FIS3/FIE* encode members of the Polycomb group (PcG) proteins and *FIS2* encodes

a transcription factor (Grossniklaus et al. 1998, Luo et al. 1999, Ohad et al. 1999). It has been shown that FIS2 together with Polycomb repressive complex 2 (PRC2) targets the type I MADS-box transcription factor AGL62 in the endosperm, a negative regulator of endosperm cellularisation (Hehenberger, Kradolfer and Kohler 2012)

1.2.2 Coordination of late stages of seed development

Studies have led to the identification of several important mutants that show a maternal effect on seed development. These mutants affect gametophytic factors that are expressed in the female gametophyte and sporophytic factors expressed in maternal sporophyte tissues. The evidence for maternal control of seed size is provided by APETALA2 (AP2), a member of the DNA binding domain class of transcription factors. Loss-of-function mutations in *AP2* cause an increase in seed size, by affecting embryo sac development and subsequently early stages of endosperm development (Ohto et al. 2009). In addition, in *megaintegumenta* (*mnt*) mutants (Schruff et al. 2006) the presence of extra integument cells before fertilisation has a maternal, sporophytic effect on seed size. In the *mnt* mutant which contains a lesion in the *ARF2* gene, mutant seeds are heavier than wild type parents, and self-pollinated *arf2* plants have low seed set. The seed coat in *mnt/arf2* mutants appears structurally normal, with only a minor effect on shape, thus communication between the embryo, endosperm, and maternal integuments is essential during seed growth (Schruff et al. 2006). The Arabidopsis *transparent testa glabra 2* (*ttg2)* mutant is defective for proanthocyanidin synthesis and mucilage deposition in the seed coat, and produces smaller, yellow seeds in comparison to the brown and bigger wild type seeds (Johnson, Kolevski and Smyth 2002). *TTG2* encodes a transcription factor of the WRKY family that is expressed strongly in the seed integument and at

low level in the endosperm (Garcia, Fitz Gerald and Berger 2005). The defective seed integuments in *ttg2* mutants have a maternal effect on endosperm growth and seed size. Another maternal regulator of seed size identified is the cytochrome *P450 KLUH (KLU)/CYP78A5*. *KLU* acts in a non-cell autonomous mode of action and is probably involved in the generation of a mobile growth stimulator. However precise identity of this mobile signal is unknown (Adamski et al. 2009)

1.3 Transmembrane receptors and peptide ligands in plant cell-cell communication

Recent biochemical and genetic studies have shown that in addition to hormones, transmembrane receptors and small secreted peptides play a pivotal role in plant cell-cell communication (Fiers, Ku and Liu 2007). Small diffusible peptides act as ligands, which activate membrane bound receptors leading to the initiation of signalling cascades in neighbouring cells thereby triggering a cellular response. Some of the identified receptors that work in conjunction with small secreted peptides are receptor–like kinases (RLKs) that transmit short-range and long-range signalling responses in plants (Dresselhaus 2006).

1.3.1 Transmembrane receptors

Analysis of multiple complete genome sequences revealed that about 20-30% of prokaryote and eukaryote genes encode transmembrane proteins (Komatsu, Konishi and Hashimoto 2007). The Institute for Genomic Research has annotated more than 27000 Arabidopsis proteins (Wortman et al. 2003) and 25% of these are predicted to be integral membrane proteins (Schwacke et al. 2003). Although the biological function of most of them is unknown, and the signals that they perceive

have not been identified, they are all potential receptors in plant cell plasma membranes that control many primary cellular functions, such as metabolite and ion transport, endocytosis, cell differentiation and proliferation.

One of the most highly conserved mechanisms for transducing extracellular signals is the G protein signalling pathway. G protein–coupled receptors (GPCRs) activate heterotrimeric G proteins by inducing the G protein alpha subunit to exchange guanosine diphosphate for guanosine triphosphate at the cell surface. A remarkable property of GPCRs which make it distinguishable from other classes of receptors is the presence of seven transmembrane (7TM) α-helical region (Chen et al. 2003). This class of receptors is not well characterized compared to GPCRs from animal systems. Arabidopsis also contain a regulator of G protein (RGS1) which was shown to stimulate the intrinsic guanosine triphosphate activity of Gα (Chen et al. 2003). RGS1 is a seven transmembrane RGS containing protein that is a putative membrane receptor for D-glucose (Johnston et al. 2007). The putative GPCR (GCRI) identified and investigated in Arabidopsis (Plakidou-Dymock, Dymock and Hooley 1998, Colucci et al. 2002, Apone et al. 2003, Warpeha et al. 2007) was identified by cDNA library screening and named GCRI (Josefsson and Rask 1997). It was shown that the *GCRI* gene is cell cycle regulated and its overexpression abolishes seed dormancy and shortens flowering time (Colucci et al. 2002). It was also shown that the *GCRI* interacts with *GPA1* in Arabidopsis and is involved in ABA signalling in guard cells (Pandey and Assmann 2004). Signal recognition by GPCRs is typically coupled by heterotrimeric G proteins to downstream effectors. Sometimes GPCRs can initiate downstream action independently of heterotrimeric G proteins in response to brassinosteroids (BR) and gibberellins (GA) in seed germination. For example, analysis of *gcr1* mutants indicated that GCR1 plays a positive role in GA

and BR regulated seed germination (Chen et al. 2004). It is also known that GPCR genetically and physically interacts with the G protein alpha subunit (GPA1) to mediate all known ABA responses in Arabidopsis. Overexpression of GPCR gives an ABA-hypersensitive phenotype (Liu et al. 2007). The GPCR also binds to ABA with high affinity at physiological concentration which leads to dissociation of the GPCR-GPA1 complex in yeast (Santner and Estelle 2009). It has been assumed that the GPCR is a new plasma membrane ABA receptor and was named GCR2 (Santner et al. 2009). Despite this, genetic analysis of *gcr2* mutants failed to detect an ABA related phenotype suggesting that GCR2 does not bind to ABA. Loss-of-function *GCR2-like* (*GCL1*) did not confer ABA insensitivity either (Gao et al. 2007). It is unlikely that GCR2 acts as a receptor for ABA in the context of seed germination and early seedling development (Guo et al. 2008). Identification of novel GPCR candidates in plants is difficult due to their low sequence conservation. On the other hand, whole proteome analysis has enabled identification of candidate GPCRs in Arabidopsis, *Oryza* and *Populus* Proteoms (Gookin, Kim and Assmann 2008). Most of them were found to interact with *GPA1* in Arabidopsis. GTG1 and GTG2 are two novel GPCR - type G-proteins that are implicated in ABA response. These proteins interact with Arabidopsis G protein alpha subunit, GPA1. Interestingly these proteins also have intrinsic GTP-binding and GTPase activity and Arabidopsis mutants lacking both *GTG1* and *GTG2* exhibit ABA hyposensitivity which indicates that both of the genes may mediate ABA responses during germination, flowering, stomatal closure and root elongation.

Another characterised transmembrane receptor, which is known to play a role in Poppy self-incompatibility is the male *S* determinant, a *Papaver rhoeas* pistil S (PrpS). *PrpS* encodes a novel 20kDa highly hydrophobic transmembrane protein

with no homology to other known proteins in plant databases (Wheeler et al. 2009). It was also shown that the predicted extracellular loop segment of PrpS interacts with the PrsS, a female pistil S determinant and is involved in *S*-specific inhibition of incompatible pollen (Wheeler et al. 2009). Interestingly *Papaver* PrpS does not contain a kinase domain or any other catalytic domains, which suggests that PrpS is not a receptor kinase. This means that the PrpS - PrsS pair differs to all other known transmembrane receptors identified to date yet has a "receptor - like" function in a specific biological response (Poulter et al. 2010).

1.3.2 Receptor Like Kinases (RLKs) in plants

In the *Arabidopsis thaliana* genome there are around 600 genes encoding RLKs which represents 2.5% of the protein coding genes (Shiu and Bleecker 2001). This is the largest gene family in Arabidopsis, which likely evolved through duplication of the genome and multiple tandem repeats (Shiu and Bleecker 2001). The protein structure of RLKs consists of three domains: an extracellular ligand binding region, single transmembrane spanning domain and a cytoplasmic kinase domain (Fig.1.1).

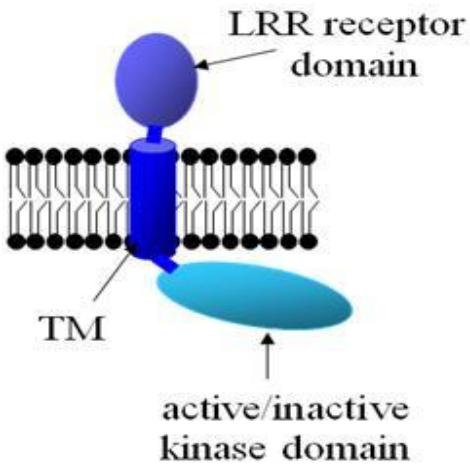

Figure 1. 4 **Diagram showing a receptor like kinase protein**
Upper blue oval represents extracellular leucine rich repeat (LRR), blue cylinder a transmembrane domain (TM), Lower pale blue oval intracellular kinase domain.

RLKs can be grouped into six major subfamilies according to the structure of their extracellular domain (Afzal, Wood and Lightfoot 2008). Among these are the S-domain, TNFR, EGF, PR and Lectin class of receptors. The largest subfamily is represented by leucine rich repeat (LRR) receptors with 200 family members. Despite the great number of receptors in this class, only a small portion have been characterized with biological function and even fewer activating peptide ligands have been identified (Jun, Fiume and Fletcher 2008). Some of the studied LRR RLKs are involved in defence mechanisms (Gomez-Gomez and Boller 2000, Gomez-Gomez and Boller 2002, Zipfel et al. 2006, Scheer and Ryan 2002); however, most RLKs regulate a wide variety of developmental processes such as cell proliferation, differentiation and maintenance of stem cells (Shpak, Lakeman and Torii 2003, Dievart et al. 2003, Gifford et al. 2005).

The first plant RLK gene to be identified was *ZEA MAYS PROTEIN KINASE 1* (*ZmPK1*) of maize (Walker and Zhang 1990) which encodes a putative serine/threonine-specific protein kinase structurally related to receptor tyrosine

kinases. The putative catalytic domain of the ZmPK1 protein is related to the *raf* family of serine/threonine protein kinases while the extracellualar domain shows high similarity to S-LOCUS GLYCOPROTEIN (SLG) (Walker and Zhang 1990)

1.3.3 Receptor Like Kinases in plant development

Various aspects of plant growth and development are regulated by *ERECTA* (*ER*) receptor like kinase (van Zanten et al. 2009). The *ER* family, consists of two other *ERECTA-LIKE 1* (*ERL1*) and *ERECTA-LIKE 2* (*ERL2*) family members (Shpak et al. 2004, Torii et al. 1996). *ERECTA* is known to affect cell proliferation as the dwarf *er* mutants have fewer, but larger cells (Shpak et al. 2003, Shpak et al. 2004). Loss of function at the three family genes results in extreme dwarfism, compact inflorescence and abnormal flowers with defects in ovule development (Pillitteri et al. 2007, Hord et al. 2008). Interestingly, *ERECTA* acts synergistically with other RLKs to regulate specific developmental processes. For example stomata development is regulated by *TOO MANY MOUTH* (TMM) RLK and *ER* (Shpak et al. 2005). ER also regulate the homeostasis of the shoo apical meristem (SAM) and its response to cytokinin (Uchida, Shimada and Tasaka 2012b).

In Arabidopsis, stem cells reside at the centre of shoot and root apical meristems (SAM and RAM, respectively). The SAM is defined by three areas: the central zone (CZ), which contains undifferentiated stem cells just above the organizing centre (OC), the peripheral zone (PZ) containing rapidly dividing initial cells derived from the CZ, and the rib zone (RZ). The SAM generates above-ground aerial organs throughout and therefore must maintain the balance between the self-renewal of a reservoir of central stem cells and organ initiation from peripheral cells. In Arabidopsis, maintenance of pluripotent cells and their differentiation is regulated by the CLAVATA signalling pathway (Clark et al. 1993), and is perhaps the best

studied mechanism of cell-cell communication in plants. The key player of that regulatory mechanism is the small peptide ligand CLAVATA3 (CLV3) (Fletcher et al. 1999). *In situ* matrix assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS) analysis identified a mature form of CLV3 (MCLV3) as a 12-amino acid peptide with two hydroxy prolines (RTVP^{h}SGP^{h}DPLHH) (Kondo et al. 2006). This peptide ligand is perceived through four separate receptors. One consists of the CLV1 RLK that forms a complex with CLV3 at the plasma membrane (Clark et al. 1993, Hirakawa et al. 2008), the second is the RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), also known as TOADSTOOL 2 (TOAD2) (Kinoshita et al. 2010), the third is the CLV2/CORYNE (CRN) receptor complex (Muller, Bleckmann and Simon 2008) and the fourth is FLS2 (Lee, Chah and Sheen 2011). CLV2 is a receptor-like protein that lacks the kinase domain and it binds to the CRN RLK to transmit a signal (Nimchuk et al. 2011). This supports the idea that RLKs act as dimers and often form complexes with other LRR receptors that lack a kinase domain. Results from biochemical studies indicate that while CLV2 and CRN readily form heterodimers and CLV1 forms homodimers, CLV1 and CLV2 do not appear to interact in the absence of CRN, although there is evidence for the formation of a CLV1/CLV2/CRN receptor complex at the plasma membrane (Bleckmann et al. 2010, Zhu et al. 2010). Two other genes regulating *WUS* expression encode protein phosphatases *POLTERGEIST* (*POL*) and *POLTERGEIST LIKE 1 (PLL1)* which are required for stem-cell maintenance. Both of them are negatively regulated by CLV1, CLV2, SOL2/CRN and CLV3 in order to promote the expression of transcription factor *WUSCHEL (WUS)* (Song, Lee and Clark 2006, Simon and Stahl 2006, Yadav et al. 2011, Nimchuk et al. 2011) through other essential components that modulate the pathway,

including KAPP (Williams, Wilson and Meyerowitz 1997), protein phosphatases and proteins involved in folding of the CLV3 signal components (Jun et al. 2008). WUS is a member of the WUSCHEL-related homeobox (WOX) family of homeodomain transcription factors (Laux et al. 1996) and *CLV3* supresses the expression of *WUS* in a non-cell autonomous manner, giving rise to a negative feedback loop that allows to balance stem cell division in the central zone and cell differentiation in the peripheral zone of the SAM (Schoof et al. 2000).

In addition to these phosphatases, a Rho GTPase-related protein (ROP) was found to associate with CLV1, which suggests an involvement of mitogen-activated protein kinase (MAPK) cascade downstream of CLV1 (Trotochaud et al. 1999). A typical MAPK cascade consists of three protein kinases: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK, which sequentially phosphorylate the corresponding downstream substrates. The activated MAPKs phosphorylate various proteins, such as transcription factors, protein kinases, metabolic enzymes and cytoskeletal proteins (Rodriguez, Petersen and Mundy 2010). In Arabidopsis, endogenous application of the synthetic CLV3 triggers the activation of MPK6, a downstream target of CLV signalling that is critical to maintain SAM homeostasis (Betsuyaku et al. 2011). Recently, it has been shown that plant-parasitic cyst nematodes secrete CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR) (CLE)-like ligand that is perceived by the CLV1, CLV2/CRN, and RPK2 receptor complex in nematode CLE perception (Replogle et al. 2012). Remarkably, nematode CLEs have been shown to functionally complement Arabidopsis *clv3* mutants, suggesting conservation of the CLE signalling pathway across kingdoms (Replogle et al. 2012).

The main root stem cell niche is specified during embryogenesis and orchestrates the fine balance of stem cell maintenance and the provision of differentiating descendants. The receptor like kinase ARABIDOPSIS CRINKLY 4 (ACR4) belongs to the CRINKLY4 family is known to play a role in these processes because of its expression pattern in differentiating root cells and mutant root tips forming supernumerary columella stem cells (De Smet et al. 2008). *CLE40* is a small secreted peptide expressed in specific root tissues which acts as a signal for ACR4 (Stahl et al. 2009), and together they control the *WOX5* expression domain and subsequently columella stem cell maintenance. ACR4 is also involved in lateral root initiation by promoting the number of initiation events and restricting the number of divisions contributing to the lateral root initiation site (De Smet et al. 2008).

Some RLKs that have been identified play a role in differentiation of epidermal tissue and other cell types along the radial axis of the Arabidopsis embryo. Among these are ACR4 (Gifford et al. 2005), ABNORMAL LEAF SHAPE2 (ALE2) (Tanaka et al. 2007), RPK1, TOAD2 (Nodine, Yadegari and Tax 2007) and GASSHO1 (GSO1-GSO2) (Tsuwamoto, Fukuoka and Takahata 2008). However there are as yet no ligands known for these RLKs. During floral development some RLKs are known to have a biological function in maintaining the outermost cell layer (L1). STRUBBELIG/SCRAMBLED (SUB/SCM) was shown to act in a non-cell autonomous manner, to control the orientation of cell divisions in L1 cells of the outer ovule integument and the L2 of floral meristems (Yadav et al. 2008). SUB/SCM regulates the formation of the outer integument and the shape of organs, such as carpels and petals, and is necessary for stem shape and height (Chevalier et al. 2005). In addition to its role in the shoot epidermis, SUB/SCM controls cell type specification of epidermal root hairs (Kwak and Schiefelbein 2007). More recently it

was also shown that SUB/SCM mediates developmental signals for leaf patterning and also is important for the balance between cell proliferation and differentiation in leaf morphogenesis (Lin et al. 2012b)

RLKs have also been shown to be involved in stomatal development. Stomata regulate gas exchange and are distributed across a leaf and stem epidermis with characteristic spacing. Stomata are formed in a series of symmetric and asymmetric divisions of the precursor cells. The meristemoid mother cell (MMC) is the first type of stomata precursor. It divides asymmetrically to produce a smaller meristemoid and a larger sister cell. Meristemoids can divide asymmetrically and eventually convert into an oval-shaped guard mother cell (GMC). The GMC divides symmetrically to produce two guard cells, which surround the stomatal pore (Casson and Gray 2008). The *TOO MANY MOUTHS* (*TMM*) gene encodes an RLK and plays a central role in the asymmetric divisions that lead to stomata formation (Geisler, Nadeau and Sack 2000). In leaves, the *tmm* mutation randomizes the plane of formative cell divisions that leads to the formation of dense stomatal clusters. Also the meristemoids divide fewer times before assuming the determinate GMC fate (Nadeau and Sack 2002). This phenotype indicates that TMM is necessary for cells to respond correctly to their position during stomatal development and that TMM participates in the intercellular signalling.

1.3.4. Receptor Like Kinases in plant reproduction

The transcriptional profiling of genes involved in plant reproduction has revealed that the majority of receptor proteins that are expressed in flowers and developing seeds belong to the RLK family (Hennig et al. 2004). This is hardly surprising since this phase of development involves many processes relying on intercellular communication, such as pollen tube growth, guidance and reception,

double fertilisation and development of the embryo and endosperm (Hennig et al. 2004). Male gametogenesis is regulated by LRR RLKs. For example mutations in *SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK1* and *SERK2*) result in male sterility due to the lack of the tapetal cell layer within the anther (Albrecht et al. 2005). The first *SERK* gene identified was reported in carrot (*Daucus carota*) suspension cultures where it was specifically expressed in cells which developed into somatic embryos (Schmidt et al. 1997). *SERK* genes were found to play a role in somatic embryogenesis in a number of species: *Arabidopsis thalina* (Hecht et al. 2001), *Medicago truncatula* (Nolan, Irwanto and Rose 2003), sunflower (*Helianthus annuus*) (Thomas et al. 2004), and *SERK1* expression was found in the Arabidopsis endosperm (Hecht et al. 2001). A member of the same family of receptors, EXTRA MICROSPOROCYTES 1/EXTRA SPOROGENOUS CELLS 1 (EMS1/EXS) (Zhao et al. 2002, Canales et al. 2002), interacts with a small protein TAPETUM DEFECTIVE 1 (TPD1) to ensure correct anther development (Jia et al. 2008, Ge, Chang and Ma 2010). In *ems1* mutants, the lack of tapetum cells results in male sterility. Similar to *EMS1,* two *CLV1*-related receptor like kinases: *BAM1* and *BAM2* are involved in anther development and establishment of cell identities (Hord et al. 2006). An additional LRR RLK, *RPK2* has also been shown to control tapetal and middle layer cell fate in anthers and is suggested to act antagonistically to the *EMS1* and *SERK* mediated signalling pathway (Mizuno et al. 2007).

Most plants are hermaphrodites and self-incompatibility (SI) is the most important out breeding mechanism that prevents self-pollination. This mechanism regulates the acceptance or rejection of pollen in many species and is controlled by a single, multiallelic *S* gene. The SI system also must have two core components: a male (pollen) *S* determinant and female (pistil) *S* determinant. Self-fertilisation is

prevented when pollen carrying an *S* allele is the same as that carried by the stigma on which it lands. Molecular and biochemical studies have identified sterility locus (*S*-locus) derived proteins, S-locus glycoprotein (SLG) and S locus receptor like kinase (SRK) which are specifically expressesed on the stigma surface (Matsubayashi 2003). SRK is a typical RLK containing an SLG like extracellular domain, transmembrane domain and cytoplasmic Ser/Thr kinase domain (Stein et al. 1991). Plants expressing *SLG* alone showed no SI specificity therefore this shows that the SRK alone regulates female (stigmatic) SI specificity and SLG has an accessory role (Suzuki et al. 2000, Suzuki et al. 2003). The plants transformed with *SRK* transgene acquire the rejection of pollen that has the same *S* – haplotype as that of the transgene (Takasaki et al. 2000). The S-locus cysteine-rich peptide (SCR) also called S-locus protein 11 (SP11), localises on the surface of the pollen and was confirmed as the ligand for SRK to play a role in triggering the self-incompatibility mechanism in *Brassica* (Kachroo et al. 2001, Takayama et al. 2001).

Genetic studies also provide evidence for the involvement of RLKs in the fertilisation process in plants. In Arabidopsis, SIRENE(SRN)/FERONIA(FER) CrRLK1L-type RLK (Rotman et al. 2003, Huck et al. 2003, Escobar-Restrepo et al. 2007) and NORTIA (NTA) function together to control pollen tube reception. Mutations in these genes prevent pollen tube rupture within the embryo sac and therefore the female gametes are not fertilised. FER/SRN contain a deletion in a gene that encodes for a RLK that is localised at the filiform apparatus of the synergid cells within the female gemetophyte. Interestingly, these cells have been shown to play a pivotal role in releasing signals in the form of small secreted peptides that are essential for correct guidance of a growing pollen tube towards the micropyle in both *Arabidopsis thaliana* (Kasahara et al. 2005) and *Torenia* species (Higashiyama et al.

1998, Higashiyama et al. 2001, Okuda et al. 2009, Kanaoka et al. 2011). Interestingly, RLKs *ANXUR1* and *ANXUR2*, are expressed in pollen tubes (Boisson-Dernier et al. 2009, Miyazaki et al. 2009). Mutant *anx1/anx2* plants exhibit defective pollen tube rupture, therefore the sperm is not released and thus unable to fertilise wild-type ovules. Although the ligands that directly interact with these RLKs have not yet been identified, the presence of homologous RLKs on both female and male gametophytes, in addition to the presence of SCR peptides, provide strong evidence for cell-cell communication in male and female reproductive organs, which is required for successful fertilisation.

1.3.5 Receptor Like Kinases in plant defence

There are a number of RLKs known to be involved in pathogen defence. Plants have the ability to recognize pathogen associated molecular patterns (PAMPs) that are found in microbial and fungal organisms but not in the host plant. The identification of plant receptors involved in the perception of various elicitors is of great value (Nurnberger and Kemmerling 2006). The first RLK identified involved in elicitor perception was the FLAGELLIN SENSITIVE 2 (FLS2) protein, an RLK with a leucine-rich repeat domain (LRR) that is involved in the perception of flg22 (Gomez-Gomez and Boller 2000). Flagellin is a highly conserved PAMP that is recognized by multiple plant species as well as animals. FLS2 was first identified by map-based cloning and was shown to be expressed in nearly all plant tissues, including flowers, leaves, stems and roots (Gomez-Gomez and Boller 2002). Another LRR-RLK that was identified as the receptor for a bacterial PAMP is the elongation factor Tu receptor (EFR). EFR is a surface receptor for elf18, a highly conserved 18 amino acid elongation factor in bacteria (Zipfel et al. 2006). Another receptor that plays a role in plant wound sites is SR160, which recognizes peptide

ligands systemin. The SR160 was first identified in tomato and it is an LRR-RLK (Scheer and Ryan 2002, Boller 2005).

Chitin is a molecule found in fungal cell walls, induces immune responses in many plant species. Recently, LYK4 receptor like kinase was identified in *Arabidopsis thaliana* as a chitin recognition receptor complex to assist chitin perception and plant innate immunity (Wan et al. 2012). Further studies of a rice *CHITIN ELICITOR RECEPTOR KINASE 1* (*OsCERK1*) homolog in Arabidopsis (*At/OsCERK1*) revealed that the ectodomain of this receptor is capable of chitin perception by itself (Shinya et al. 2012).

1.3.6 Secreted peptides as signalling molecules

There are over 1000 genes in *Arabidopsis thaliana* that encode putative secreted peptides (Butenko et al. 2009). However there is limited information available to link these potential peptide ligands with their receptors and cellular signalling. Moreover, recent experimental work shows that there is functional redundancy between closely related potential peptide ligands. Genetic redundancy complicates the functional analysis of peptide ligands (Butenko et al. 2009). Several secreted and non-secreted peptides are produced by selective proteolitic cleavage from larger propeptides. The Arabidopsis genome has >550 putative protease-encoding genes representing all catalytic types (Beers, Jones and Dickerman 2004). Among the proteases identified in Arabidopsis there are the subtilisin-like serine protease (AtSBT1.1) (Srivastava, Liu and Howell 2008), active serine carboxypeptidase BRASSINOSTEROID-INSENSITIVE1 (BRI1) SUPRESSOR (BRS1) (Li et al. 2001, Zhou and Li 2005), STOMATAL DENSITY AND DISTRIBUTION1 (SDD1) and ABNORMAL LEAF SHAPE (ALE1), which are involved in signalling pathways that regulate the development of stomatal guard

cells (Berger and Altmann 2000) and epidermal formation in embryos and juvenile plants (Tanaka et al. 2001), respectively.

Secreted peptides can be categorized into three groups. In the first group are peptides that are subjected to proteolytic processing, and often undergo complex post-translational modification such as hydroxylation, glycosylation and sulfation, e.g. PHYTOSULFOKINE (PSK), CLAVATA3/ENDOSPERM SURROUNDING REGION (CLE), TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), C-TERMONALLY ENCODED PEPTIDE 1 (CEP1) and ROOT MERISTEM GROWTH FACTOR 1 (RGF1). In the second group there are peptides that form intramolecular disulfide bonds followed by proteolytic processing including RAPID ALKALINIZATION FACTOR (RALF), and EPIDERMAL PATTERNING FACTOR (EPF). The third group consists of peptides that form multiple intramolecular disulfide bonds without proteolytic processing, such as *S*-LOCUS CYSTEINE-RICH PROTEIN/*S*-LOCUS PROTEIN 11 (SCR/SP11), TAPETUM DETERMINANT 1 (TPD1) and LUREs (Fukuda and Higashiyama 2011).

There are many different plant peptides families and only a few of them have been identified with a known function and potential receptor. The first functional plant peptide to be identified was tomato systemin, which is involved in plant wounding response (Pearce et al. 1991, Constabel, Bergey and Ryan 1995). This peptide ligand is mobile within plants (Ryan and Pearce 1998) and systemin receptor represents LRR RLK TOMATO BRASSINOSTEROID INTENSIVE1 (tBRI1) (Scheer and Ryan 1999, Scheer and Ryan 2002, Montoya et al. 2002). Prosystemin orthologs are also found in potato, black nightshade and bell pepper (Constabel, Yip and Ryan 1998).

The study of stomata development has also revealed the key role of plant secreted peptides. Overexpression of *EPF1* specifically affects stomatal development and leads to decreased stomatal density. Thus the number of stomatal cells strictly depends on the frequency of asymmetric cell division, which is mediated by the EPF1 peptide (Hara et al. 2007). *EPF2* limits the final density of epidermal cells by acting as a key component of a negative feedback loop that limits the number of cells entering the stomatal lineage. *EPF2*, like *EPF1*, also affects asymmetric cell divisions during stomatal development and controls stomatal density (Hunt and Gray 2009, Shimada, Sugano and Hara-Nishimura 2011, Lee et al. 2012a). Genetic evidence suggests that *EPF1* and *EPF2* act in the same genetic pathway as the *TMM* receptor and therefore are its best peptide ligand candidates in the process of maintaining stomatal density and pattern (Hara et al. 2009). In addition, *EPF1* and *EPF2* acts upstream of the *ERECTA*-family receptor like kinases (Shpak et al. 2005) and were found to regulate stomatal development (Lee et al. 2012b). The *EPFL9/STOMAGEN* secretory peptide expressed in the inner tissue of immature leaves (mesophyll), also acts as a positive regulator of stomatal density (Sugano et al. 2010) requiring TMM to function (Shimada et al. 2011). In Arabidopsis there are eleven *EPF LIKE* (*EPFL)* genes identified that play a role in stomatal development and other processes (Shimada et al. 2011). Two of them, *EPFL4* and *EPFL6/CHALLAH (CHAL)* are expressed in the endodermis and physically associate with *ERECTA* in planta where they ensure proper inflorescence development (Uchida et al. 2012a).

Another plant peptide ligand phytosulfokine (PSK) was identified as a growth factor, and is responsible for dedifferentiation and callus growth (Matsubayashi et al. 1999). This growth factor was purified from conditioned

medium derived from suspension culture of dispersed asparagus mesophyll cells (Matsubayashi and Sakagami 1996). PSK is a five-residue peptide that undergoes posttranslational sulfation on its tyrosine residues. In addition its role in de-differentiation and callus growth PSK also stimulates tracheary element differentiation of *Zinnia* mesophyll cells without intervening cell division (Matsubayashi et al. 1999). Five paralogous genes encoding 80-residue precursors of PSK were identified in Arabidopsis, with each predicted protein having a probable secretion signal at the N-terminus and a single PSK sequence close to the C-terminus (Yang et al. 2001). In addition there are dibasic amino acid residues immediately upstream of the PSK domain. The families of genes encoding putative PSK precursors also exist in many other plant species including rice, carrots and asparagus with only a few residues being conserved throughout the family. The expression of the gene is not limited to the region in which individual cells actively divide since PSK mRNA is found not only in callus cells but also in the leaves and roots of intact plants (Yang et al. 2001). Acidic amino acid residues flanking the mature PSK sequence in PSK precursors are thought to be involved in tyrosine sulfation, which is catalysed by a tyrozyl protein sulfotransferase in the Golgi apparatus (Hanai et al. 2000). PSK receptor (PSKR1) is a typical LRR RLK (Matsubayashi and Sakagami 2000, Matsubayashi et al. 2002).

Another class of plant peptides involved in floral abscission are INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) small secreted peptides. IDA together with five additional IDA-LIKE members (IDL1-5) belongs to one family that possesses a conserved C terminal domain containing extended PIP motif (EPIP) that has been shown to be the functional unit of IDA (Stenvik et al. 2008, Butenko et al. 2003). The floral abscission defect of the *ida* mutant can be partially

or completely complemented by the EPIP motif of IDL proteins, implying some degree of functional redundancy (Stenvik et al. 2008). The phenotypes of overexpression of IDA and IDL proteins are similar and lead to premature floral abscission and ectopic abscission of additional plant organs (Stenvik et al. 2006) which also implies functional redundancy between two proteins. Genetic analyses have shown HAESA, (HAE) LRR RLK to be involved in controlling abscission (Jinn, Stone and Walker 2000, Butenko et al. 2003). HAESA acts in the same pathway as IDA (Cho et al. 2008, Stenvik et al. 2008), represses the KNAT2 and KNAT6 transcription factors (Shi et al. 2011, Butenko, Shi and Aalen 2012) and thus regulates floral abscission. The IDA signalling pathway shares components of the CLV3 pathway (Butenko et al. 2009). Once CLV3 is secreted and cleaved, the mature peptide ligand binds CLV1 in underlying meristem cells to initiate a mitogen-activated protein kinase (MAPK) cascade, which includes MAPK kinases 3,4,5 and 6 (Jun et al. 2008, Cho et al. 2008).

The best studied signalling peptides in plants are CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR) related (CLE) proteins. The first *CLE* gene reported was the endosperm-specific gene, *ESR* in *Zea mays* (maize) (Opsahl-Ferstad et al. 1997). In Arabidopsis there are 32 *CLE* genes (Cock and McCormick 2001) that encode small (~100 amino acids long) proteins that have a conserved structure that includes a putative N-terminal secretory signal peptide and a conserved 14-amino acid CLE domain at the C-terminus (Sharma, Ramirez and Fletcher 2003, Strabala et al. 2006, Jun et al. 2008). Post-translational regulation of CLE peptides is important for CLE function. For example, overexpression of the *CLE1* gene induces short root phenotypes (Strabala et al. 2006), whereas application of a synthetic CLE1 peptide does not induce any visible phenotypes (Kinoshita et al.

2007), suggesting that posttranslational modification of the CLE protein may be essential for CLE1 function in plants. Two representatives in this gene family, *CLV3* and *CLE40* are responsible for the maintenance of the SAM and the functioning of the RAM, respectively (Hobe et al. 2003).

In the vascular meristem another representative of the CLE family, TDIF is perceived by a receptor *TDIF RECEPTOR/PHLOEM INTERCALATED WITH XYLEM* (*TDR/PXY*) in order to promote proliferation of procambial cells suppresses their xylem differentiation, and regulate the expression of *WOX4* (Hirakawa et al. 2008, Hirakawa, Kondo and Fukuda 2010). In young embryo and endosperm the recently identified *CLE8* (Fiume and Fletcher 2012) regulates seed size via embryo and endosperm proliferation. Together with *WOX8*, *CLE8* transduces a signal that coordinates the growth of seed tissues at early stages of development (Fiume and Fletcher 2012).

Secreted peptides are also known to regulate self-incompatibility (SI) mechanism as previously mentioned. The male determinant of the Brassica SI system comprises of a small gene called *S locus cysteine rich protein (SCR)* or *S LOCUS PROTEIN 11 (SP11)* (Schopfer, Nasrallah and Nasrallah 1999, Takayama et al. 2000). This is a highly polymorphic peptide containing a putative signal peptide cleavage site and is expressed predominantly in the anther. *S* haplotype specific ligand – receptor interactions were demonstrated by experiments that used synthetic radio labelled SP11 (Takayama et al. 2001) and also a SP11 peptide that induced autophosphorylation of its cognitive SRK receptor (Kachroo et al. 2001). In *Papaver rhoeas* the female determinant of SI, called PrsS (*Papaver rhoeas* stigma S determinant) (Foote et al. 1994) encodes a small (~ 15kDa) peptide secreted by the stigmatic papilla cells and belongs to the large family of *S-PROTEIN*

HOMOLOGUE (*SPH*) genes (Foote et al. 1994). The *SPH* family consists of 84 genes and most of them are floral expressed (Wheeler, Vatovec and Franklin-Tong 2010). The identified male S determinant of *Papaver* SI is a transmembrane PrpS receptor (Poulter et al. 2010, Wheeler et al. 2009). Incompatible interactions trigger a rapid increase in cytosolic free Ca^{2+} in incompatible pollen tubes (Franklin-Tong 1993) and also activate a downstream signalling pathway resulting in programmed cell death (PCD)(Bosch and Franklin-Tong 2007).

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1.4 Coordination of seed formation

Mature seed formation requires coordinated development of its components: embryo, cellular endosperm and expansion of integuments. The early growth phase of the Arabidopsis endosperm is under the control of the *HAIKU* genetic pathway (Luo et al. 2005). Two mutants named *iku1* and *iku2*, cause a reduction in endosperm size, which correlates with a decrease in cell elongation in the maternal integuments (Garcia et al. 2003). *IKU1* encodes a protein of unknown function containing a VQ motif and is preferentially expressed in the early endosperm before cellularisation (Wang et al. 2010). *IKU2* encodes an LRR RLK and shows a low level of expression in the endosperm (Luo et al. 2005). A third gene in the *HAIKU* pathway, *MINI3*, encodes a WRKY transcription factor and mutation in this gene causes a reduction in seed size (Luo et al. 2005). The seed phenotype of double mutants *iku2/iku1*, *mini3/iku1* and *mini3/iku2* are similar to that of the single mutants suggesting that these genes operate in the same genetic pathway to regulate seed growth and final size (Luo et al. 2005). Both *MINI3* and *IKU2* show a decreased level of expression in the loss of function mutant *iku1*. *IKU2* expression is reduced in the *mini3* background, whereas *MINI3* expression levels remain unaltered in the *iku2* mutant.

This clearly indicates that *IKU1* acts upstream of *MINI3* and that *MINI3* operates upstream of *IKU2* in the same genetic pathway (Luo et al. 2005).

Similarly to *IKU1* and *IKU2*, *MINI3* is expressed in the early developing endosperm before cellularisation. A yeast-two-hybrid screen indicated that both IKU1 and MINI3 form a complex in the nucleus and regulate *IKU2* downstream (Zhou et al. 2009). Interestingly *IKU2* and *MINI3* are regulated by SHORT HYPOCOTYL UNDER BLUE1 (SHB1) which is a protein that binds to their promoter (Zhou et al. 2009, Johnson et al. 2002). The transcript level of *IKU2* is higher in *shb1-D* dominant gain of function mutants. In *shb1-D* mutants, endosperm cellularisation and embryo growth are delayed in comparison to wild-type and the size of the seed effectively increases. Interestingly, double *shb1-D/iku2* seeds are similar to *iku2* suggesting the epistatic role of these two genes to regulate seed development (Zhou et al. 2009). Recently it has been found that *MINI3* interacts *in vitro* with MED25, a component of the mediator complex (Cevik et al. 2012) (Fig.1.5).

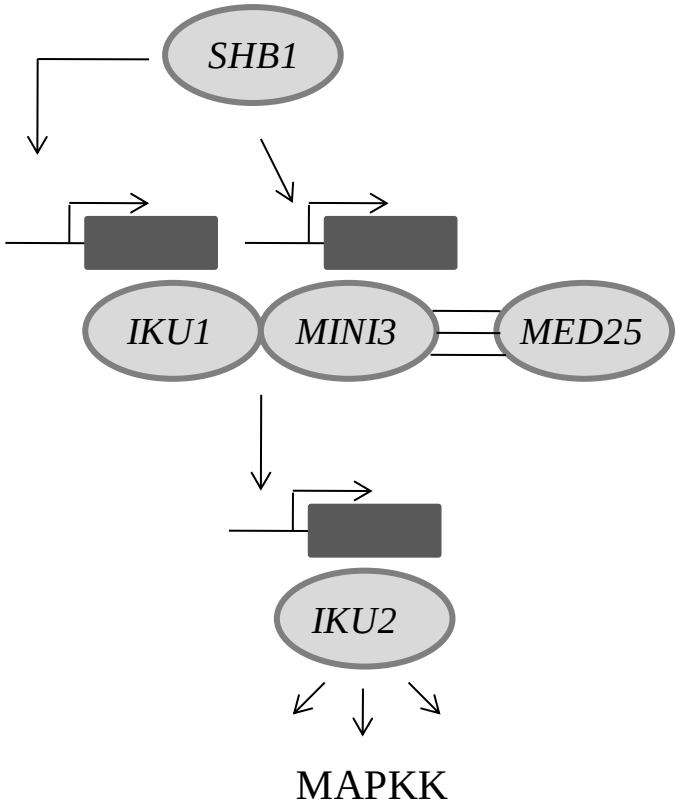

Figure 1. 1 **Diagram of the *IKU2* signalling pathway**

SHB1 binds to promoter of *IKU1* and *MINI3* to regulate their transcriptional activity. *IKU1 and MINI3* physically interact to bind IKU2 promoter to regulate its transcriptional activity. MED25 acts as a mediator protein of *MINI3* transcription factor. IKU2 could activate a MAPKK signalling pathway. Grey ovals represent the genes, grey boxes coding sequences, lines promoters of the genes. Arrows indicate the pattern of regulation of gene expression in *IKU* pathway.

In addition to *IKU2*, *TTG2* has also been found to regulate seed size (Garcia et al. 2005). Developing seeds of double homozygous *iku2/ttg2* mutants are extremely reduced in size compared to either single *iku2* or *ttg2* mutants. The additive reduction of integument cell elongation, endosperm growth and seed size when *iku2* and *ttg2* mutations are combined indicates that each gene acts in a distinct genetic pathway but with common effectors (Garcia et al. 2005). Since the two genes

are regulators of integument and endosperm development, interplay must exist between the maternal integuments and the endosperm.

1.5 Thesis aim and objectives

From the reviewed literature, it is clear that co-ordination of seed growth and development is a highly complex and tightly orchestrated process involving many different signalling molecules. Interestingly, CLE signalling peptides have been identified in the embryo surrounding region of Arabidopsis and maize (Fiume and Fletcher 2012, Opsahl-Ferstad et al. 1997) which is suggestive of a conserved and high level of communication between the endosperm and embryo. However, the corresponding partner receptor proteins for these peptides remain thus far unknown. The most interesting candidate responsible for this cross-talk in Arabidopsis is the *IKU2* LRR RLK, which plays a role in seed size regulation and is expressed in the micropylar endosperm at the early stage of seed development (Luo et al. 2005). However, its interacting protein partners and its specific activating ligand(s) remain unknown. Therefore the aim of this thesis is to carry out a detailed genetic, phenotypic and biochemical characterisations to further explore the putative role of *IKU2* in cell-cell communication during early seed development in *A. thaliana*. To this aim, the following objectives will be carried out:

- Test the genetic relationship between *IKU2* function and its closest homolog and other RLKs expressed in reproductive tissues, specially during early seed development,
- Assess the role of putative *IKU2* interacting factors in early seed development

- Define the biochemical properties of the IKU2 including structural conformation of the kinase domain, its phosphorylation activity and role in IKU2 function

Chapter 2

General Methods

2.1 Plant material and growth conditions

2.1.1 Plant material

The wild-type plants used in this study were *Arabidopsis thaliana* ecotypes Columbia and Landsberg *erecta*. All mutants are in the Columbia background (Col-0) unless stated otherwise. *iku2-4* (Col-0), *iku2-3* (L*er*), *iku2-1* (L*er*), *serk1-1/serk2-1/+*(Col-0), *ems1*, *er105* (Col-0) mutant seeds were provided by collaborators (sacco de Vries, Univeristy of Wagenigen, The Netherlands, Ming Luo, CSIRO, Australia, Hugh Dickinson, University of Oxford). The *iku2like1, iku2like2* T-DNA lines were obtained from Nottingham Arabidopsis Stock Centre (NASC).

2.1.2 Plant growth conditions

Seeds were surface sterilized with 70% ethanol and sown on plates with Murashige and Skoog salt (Sigma-Aldrich®) containing ammonium nitrate, boric acid, calcium chloride anhydrous, Cobalt chloride • $6H_2O$, Cupric sulfate • $5H_2O$, Na_2-EDTA, Ferrous sulfate • $7H_2O$, Magnesium sulfate, Manganese sulfate • H_2O, Molybdic acid (sodium salt) • $2H_2O$, Potassium iodide, Potassium nitrate, Potassium phosphate monobasic at the concentration of 4.3% with 0.8% agar (Melford-Micro® cat no. M1002). The seeds were stratified in the dark at 4°C for three days and then transferred to growth chamber. 10 days later germinated seedlings were transplanted on to potting mixture (2:1 ratio of Levington F2 (Seed and Modular Compost) soil to vermiculite) and grown in a glasshouse or in the controlled environment unit delivering 300 micro-moles of supplemental light until maturation (16h light/8 dark, 20°C).

2.1.3 Arabidopsis pollinations

The flowers taken for crosses were in the pre-anthesis developmental stage. Sepal, petals and immature anthers were removed from the flower buds of the female parent plant using forceps leaving an unfertilized stigma for two days. Stamens bearing dehiscent pollen were taken from the male parent plant and its anther was rubbed directly onto the stigma of the female reproductive organ. Stigmas were labeled and mature siliques were collected approximately three weeks after fertilization.

2.1.4 Media

2.1.4 A. L-Broth

- 10g/L of Bacto-tryptone
- 5g/L of yeast extract
- 10g/L of NaCl

The pH was adjusted to 7.5 with 10M NaOH.

2.1.4 B. YEB (Agrobacterium growth medium)

- 5g/l sucrose
- 5g/l peptone
- 5g/l beef extract
- 1g/l yeast extract
- 0.049g $MgSO_4$

The pH was adjusted to 7.2; 15 g of agar per litre was added for solid media

2.1.4 C SOC Medium

- 20g/l Bacto tryptone
- 5g/l Bacto yeast extract
- 10mM sodium chloride
- 2.5mM potassium chloride
- 10mM magnesium chloride
- 10mM magnesium sulphate
- 2g/l glucose

2.1.4 D LB-Agar

15g of agar (Melford-Micro® cat no. M1002) in LB, adjusted with distilled water to 1l and sterilised by autoclaving.

2.1.4 E. YEB – Agar

15g of agar (Melford-Micro® cat no. M1002) in YEB, adjusted with distilled water to 1l and sterilised by autoclaving.

2.1.4 F MS media for Arabidopsis seed germination

- 1x MS salts (Murashige and Skoog - Sigma Aldrich)/l
- 8g/l of Agar (Melford-Micro® cat no. M1002)

pH adjusted with 5M KOH to 5.7 and sterilized by autoclaving.

2.1.5 Genotypes of bacterial strains

E.coli DH5α : F^- end A1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–

E. coli Rosetta-2 pLys: F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE2 (Cam^R)

2.2 General molecular methods

2.2.1 Preparation of genomic DNA

Genomic DNA was extracted from leaf material with a method (Edwards, Johnstone and Thompson 1991). High-throughput genotyping was performed in 96 well blocks. For this, the plant tissue was ground with the Qiagen Mixer Mill. The DNA was isolated from leaf tissue by adding extraction buffer (100mM Tris pH 7.5, 50mM EDTA, 500mM NaCl, 10mM β-mercaptoethanol) and vortexing the sample for 5s. Afterwards SDS was added to samples to 0.5%. The extracts were centrifuged for 1 minutes and 300µl of supernatant was transferred to a fresh 96well block.

300µl of isopropanol was added to the mixture and precipitated at -20ºC for 1 hour. Following centrifugation for 10 minutes the supernatant was removed and the pellet washed with 70% ethanol, air dried for 1hour and re-suspended by pipetting in 100µl TE buffer.

2.2.2 PCR methods

Standard PCR reactions were set up in 40µl volumes containing 1.25 units of DNA *Taq* Polymerase (Invitrogen®), 4µl (10pg – 1µg) of DNA template, 0.1 – 1µM of gene specific forward and reverse primers, 0.2mM of each dNTP, 1-4mM of $MgCl_2$. Standard PCR reactions were carried out with an initial denaturation step of 94°C for 1 minutes followed by cycles of denaturation at 94°C for 30s, annealing at a temperature deemed appropriate for the primer pair for 30s and extension at 72°C for 1 minutes per kb of product.

2.2.3 Genotyping of bacterial (E.coli) colonies

The presence of the desired fragment in the expression vector was confirmed by PCR using gene specific primers or in case of pGEM-T Easy M13For and M13Rev primers (Appendix 1). Toothpicks containing single colonies were placed in 5µl of water, and the PCR reagents were added up to the reaction volume of 40µl (1.25units of DNA *Taq* Polymerase (Invitrogen®), 0.1 – 1µM of gene specific forward and reverse primers, 0.2mM of each dNTP, 1-4mM of $MgCl_2$). PCR reactions were carried out with an initial denaturation step of 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30s, annealing at a temperature deemed appropriate for the primer pair for 30s and extension at 72°C for 1 minutes per kb of product.

2.2.4 Genotyping of LRR RLK mutants and T-DNA lines

A list of oligonucleotide sequences used to genotype the mutants and T-DNA lines are available in Appendix 1. Standard PCR conditions were used for genotyping plants unless stated otherwise in the relevant results section.

2.2.5 Agarose gel electrophoresis

Following PCR, agarose gel electrophoresis was carried out to visualise products. For a standard 40µl reaction, 3µl of Orange G ($C_{16}H_{10}N_2O_7S_2Na_2$, Sigma-Aldrich®, Cat. No. O3756) was added prior to loading. The size of products was estimated by loading 5µl (0.7µg/lane) of 1 kb Plus DNA ladder (Invitrogen Ltd., Cat. No. 10787). The separation of PCR products was visualized on gels made up using 1 x TAE buffer (VWR International, Cat. No. 44125D) containing either 1% or 1.5 % agarose and stained with ethidium bromide (final concentration 0.5µg/ml). Gels were run in tanks containing 1 x TAE buffer at approximately 100 mA (300V) for 20-60 minutes depending on the size of the products. Photographs were taken using a G:BOX gel documentation system (Syngene) and the images were analyzed using Gene Tools software.

2.2.6 Quantification of DNA

DNA concentrations were measured using a Nano-Drop spectrophotometer (Thermo Scientific). The measuring point was cleaned with Millipore water and soft tissue. The instrument was blanked using 1µl of distilled water on the measuring

point (blank measurement). To determine the concentration of the DNA, 1µl sample was loaded and the concentration was read on the PC monitor.

2.2.7 Preparation of cDNA from total RNA extracted from young seeds

Hot borate extraction buffer was made up of 200mM sodium borate decahydrate (Borax) (pH 9), 30mM ethylene glycol bis (beta-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 1% (w/v) sodium dodecyl sulphate (SDS), 1% (w/v) sodium deoxycholate, 2% (w/v) polyvinylpyrrolidone (PVP) (*M*r 40,000), 0.5% (v/v) Nonidet-40 (NP-40), and 10mM dithiothreitol (DTT) added just before grinding. The buffer was made with 0.1% DEPC (diethyl pyrocarbonate)–treated water and then autoclaved.

Approximately 100mg of frozen Arabidopsis early developed seeds dissected out of the siliques were homogenized in 1.5ml microcentrifuge tubes containing glass beads using a Simal S6 shaker. 1ml of 80°C heated extraction buffer and 60µl of Proteinase K (Progen Industries Limited, 25 mg/mL in H_2O) was then added to the frozen homogenized tissue and mixed by vortexing. The sample was transferred to a fresh 15ml tube and another 1ml of extraction buffer was added to a grinding tube to wash the remaining tissue. The tissue-buffer mix was loaded on two Qiashredder spin columns and microcentrifuged for 1minutes at 13000 rpm. The flow through was transferred to a fresh sterile tube and the loading and centrifugation step was repeated until the whole tissue was processed. 0.5 volume of absolute ethanol was added to the collected flow through and mixed by inverting several times. The sample was loaded on two Qiagen RNeasy mini columns and centrifuged for 1minutes at 13000rpm and the flow through discarded. An on-column DNase digestion was performed with the RNAase-Free DNase Set (Qiagen). For washing and drying the columns the RNeasy Mini Protocol for RNA cleanup

was followed. Samples were re-suspended in 40µl of RNase-free water and stored at -80°C. The first strand cDNA synthesis was performed using 200 units of Superscript™ II Reverse Transcriptase (Invitrogen Ltd., Cat. No. 18064-14), 50-250ng of random hexamers and 0.1M DTT following the manufacturer's guidelines.

2.2.8 Phenol:chlorophorm plasmid DNA extraction

50µl of PCR product was made up to 100µl total volume with sterile water before adding 100µl of phenol/chloroform. The samples were vortexed and centrifuged at 13000g for 5 minutes and the supernatant transferred to a new 1.5ml tube. Twice the initial volume of 100% ethanol (400µl) and 1µl of glycogen (10-20 mg/ml) was added before placing the sample at -20°C for 30minutes. The samples were centrifuged again for 10minutes 13000g before washing twice with 70% (v/v) ethanol. The pellet was air dried and re-suspended.

2.2.9 DNA sequence analysis

Sequencing reactions were performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Each reaction contained the appropriate primer (3.2 pmol), 4 µl of the Terminator Ready Reaction Mix, 2µl of Big Dye Sequencing Buffer (5x) and 200-500 ng double stranded DNA or 30-90ng PCR products DNA. Each reaction was made up to 10 µl with sterile distilled water. PCR cycles (25) were as follows: 96ºC for 10 s, 50ºC for 5 s and 60ºC for 4 minutes. Sequencing electrophoresis was performed using an ABI automated sequencer (Applied Biosystems). Nucleotide sequence data was edited using EditSeq package within DNASTAR (DNASTAR, Inc).

2.3 General cloning strategy

2.3.1 Cloning into pGEM-T Easy vectors (Promega)

DNA amplified by PCR amplification using Pyrobest DNA polymerase was cloned into a pGEM-T Easy vector (Promega Ltd., Cat.No. A1360) is linearised with a single 3´-terminal thymidine at both ends. To enable the ligation of the blunt ended PCR amplified insert into a pGEM-T Easy the "add A-tailing" procedure was performed by adding 1µl of 10mM dNTPs and 1µl of *Taq* polymerase to PCR mixture and subsequently incubating it at 72°C for 30minutes. The fragments to be cloned were gel purified using a Qiagen Gel Extraction Kit. To facilitate the ligation a 3:1 ratio of insert to vector was maintained. The reaction components for ligation protocol consisted of: 5µl of 2x Rapid ligation buffer, 3µl of insert (150ng/µl), 1µl of pGEM T-Easy vector (150ng/µl) and 1µl of T4 DNA ligase (3 Weiss units/µl). The ligations into pGEM T-Easy vector were performed overnight at 4ºC. 3µl of the ligation was used to transform 50µl of DH5α *E.coli* cells and spread on LB agar (Melford-Micro® cat no. M1002) plates containing ampicilin (100µg/1ml), 40µg/ml of (Isopropyl β-D-1-thiogalactopyranoside) IPTG, 40µg/ml of (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) XGal. The plasmid was extracted using Qiagen plasmid mini-prep kit according to manufacturers' instruction.

2.3.2 Gateway cloning

Gateway technology is a universal cloning technology which takes advantage of the site specific recombination properties of bacteria lambda. The first step of this cloning is inserting a gene of interest into the Gateway, pENTRY vector. In order to produce the pENTRY vector a "BP" reaction was performed using 1:1 ratio of the

expression clone (pGEM-T Easy) with the gene of interest flanked with *att*B1 and *att*B2 sites and pDONOR vector (pDONR 207) containing *att*P sites. The 5µl BP reaction consisted of 0.5µl of 150ng/µl of pGEM-Teasy vector with the gene of interest, 0.5µl of 150ng/µl pDONR207 vector, 3µl of TE buffer, 1µl of BP clonase enzyme mix (Invitrogen). The mixture was incubated on the bench (~20°C) overnight, followed by incubation with Proteinase K (1µl of Proteinase K at 37°C for 10minutes). 2µl of BP reaction was used to transform 50µl of *E.coli* DH5α cells (heat shock) and spread on LB agar (Sigma Aldrich) plates containing gentamicin (15µg/ml), the colonies were inoculated in to 5ml LB containing 15µg/ml of gentamicin overnight with shaking (200rpm) at 37°C. The plasmid was extracted using a Qiagen plasmid mini-prep kit.

In the second step in Gateway cloning the gene cassette in the pENTRY vector is moved into a Gateway Destination vector (expression vector). In order to generate an expression vector, a 5µl LR reaction was performed between the pENTRY clone containing the gene of interest flanked by *att*L sites and a destination vector containing *att*R sites. The mix was prepared as follows: 0.5µl of 150ng/µl of pENTRY clone, 0.5µl of 150ng/ng of destination vector, 3µl of TE buffer and 1µl of LR clonase. The reaction was performed at 25°C overnight and followed by a Proteinase K step next day (1µl of Proteinase K at 37°C for 10minutes). 2µl of the plasmid was used to transform DH5α *E.Coli* cells and selected on the LB agar (Melford-Micro® cat no. M1002) plate with the appropriate antibiotic. Plasmids were inoculated in 5ml of LB at 37°C overnight and plasmid DNA was extracted using Qiagen plasmid mini-prep kit.

2.3.3 Heat shock transformation of E.coli DH5α cells

Unless stated otherwise all vectors were transformed into DH5α *E.coli* cells before selection on LB agar (Melford-Micro® cat no. M1002) plates with the addition of the appropriate antibiotics. 3µl of the appropriate plasmid was added to 50µl of DH5α competent cells, mixed and kept on ice for 30minutes. The transformation of cells was performed at 42°C for 1minutes (heat shock) and kept on ice for 3minutes. 1 ml of liquid LB media was added immediately after heat shock and incubated at 37°C for 1hour. The cells were collected by gentle centrifugation (600rpm) and spread on LB agar (Melford-Micro® cat no. M1002) plates containing appropriate antibiotics. Plasmids from resistant *E.coli* were grown overnight and recovered using Qiagen plasmid mini-prep kit.

2.3.4 Antibiotic selection on LB agar Plates

The plates were prepared using LB – Broth Miller (MERCK) 25g/L, agar (Melford-Micro® cat no. M1002) 15g/L with the addition of the appropriate antibiotic selection (ampicillin 100µg/ml, kanamycin 50µg/ml, streptomycin 50 µg/ml, chloramphenicol 170µg/ml, gentamycin 15 µg/ml) after autoclaving.

2.3.5 β galactosidase selection

1ml/L 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside(X-Gal) (20mg/ml in dimethylformamide), 500µl/L isopropylthio-ß-D-galactoside (IPTG) was added to 1litre of LB (IPTG, 2mg/8ml sterile water adjust volume to 10mls then filter sterilised through a 0.22 micron disposable filter) and ampicillin (Sigma)100mg/ml.

2.3.6 pCAMBIA 1300 vector

The pCAMBIA vector (Fig. 2.1) backbone is derived from the pPZP vectors. The characteristics of the vector consists of several features: high copy number in *E.coli* for high DNA yields, pVS1 replicon for high stability in *Agrobacterium*, small size, 7-12kb depending on which plasmid, restriction sites designed for modular plasmid modifications and small but adequate poly-linkers for introducing the DNA of interest, bacterial selection with chloramphenicol or kanamycin, plant selection with hygromycin B or kanamycin and simple means to construct translational fusions to *gusA* reporter genes.

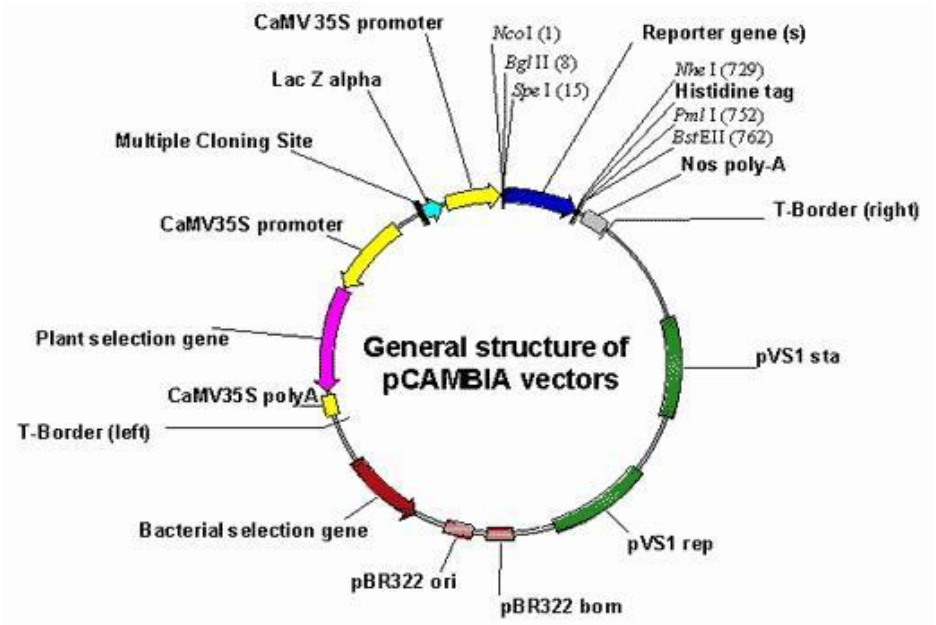

Figure 2. 1 **Plasmid map of pCAMBIA vector (from http://www.cambia.org/daisy/cambia/home.html**

2.3.7 pGEX-4T-1 vector

pGEX-4T-1 is a 4900bp bacterial plasmid (Fig. 2.2) of pBR322-type ColE1 origin of replication. It is a pGEX-series GST (glutathione S-transferase) fusion

vector with Ptac promoter, lacI repressor, thrombin cleavage site ORF; ampicillin resistance; restriction enzyme cloning.

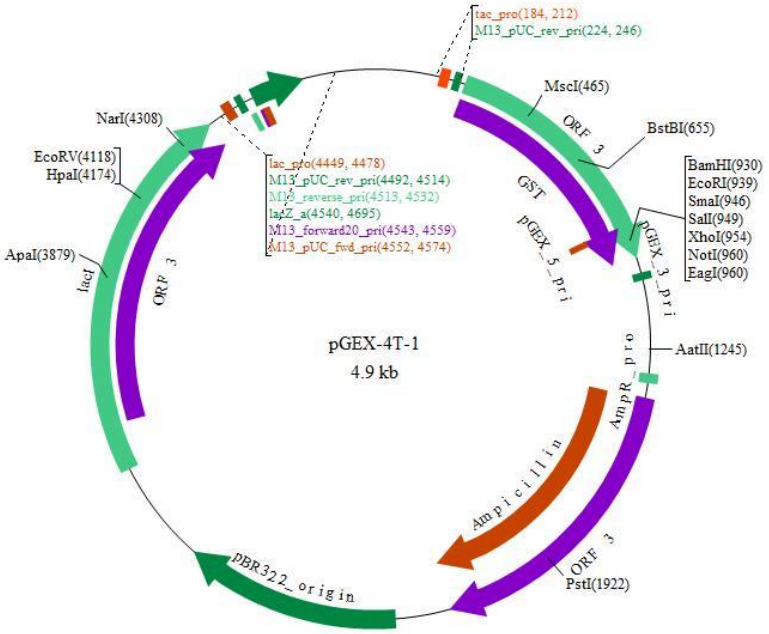

Figure 2. 2 **Map of pGEX-4T-1 plasmid (from http://www.biovisualtech.com/bvplasmid/pGEX-4T-1.htm)**

2.3.8 Rosetta-2 pLys competent cells

Rosetta host strains are BL21 derivatives designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. These strains supply tRNAs for AGG, AGA, AUA, CUA, CCC, GGA codons on a compatible chloramphenicol-resistant plasmid. Thus the Rosetta strains provide for "universal" translation which is otherwise limited by the codon usage of *E. coli*. The tRNA genes are driven by their native promoters.

2.3.9 Genotypes of bacterial strains

E.coli DH5α - F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–

E.coli Rosetta-2 pLys - F^- ompT $hsdS_B(R_B^-$ $m_B^-)$ gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R)

2.3.10 Agrobacterium transformation

1µl of plasmid DNA (100-300µg/µl) was added to 40µl of electro-competent *Agrobacterium* GV3101 cells and incubated on ice for 10minutes. Electroporation was carried out in an ice-chilled electroporation cuvette and 1ml of YEB liquid media was added immediately after the electroporation in the Biorad Micro Pulser. The bacterial suspension was incubated for 2-3 hours at 28°C with gentle agitation. The cells were collected by gentle centrifugation (600rpm) and spread on YEB agar plates containing appropriate antibiotics. Colonies were incubated for 2-3 days at 28ºC.

2.4 Other specific methods

2.4.1 Arabidopsis floral dipping transformation

The Agrobacterium strain harbouring the gene of interest in a binary vector was inoculated with shaking in 5ml liquid YEB containing appropriate antibiotics. The culture was incubated at 28°C for two days. This feeder culture was inoculated into 500ml liquid YEB at 28°C for 24h. *Agrobacterium* cells were collected by centrifugation at 4000g for 10minutes at room temperature and gently re-suspended

in 1 volume of freshly made 5% (w/v) sucrose solution. 200µl of Silwett L-77 was added before dipping to a concentration of 0.02% (v/v). The early developing inflorescences of *Arabidopsis* plants were dipped in the *Agrobacterium* cell suspension for 10s with gentle agitation (Zhang et al. 2006).

2.4.2 Screen for the primary transformants

Seeds were stratified in 0.1% agarose at 4°C for three days. Screening of primary transformants (T_0) was carried out on Rockwool soaked with $^1/_2$ strength MS and 100µg/ml BASTA herbicide or other appropriate antibiotic (hygromycin, kanamycin). Total number of transformants was counted and after 2-3 weeks ~20 seedlings were transferred to soil and grown to maturation and T_1 seeds collected. The independent transformant T_1 lines were grown to maturation and T_2 seeds were collected and grown in soil in a controlled environment for phenotypic analysis. The homozygosity or heterozygosity of the transformants was assessed using a PCR genotyping assay.

2.4.3 Kinase phosphorylation assay

2.4.3 A Cloning of recombinant protein into pGEM-T Easy

The WT and R953K kinase domains were amplified in 100µl reaction with Pyrobest *Taq* DNA polymerase using iku2.kin.For(*XhoI*) and iku2.kin.Rev(*NotI*) primers (list of primers in Appendix 1). To ligate the blunt ended products into a pGEM-T Easy vector that has T overhangs, the "A" tails were added according to the procedure described in general methods. The PCR fragments were gel purified using Qiagen Extraction Kit and ligated overnight at 4°C into pGEM-T Easy. The ligations were used for transformation of *E.coli* DH5α cells and a blue/white selection was performed on the agar plates to identify recombinants. The plasmid

DNA was extracted with QiagenMiniprep Kit and sequenced to confirm the nucleotide sequence.

2.4.3.B IKU2 mutagenesis and cloning into PGEX 4T-1 vector

In order to engineer an inactive version of IKU2 kinase, PCR mutagenesis was performed in 100µl mixture containing double stranded WT kinase domain DNA as a template, *Pfu* DNA polymerase and iku2.mut.kin.For and iku2.mut.kinase.Rev primers (Appendix 1). The PCR program consists of denaturation step at 94°C for 2 minutes and 12 cycles of 94°C for 30s, 60°C for 30s, 68°C for 12minutes and final elongation at 68°C for 15minutes. The PCR product was subsequently digested with *DpnI* restriction enzyme (37°C for 1.5h). Plasmid DNA was extracted with phenol:chloroform method. The samples (50µl of *Dpn*I digested PCR product) were made up to 100µl total volume with sterile water before adding 100µl of phenol/chloroform (1:1). The samples were vortexed and centrifuged at 13000g for 5minutes and the supernatant transferred to a new 1.5ml tube. 2 x of the initial volume of 100% ethanol and 1µl of glycogen was added before placing the sample at -20°C for 30minutes. The samples were centrifuged again for 10minutes at 13000g before washing twice with 70% (v/v) ethanol. The pellet was air dried and re-suspended. Plasmid DNA was transformed into *E.coli* DH5α cells. Selected colonies were sequenced to confirm the appropriate nucleotide sequence. Full length nucleotides sequences for the three types of kinases: IKU2 wild type, IKU2-R953K, IKU2-K699R were digested with *Xho*I and *Not*I restriction enzymes and ligated into PGEX 4T-1 vector and transformed into *E.coli* Rosetta^{TM}2 pLysS cells (Novagen). 1µl of plasmid was mixed with 15µl Rosetta cells and kept for 30minutes on ice. The transformation of cells was performed at 42°C for 1minutes and again kept on ice for 3minutes. 1ml of LB media was added

immediately after transformation and incubated at 37°C for 1hour. The cells were selected on LB media plates for ampicilin (100µg/ml) and chloramphenicol (CAM) (170µg/ml) resistance. The colonies were then incubated overnight with shaking at 37°C in 5ml liquid LB media and appropriate antibiotics. The overnight culture was diluted 1:20 in two falcon tubes with 5ml LB + antibiotic and grown at 37ºC until OD600=0.6. The induction of protein expression was carried out with two different concentration of IPTG (0.1mM and 1mM) to optimize the conditions. Three hours later 1ml of each sample was transferred to a fresh 1.5ml tube, centrifuged at room temperature at 13000g for 30s and the supernatant removed. The crude protein was heated to 95ºC with sample buffer (106 mM Tris HCl, 141 mM Tris Base, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Phenol Red, pH 8.5) and resolved/ separated on 7.5% SDS PAGE gel.

2.4.3.C Protein expression and native protein purification

5ml cultures with all three kinases in pGEX4T-1 were grown overnight at 37ºC and subsequently added to 200ml of LB. The growth of *E.coli* was carried out in 1.5L conical flasks until the OD600 = 0.5-0.6. After addition of IPTG to 0.1mM, the culture was grown for another 3h at 37°C at 220rpm. To pellet the cells, the cultures were transferred to fresh 200ml plastic bottles and centrifuged at 7000rpm for 20minutes at 4°C. The pellet was resuspended by vortexing in lysis buffer (10ml of freshly made phosphate buffered saline pH7.5 mixed with 1mg/ml of lysozyme, 1mM PMSF, 1 Complete Mini EDTA-free tablet (Roche - 11863170001) in a 50ml tube. The resuspended samples were incubated on ice for 5minutes and passed two times through freeze thaw cycles using liquid nitrogen. The freezing was performed by immersing the tube in liquid nitrogen and the thawing took place on the bench at room temperature. The samples were then sonicated three times for 12s placing the

samples on ice between the pulses at medium intensity. Afterwards 1% of Triton X-100 and 5µg/ml of DNase1 were added and the samples were rotated for 30minutes at 4ºC. Subsequently the samples were centrifuged at 19000rpm for 50 minutes at 4°C. In order to prepare a glutathione-agarose slurry, 50µl of glutathione sepharose TM 4B was washed three times with PBS/1% Triton buffer at 500rpm and the supernatant was discarded. The clear protein lysate/ the supernatant was added to the glutathione resin and incubated on the rotor for 1h at 4°C. Afterwards the samples were centrifuged at 500rpm for 1minutes and the supernatant was removed. The pellets were transferred to fresh microcentrifuge tubes, centrifuged for 1minutes at 500rpm and the supernatant was removed. 50µl of 50mM Tris pH 8.0, glutathione 10mM was added and the samples were incubated at room temperature for 10minutes. The sample was centrifuged at 1000rpm for 30s and the supernatant was transferred to a fresh tube. The step was repeated two times collating the supernatans in the fresh tube. 2 x volume of storage buffer (106 mM Tris HCl, 141 mM Tris Base, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, 0.175 mM Phenol Red, pH 8.5) was added to the sample, vortexed briefly and stored at -20°C.

2.4.3.D Autophosphorylation assay

The kinase assay was performed in 20µl volume containing 2µl of 10 x kinase buffer (2mM CaCl2, 100mM MgCl2, 10mM DTT, 200mM TrispH7.5, 250µM of "cold" ATP), the 5µl of protein and 25µCi of γ^{32}P-ATP added to each reaction. The fusion proteins were phosphorylated at room temperature for 1 hour. The reactions were stopped by adding SDS-PAGE sample buffer (NuPAGE Sample Reducing Agent 10x, NuPAGE LDS sample buffer 4x) and the samples were heated for 2 minutes at 95°C. The phosphorylated proteins were resolved on 7.5% SDS-

PAGE (Laemmli 1970) and the labelled proteins were visualised by autoradiography.

2.4.4 Generation of pIKU2:ERECTA$_{myc}$ transgenic line

A DNA fragment containing *IKU2* promoter sequence was PCR amplified from genomic DNA in a 100µl PCR mixture with Pyrobest *Taq* polymerase and iku2pKpn*I*Asc*I*.For and iku2pBH*I*.Asc*I*.Rev oligonucleotides (primers listed in Appendix 1). The "add A" tailing procedure was performed and the fragment was subcloned into pGEM-T Easy vector and sequenced with *IKU2* gene specific primers to confirm the appropriate DNA insertion and presence of the restriction sites. Subsequently, *IKU2* promoter was digested with *Kpn*I and *Bam*HI restriction enzymes and subcloned into the pCAMBIA1300 vector. The vector was subsequently linearised using *Pst*I restriction enzyme and the sticky ends blunt ended using Klenow DNA polymerase to enable the introduction of a Gateway cassette (GW) with the *ERECTA* gene. To introduce the *ERECTA* gene into a GW cassette the gene was amplified and a *myc* tag added in the two-step PCR. In the first round of PCR the *ERECTA* gene was amplified using *ERECTA* cDNA as a template using erATGForGW and er.Rev.GW primers in a 100ul PCR mixture using Pyrobest DNA polymerase. The thermal cycling program used for the PCR consisted of 4 cycles of 95°C for 30s, 55°C for 30s, 72°C for 8.25s and subsequent 20 cycles consisting of 95°C 30s, 65°C 30s and 72°C 30s. The fragment was separated on 1% gel and extracted with Qiagen Gel Extraction Kit. The eluted DNA was used as a template for a second PCR amplified with erATGFor.GW and Ara.mycGW primers. Amplification cycles were as follows: 4 x 95°C for 30s, 55°C for 30s, 72°C for 8.25s and 20 x 95°C for 30s, 65°C for 30s and 72°C for 8.25s. The fragment was gel purified and ligated into pGEM-Teasy vector and sequenced to confirm the right

insertion and presence of a *myc* tag and *att*B flanking sites. The vector with the erecta.myc fusion was subcloned into pDONOR207 vector in a 5µl BP reaction. In order to generate the final clone pIKU2:ERECTA$_{myc}$, a 5µl LR reaction with 1:1 ratio of iku2pGW and erecta in pDONR207 was used.

2.4.5 Generation of pIKU2:IKU2ΔKIN$_{myc}$ transgenic line

To engineer an IKU2 lacking the cytoplasmic domain, the DNA fragment containing -800 to +1887bp of *IKU2* gene was PCR amplified from genomic DNA. 100µl PCR reaction mixture with Pyrobest *Taq* polymerase was used to amplify the DNA fragment in the two step PCR strategy (Fig. 2.3). In the first PCR reaction iku2gForGW (Fig. 2.3) and iku2gRev.myc (Fig. 2.3) oligonucleotides were used (see list in Appendix 1). The PCR program consists of denaturation step at 94°C for 2 minutes and initial 4 cycles of 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 4minutes followed by 24 cycles of 94°C for 30s, annealing at 65°C for 30s and extension at 72°C for 4 minutes. The 2.7 kb PCR product was separated on 1% agarose gel (Fig.2.3) and cleaned with Qiagen Gel Extraction Kit. The eluted DNA (1:300) was used as a template for the second step in 100µl PCR reaction with iku2gFor.GW (Fig. 2.3F1) and Ara.Rev.myc (Fig. 2.3R2) primers. The PCR conditions consisted of denaturation step at 94°C for 2 minutes and initial 4 cycles of 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 4minutes followed by 24 cycles of 94°C for 30s, annealing at 65°C for 30s and extension at 72°C for 4 minutes.

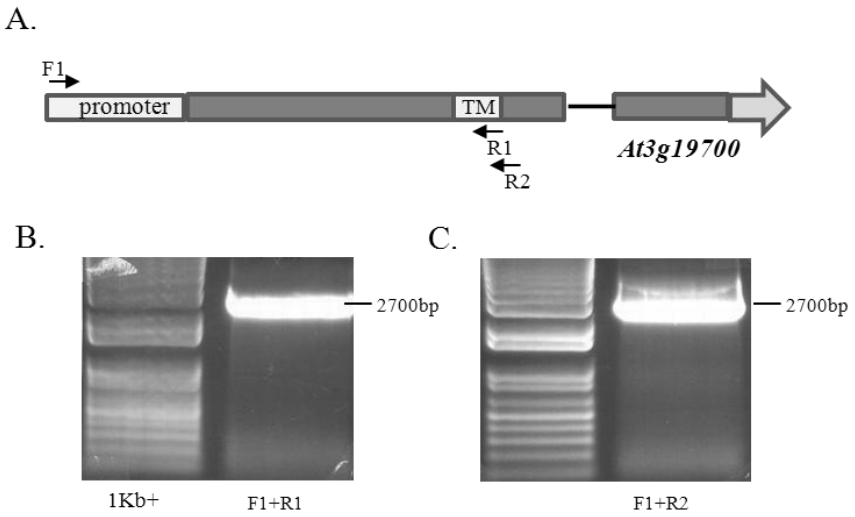

Figure 2. 3 **Molecular cloning of pIKU2:IKU2ΔKIN$_{.myc}$ construct**
(A) Schematic representation of *IKU2* gene and position of oligonucleotides used for amplification of part of the gene used for cloning. Exons are depicted as solid grey bars, promoter as a white bar, UTRs: light grey bars, intron regions as solid lines, and positions for oligonucleotide primers are indicated by arrows. TM: transmembrane domain. (B) In the first PCR reaction a 2.7Kb product from-800 to +1887bp was amplified using F1 and F2 primers. (C) In the second PCR round the diluted product of PCR A was used as a template for amplification with F1 and R2 primer pairs.

To ligate the blunt ended products into a pGEM-T Easy vector that has the T overhangs, the "A" tails were added by incubation of the mixture at 72°C for 30minutes with 1µl of 10mM dNTP mix and 1µl of *Taq* polymerase (Invitrogen). The amplified product was visualised by electrophoresis, extracted with Qiagen Gel Extraction Kit. For ligation 1µl of pGEM-T Easy vector and 3µl of insert was used. The ligation was carried out overnight at 4°C. 2µl of ligation was used to transform DH5α competent *E.coli* cells and blue/white selection was performed on the LB agar (Melford-Micro® cat no. M1002) plates contained ampicilin (100µg/1ml) , 40µg/ml of IPTG, 40µg/ml of XGal. White colonies were screened by PCR (Fig.2.4) with the internal *IKU2* specific primers (iku2-1For/iku2-1Rev) and inoculated overnight at 37°C in 5ml of LB.

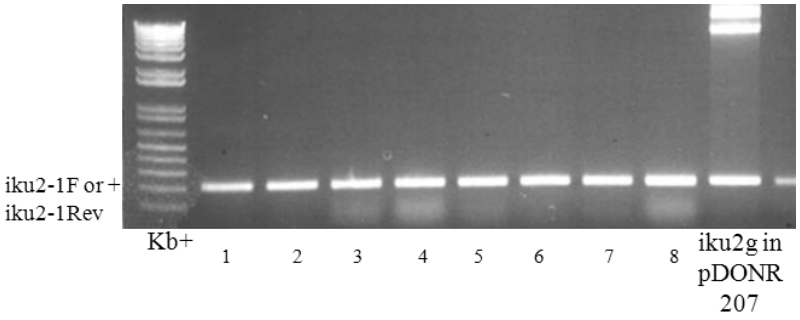

Figure 2. 4 **Colony PCR of pIKU2:IKU2ΔKIN$_{.myc}$ insert in pGEM-T Easy vector**

Internal *IKU2* gene specific primer pairs iku2-1For + iku2-1Rev (200bp) were used to screen individual white colonies (1-8) for the correct insertion of *IKU2* DNA fragment. Iku2g in pDONR207: positive control.

Plasmid DNA was extracted with QiagenMiniprep Kit. Subsequently the clone was verified by sequencing using *IKU2* specific primers and was named pIKU2::IKU2ΔKIN$_{.myc}$. The ligation of the clone in pGEM-T Easy into a pDONR207 (Invitrogen) entry vector was carried out in room temperature for 1hour, DH5α cells were transformed and selected for gentamycin resistance. The colonies were screened by PCR, the plasmid DNA (iku2fullmyc in pDONR207) was used to clone into the Gateway compatible pMDC123 complementation vector (Curtis and Grossniklaus 2003) using DH5α competent cells and kanamycin selection.

2.4.6 Spectrophotometry

The absorbance of a sample was measured at 600nm (OD600). Prior to the optical density measurements the spectrophotometer was normalized by a "blank" measurement (the solute without the bacteria).

2.4.7 Database search

The *IKU2* (*At3g19700*) nucleotide and amino acid sequence was compared to TAIR database http://www.arabidopsis.org/ with the BLASTN and BLASTP

algorithm respectively, resulting in identification of *IKU2-LIKE* gene (*At1g09970*). Multiple protein alignment was performed with Jalview http://www.jalview.org. The preliminary expression profile of the genes of interest (*IKU2*, *IKU2-LIKE*, *EMS1*, *SERK1*, *SERK2*) was investigated with Gene Networks in Seed Development database (http://seedgenenetwork.net/).

2.4.8 Structural protein modelling

Homology modelling was made by submitting the entire IKU2 and IKU2-3 kinase domain sequences to the web-based Swiss-Model workspace (http://swissmodel.expasy.org/workspace/) (Arnold et al. 2006, Schwede et al. 2003) using automated mode and default settings. The algorithms generated two models, one for the IKU2 and one for the IKU2-3 kinase domain. The templates was the kinase domain of IRAK-4 (Wang et al. 2006). Models were saved as protein data bank (.pdb) files and molecular graphics images were produced using a Deep View – Swiss Pdb Viewer (http://spdbv.vital-it.ch/disclaim.html).

2.4.9 Feulgen staining

Tissue (4 and 6 days after pollination) was collected and both ends of the siliques were cut off and placed in a microcentrifuge tube containing 1ml of fixative (3:1 ethanol:acetic acid (v/v) prepared fresh on the day and fixed at 4°C overnight. The samples were then transferred to 70% (v/v) ethanol and stored at 4°C. The tissue was subsequently washed three times with distilled water and hydrolysed in 5N HCl (for each 10ml: 4.3ml 36% (v/v) HCl and 5.7ml of distilled water) for 30 minutes. The samples were washed three times with distilled water and stained in Schiffs Reagent for 30 minutes in the dark. Afterwards three washes with cold tap water were performed. The subsequent washes consist of 70%, 95% and 100% ethanol (v/v), 10 minutes each. The final wash with 100% ethanol was repeated and the

samples stored at 4°C overnight. The following day the ethanol was changed three times every hour until it stopped turning pink. Afterwards the ethanol was replaced with LR White resin twice and kept overnight. On the third day, a small drop of Accelerator was added to 2-3ml of resin and a pea-size blob was pipetted onto the middle of a microscope slide between two coverslips. Seeds were dissected from the siliques, placed in the resin and a coverslip was placed on the top with its edges supported by two coverslips already on the either side of the sample.

2.4.10 Glucoronidase (GUS) staining protocol

Arabidopsis siliques were placed in a 90% (v/v) acetone solution and incubated on ice for 40 minutes. Subsequently the siliques were washed for 5 minutes with 0.05M sodium phosphate buffer. The tissues were transferred to GUS staining solution (50mM sodium phosphate buffer (pH 7.0), 10mM EDTA (pH 7.0), 0.5mM potassium ferricyanide, 0.5mM potassium ferrocyanide, 1mM X-Gluc A, 0.1% (v/v) of Triton-X100), vacuum infiltrated for 20-30minutes and incubated at 37°C overnight. The next day the samples were washed with 70% (v/v) ethanol until chlorophyll was removed

2.4.11 Confocal microscopy

Transgenic *Arabidopsis* seeds at early developmental stages were mounted for microscopic observation in water under glass coverslips. The seeds were examined using a Zeiss LSM 710 confocal microscope at 10x and 40x magnification. The argon laser excitation wavelength was 488 nm GFP emission was detected with the filter set for FITC (505–530 nm). The fluorescence of the images was assessed using the ZEN 2009 LE imaging software.

2.5 Seed measurements and statistical data analysis

2.5.1 Phenotypic analysis of seed size

To measure dry seeds with the ImageJ software (http://rsbweb.nih.gov/ij/), seed images were first taken with a camera attached to a dissecting microscope using a graticule to allow a scale to be set for all measurements. Images were then converted to greyscale (16bit) and a threshold was set using an automated routine. The seed area was measured and converted to mm² using the "analyse particles" tool pack from ImageJ.

2.5.2 Statistical data analysis of seed measurements

Dry mature seed sizes data and measurements taken at globular stage of seed development were analysed using general analysis of variance (ANOVA) tests (Armstrong, Slade and Eperjesi 2000). For dry mature seeds, the seeds were compared for variation in order to assess the differences between the seeds sizes derived from individual plants within the same genotype. A Tukey test (Antonello, Clark and Heyse 1993) was applied to test the differences between the seed sizes from individual plants within the same genotype. One-way ANOVA was carried out on the pooled seeds to assess the significance of any differences in the seed sizes produced by different mutant lines. Unless otherwise stated, least significant values were used from the ANOVA output to compare the significance of the mean seed sizes between the lines. For seeds at the globular stage of development seed length, width and integument thickness were measured using laser scanning confocal microscopy images taken from early seeds stained with Feulgen (section 2.4.9). ANOVA was carried out to assess the significance of any differences in seed length, width and integument thickness produced by different mutants and transgenic lines.

All statistical calculations were carried out using the Genstat for Windows version 12 (VSN International, Herts, UK) and Microsoft Excel for Windows.

Chapter 3

Results

Chapter 3.1

Genetic analysis of *IKU2* RLKs involved in plant reproduction

3.1.1 Identification of candidate RLKs for genetic analysis with *IKU2*

RLKs that are evolutionary related and share high structural similarity are known to be functionally redundant as they very often act in the same or parallel signalling pathways to regulate various biological responses (Shiu and Bleecker 2001, Shpak et al. 2004). The closest IKU2 homolog (Shiu and Bleecker 2001) is a gene annotated as an RLK of unknown function (*At1g09970)*, here named *IKU2-LIKE* (*IKL*). Further, RLKs are thought to act in homo, hetero and oligomers (Lemmon and Schlessinger 1994), thus to narrow the selection of other RLKs (600 possible candidates in Arabidopsis) that could act with IKU2, only those with known functioning were selected for the analysis. The chosen RLKs: *SERK1/SERK2* and *EMS1/EXS* control anther development (Zhao et al. 2002, Albrecht et al. 2005) but most importantly affect embryogenesis (Schmidt et al. 1997, Thomas et al. 2004, Canales et al. 2002) therefore pose as suitable candidates to genetically interact with *IKU2* during seed development. The double *SERK1/SERK2* and single *EMS1/EXS* recessive mutant seeds (Col-0) were obtained from collaborators (Sacco de Vries, University of Wagenigen and Hugh Dickinson, University of Oxford, respectively), and the *IKL* mutant seeds were obtained from European Arabidopsis Stock Centre (NASC).

3.1.2 Genetic relationship between *IKU2* and *IKU2-LIKE* function

To identify the closest homolog of *IKU2*, the Arabidopsis genome was screened using *IKU2* nucleotide and amino acid sequence (see general methods). As a result of BLAST homology searches (Fig. 3.1.1), a putative *IKU2* homolog gene, named *IKU2-LIKE (IKL)* (*At1g09970*) was identified. *IKL* shares high nucleotide (51%) and amino acid (59%) sequence identity with *IKU2* (Fig.3.1.2).

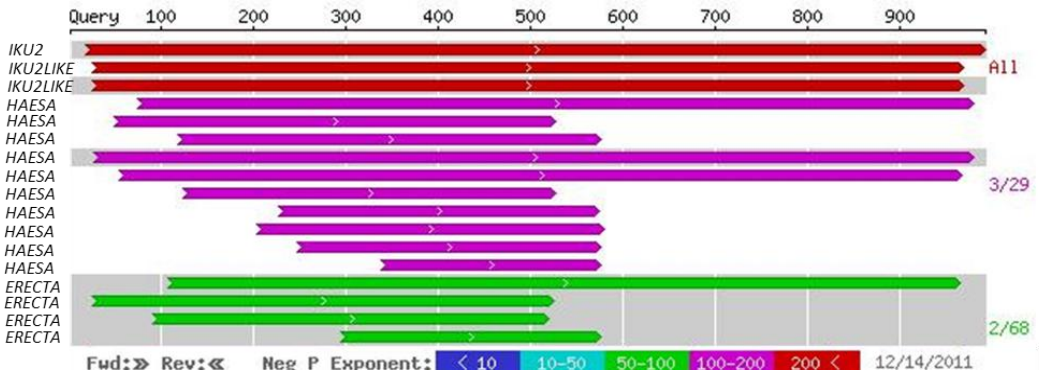

Figure 3.1. 1 **Identification of *IKU2* closest homolog *IKU2-LIKE* (*IKL*)**
Graphical representation of amino acid homology between IKU2 and the closest Arabidopsis: *IKU2LIKE* (in red), *HAESA* (in pink), *ERECTA* (in green)

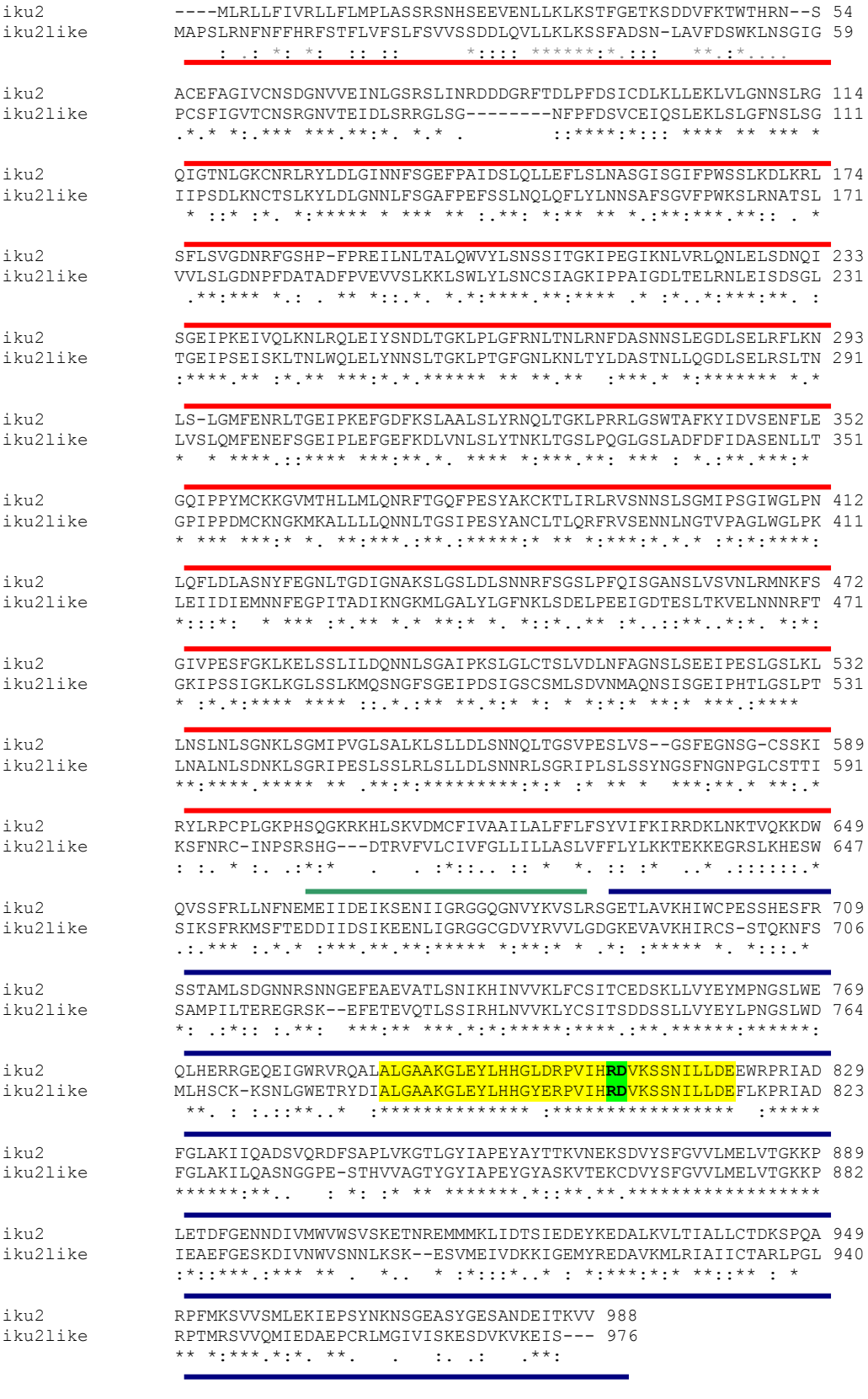
iku2 ----MLRLLFIVRLLFLMPLASSRSNHSEEVENLLKLKSTFGETKSDDVFKTWTHRN--S 54
iku2like MAPSLRNFNFFHRFSTFLVFSLFSVVSSDDLQVLLKLKSSFADSN-LAVFDSWKLNSGIG 59
iku2 ACEFAGIVCNSDGNVVEINLGSRSLINRDDDGRFTDLPFDSICDLKLLEKLVLGNNSLRG 114
iku2like PCSFIGVTCNSRGNVTEIDLSRRGLSG--------NFPFDSVCEIQSLEKLSLGFNSLSG 111
iku2 QIGTNLGKCNRLRYLDLGINNFSGEFPAIDSLQLLEFLSLNASGISGIFPWSSLKDLKRL 174
iku2like IIPSDLKNCTSLKYLDLGNNLFSGAFPEFSSLNQLQFLYLNNSAFSGVFPWKSLRNATSL 171
iku2 SFLSVGDNRFGSHP-FPREILNLTALQWVYLSNSSITGKIPEGIKNLVRLQNLELSDNQI 233
iku2like VVLSLGDNPFDATADFPVEVVSLKKLSWLYLSNCSIAGKIPPAIGDLTELRNLEISDSGL 231
iku2 SGEIPKEIVQLKNLRQLEIYSNDLTGKLPLGFRNLTNLRNFDASNNSLEGDLSELRFLKN 293
iku2like TGEIPSEISKLTNLWQLELYNNSLTGKLPTGFGNLKNLTYLDASTNLLQGDLSELRSLTN 291
iku2 LS-LGMFENRLTGEIPKEFGDFKSLAALSLYRNQLTGKLPRRLGSWTAFKYIDVSENFLE 352
iku2like LVSLQMFENEFSGEIPLEFGEFKDLVNLSLYTNKLTGSLPQGLGSLADFDFIDASENLLT 351
iku2 GQIPPYMCKKGVMTHLLMLQNRFTGQFPESYAKCKTLIRLRVSNNSLSGMIPSGIWGLPN 412
iku2like GPIPPDMCKNGKMKALLLLQNNLTGSIPESYANCLTLQRFRVSENNLNGTVPAGLWGLPK 411
iku2 LQFLDLASNYFEGNLTGDIGNAKSLGSLDLSNNRFSGSLPFQISGANSLVSVNLRMNKFS 472
iku2like LEIIDIEMNNFEGPITADIKNGKMLGALYLGFNKLSDELPEEIGDTESLTKVELNNNRFT 471
iku2 GIVPESFGKLKELSSLILDQNNLSGAIPKSLGLCTSLVDLNFAGNSLSEEIPESLGSLKL 532
iku2like GKIPSSIGKLKGLSSLKMQSNGFSGEIPDSIGSCSMLSDVNMAQNSISGEIPHTLGSLPT 531
iku2 LNSLNLSGNKLSGMIPVGLSALKLSLLDLSNNQLTGSVPESLVS--GSFEGNSG-CSSKI 589
iku2like LNALNLSDNKLSGRIPESLSSLRLSLLDLSNNRLSGRIPLSLSSYNGSFNGNPGLCSTTI 591
iku2 RYLRPCPLGKPHSQGKRKHLSKVDMCFIVAAILALFFLFSYVIFKIRRDKLNKTVQKKDW 649
iku2like KSFNRC-INPSRSHG---DTRVFVLCIVFGLLILLASLVFFLYLKKTEKKEGRSLKHESW 647
iku2 QVSSFRLLNFNEMEIIDEIKSENIIGRGGQGNVYKVSLRSGETLAVKHIWCPESSHESFR 709
iku2like SIKSFRKMSFTEDDIIDSIKEENLIGRGGCGDVYRVVLGDGKEVAVKHIRCS-STQKNFS 706
iku2 SSTAMLSDGNNRSNNGEFEAEVATLSNIKHINVVKLFCSITCEDSKLLVYEYMPNGSLWE 769
iku2like SAMPILTEREGRSK--EFETEVQTLSSIRHLNVVKLYCSITSDDSSLLVYEYLPNGSLWD 764
iku2 QLHERRGEQEIGWRVRQALALGAAKGLEYLHHGLDRPVIHRDVKSSNILLDEEWRPRIAD 829
iku2like MLHSCK-KSNLGWETRYDIALGAAKGLEYLHHGYERPVIHRDVKSSNILLDEFLKPRIAD 823
iku2 FGLAKIIQADSVQRDFSAPLVKGTLGYIAPEYAYTTKVNEKSDVYSFGVVLMELVTGKKP 889
iku2like FGLAKILQASNGGPE-STHVVAGTYGYIAPEYGYASKVTEKCDVYSFGVVLMELVTGKKP 882
iku2 LETDFGENNDIVMWVWSVSKETNREMMMKLIDTSIEDEYKEDALKVLTIALLCTDKSPQA 949
iku2like IEAEFGESKDIVNWVSNNLKSK--ESVMEIVDKKIGEMYREDAVKMLRIAIICTARLPGL 940
iku2 RPFMKSVVSMLEKIEPSYNKNSGEASYGESANDEITKVV 988
iku2like RPTMRSVVQMIEDAEPCRLMGIVISKESDVKVKEIS--- 976

Figure 3.1. 2 **Amino acid alignment of IKU2 and IKL proteins.**
Clustal-W alignment of amino acid sequences "*" identical residues between the two sequences, ":"conserved substitution, "." Semi conserved substitution. Underlying red lines correspond to the extracellular leucine rich repeat domain, green line represents the transmembrane domain, blue lines underlie cytoplasmic kinase domain. In yellow, the conserved seronine/threonine (D-type) active site of the kinase RD domain with conserved arginine (R) and aspartic acid (D) residues.

To determine whether there is an overlap in the expression of these two genes, the *in silico* expression profiles were analysed using the Gene Networks in Seed Development database (http://seedgenenetwork.net/). Analysis of *IKL* expression profile during different stages of seed development (Fig.3.1.3) showed that initially *IKL* transcripts are detectable in the preglobular stage embryo proper and in the micropylar part of the endosperm. In seeds containing globular staged embryos, *IKL* expression was only detected in the peripheral endosperm. During heart stage embryo development, *IKL* transcripts were present in the micropylar and peripheral parts of the endosperm. *IKL* expression remains in the peripheral endosperm throughout the cotyledon staged embryo and in the endosperm and seed coat of mature seeds. Since *IKU2* transcripts are present only in micropylar endosperm in globular staged seeds the spatial expression of these two genes seems not to overlap precisely in expression pattern.

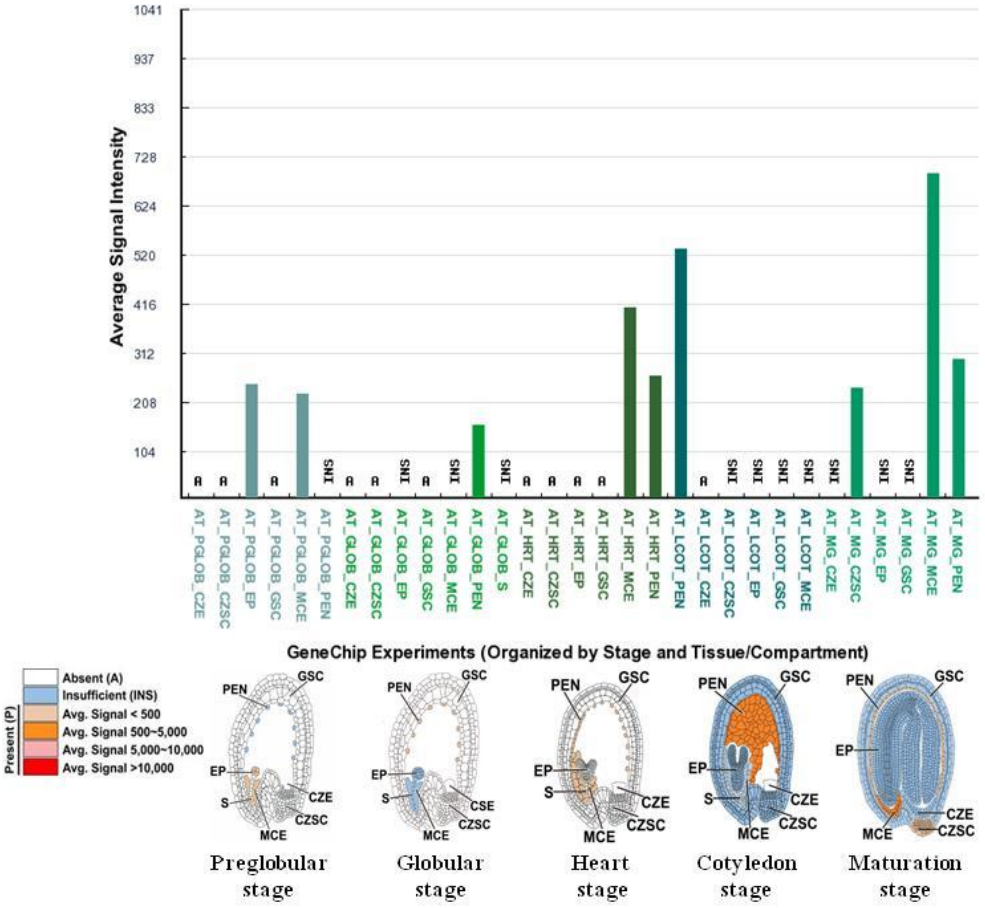

Figure 3.1. 3 ***In silico* expression profile of *IKL***

Bar chart showing average signal intensities of *IKL* for all seed compartments: CZE, chalazal endosperm; CZSC, chalazal seed coat; EP, embryo proper; GSC, general seed coat; MCE, micropylar endosperm; PEN, peripheral endosperm. Abbreviation: A, Absent; INS, insufficient. The developmental stages: PGLOB, pre-globular, GLOB, globular, HRT, heart; LCOT, linear cotyledon; MG, mature green.

The images below represent accumulation of the mRNA in different tissue compartments at different stages of seed development. Colours correspond to the average signal intensity of biological replicates.

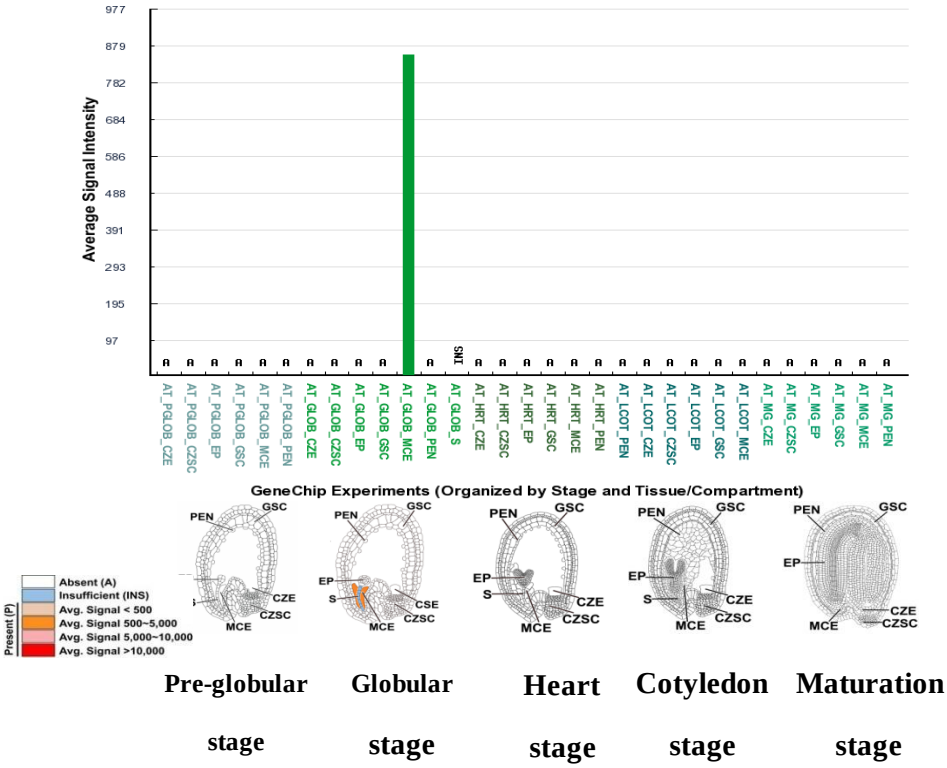

Figure 3.1. 4 ***In silico* expression profile of *IKU2***

Bar chart showing average signal intensities of *IKU2* for all seed compartments: CZE, chalazal endosperm; CZSC, chalazal seed coat; EP, embryo proper; GSC, general seed coat; MCE, micropylar endosperm; PEN, peripheral endosperm. Abbreviation: A, Absent; INS, insufficient. The developmental stages: PGLOB, pre-globular, GLOB, globular, HRT, heart; LCOT, linear cotyledon; MG, mature green.

The images below represent accumulation of the mRNA in different tissue compartments at different stages of seed development. Colours correspond to the average signal intensity of biological replicates.

To investigate if *IKU2* and *IKL* overlap in their function, I carried out a genetic analysis of *iku2/ikl* double mutants. To this aim, two T-DNA *ikl* alleles were identified in the SIGnAL mutant collection as salk_094492 (*ikl-1*) and salk_120595 (*ikl-2*). Screening and isolation of *ikl-1* (salk_094492) and *ikl-2* (salk_120595) mutant lines was performed as described by the SIGnAL primer design tool (http://signal.salk.edu/tdnaprimers.). The T-DNA insertions isolated were located in exon1 and a PCR based assay (Fig. 3.1.5) was applied to identify homozygous *ikl-1* and *ikl-2* plants. To isolate *ikl-1* homozygous plants, salk_094492F (Fig. 3.1.6F1),

salk_094492R (Fig. 3.1.6R1) gene specific primers were used to amplify the wild type copy of *IKL* and Lba1salk with R1 were used to confirm the presence of T-DNA insertion in two PCR reactions (Fig. 3.1.6). Similarly to genotype *ikl-2* lines: salk_120595F (Fig.3.1.6 F2), salk_120595R (Fig. 3.1.6R2) were used to amplify the wild type copy of *At1g09970* while the presence of the T-DNA insertion was confirmed by using Lba1salk and salk_120595R (Fig.3.1.6R2) primer pairs (Fig. 3.1.6).

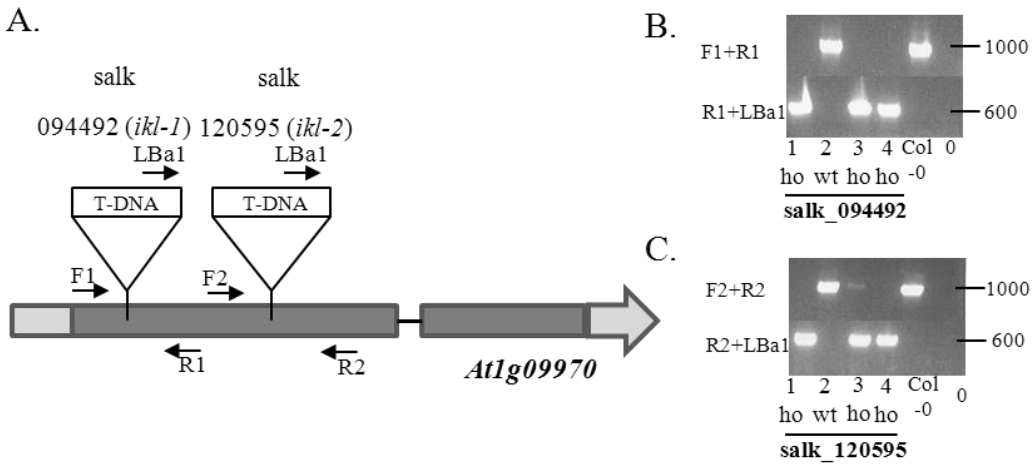

Figure 3.1. 5 **Screening and isolation of *At1g09970 (IKL)* T-DNA insertion mutants**

(A) Schematic representation of two T-DNA insertion sites in exon 1 of *At1g09970.*Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines, and positions for oligonucleotide primers are indicated by arrows. (B) *ikl-1*/*ikl-1* plants were characterized by PCR genotyping using gene specific (F1+R1, F2+R2) and T-DNA specific left border (LBa1) primer combinations. In homozygous (ho) *ikl-1* (salk_094492) gene specific R1primer in combination with the left border primer (LBa1) amplified products of 600bp. In wild type (wt) a 1000bp PCR product is detected using a gene -specific primer pairs (F1+R1), whereas the same product is absent in homozygous *ikl- 1* plants. (C) In homozygous (ho) *ikl-2* line (salk_120959) gene specific R2 in combination with the LBa1 primers also amplify product of 600bp. In wild type (wt) the F2 and R2 primers amplify a product of 1000bp which is absent in homozygous (ho) *ikl-2* plants. The full view of gel pictures is available in Appendix 2.

The PCR reactions were carried out with the initial step of denaturation at 94ºC for 1minutes, followed by cycles of denaturation at 94ºC for 30s, annealing at 58ºC for 30s and extension at 72ºC for 1minute. The predicted sizes of PCR products are represented in Fig. 3.1.5 and further details are shown in Appendix 2.

Salk_120595 and salk_094492 insertions are likely to be *IKL* loss-of-function alleles of since they cause a premature truncation of the protein. In order to assess any visible seed and plant abnormalities *ikl-1/ikl-1* and *ikl-2/ikl-2* plants were allowed to self-fertilise, and the F2 generation were PCR genotyped (Fig. 3.1.6) to select homozygous plants for analysis. The plant morphology of single homozygous mutant lines was indistinguishable from wild-type plants during the vegetative growth phase (data not shown). Flowers and siliques of these plants were morphologically similar to wild-type, however developing and mature seeds of *ikl-1/ikl-1* displayed phenotypic abnormalities (Fig. 3.1.7). To allow further analysis of double mutants, *ikl-1/ikl-1*and *ikl-2/ikl-2* plants were crossed with the *iku2-4* mutant line. The F_2 plants were genotyped by PCR in order to identify double homozygous *iku2-4/iku2-4;ikl-1/ikl-1*and *iku2-4/iku2-4;ikl-2/ikl-2* plants which were selected for further phenotypic analysis.

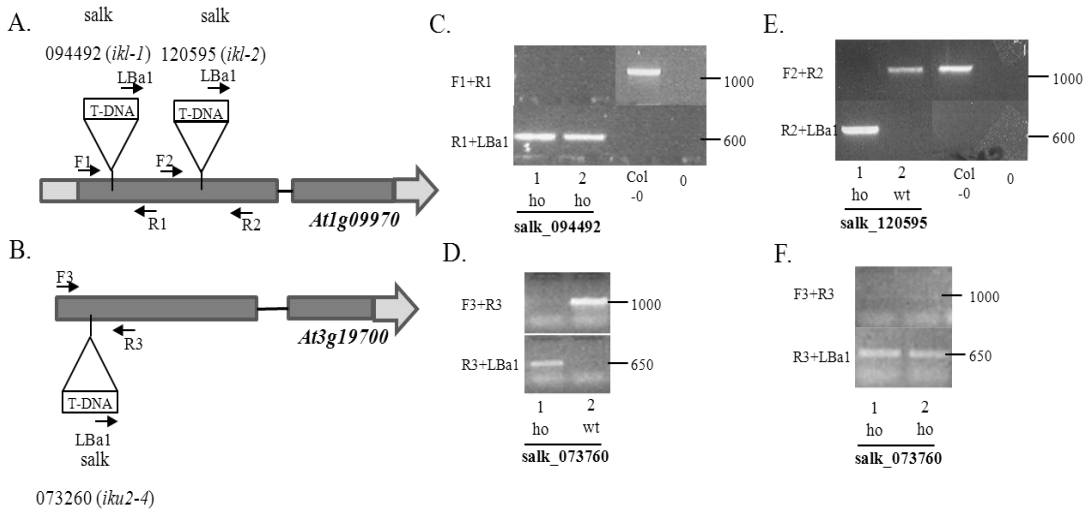

Figure 3.1. 6 **Representation of PCR genotyping of T-DNA insertions in *iku2/ikl* double mutants**

(A) Schematic representation of two T-DNA insertion sites in exon 1 of*At1g09970.* (B) Insertion site of the T-DNA in exon 1 of *At3g19700.*Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines, and positions for oligonucleotide primers are indicated by arrows. (C) In double homozygous (ho) mutant *iku2-4*/*ikl-1* (salk_73260 and salk_094492 respectively) gene specific R1 in combination with left border primer (LBa1) amplify products of 600bp. In wild type (wt) for the *ikl-1* allele a 1000bp PCR product is detected by a gene specific primer pair (F1+R1), whereas the same product is absent in double homozygous *iku2-4*/*ikl-1* (D) Insertion of T-DNA in *At3g19700* (*IKU2*) gene in *iku2-4/ikl-1* double mutants was confirmed with R3 and Lba1 primer pair (650bp) and the wild type allele of the gene with F3+R3 (1000bp). (E) In double homozygous (ho) *iku2-4*/*ikl-2* mutant (salk_07260 and salk_120959 respectively) insertion of T-DNA was confirmed with gene specific R2 in combination with the LBa1 primers that amplify a product of 600bp and the presence of the wild type allele (no T-DNA insertion) with F2 and R2 primers that amplify products of 1000bp. The same product is absent in homozygous (ho) *ikl-2* (F) Insertion of T-DNA in *At3g19700* (*IKU2*) gene in *iku2-4/ikl-2* double mutants was confirmed with R3 and Lba1 primer pair (650bp) and the wild type allele of the gene with F3+R3 (1000bp). The full view of gel pictures available in Appendix 2.

Four independent plants of each genotype: Col-0, *iku2-4/iku2-4, ikl-1/ikl-1, ikl-2/ikl-2, iku2-4/iku2-4;ikl-1/ikl-1, iku2-4/iku2-4;ikl-2/ikl-2* were taken for further analysis. There were no visible vegetative developmental abnormalities present in any of the double homozygous *iku2-4/iku2-4;ikl-1/ikl-1* and *iku2-4/iku2-4;/ikl-2/ikl-2* lines. The reproductive tissues, flowers and siliques, were also indistinguishable from wild-type. The seed progeny were collected from four individual plants of each genotype and seed sizes were phenotypically analysed for changes in seed size and

shape. To determine the seed sizes from individual plants, the seed areas of dry mature seeds were measured and analysed using ImageJ and statistical methods (see general methods). The seed sizes from individual plants were compared for variation using ANOVA. (Table 3.1.1) The plants derived from the same genotype/line are not expected to differ amongst themselves significantly.

	Col-0		***iku2-4***		***ikl-1***	
Source of variation	d.f.	m.s.	d.f.	m.s.	d.f.	m.s.
Plant	3	0.0033040***	3	0.0011671**	3	0.0063652***
Residual	1378	0.0002952	1433	0.0003073	1687	0.0006664
	ikl-2		***iku2-4/ikl-1***		***iku2-4/ikl-2***	
Source of variation	d.f.	m.s.	d.f.	m.s.	d.f.	m.s
Plant	3	0.0092617***	3	0.0005802	3	0.0026661*
Residual	1478	0.0003373	1968	0.0005561	2395	0.0007971

Table 3.1. 1 **Mean squares from analysis of variance (ANOVA) Seed areas of individual plants derived from particular genotypes: Col-0*, iku2-4/iku2-4, ikl-1/ikl-1, ikl-2/ikl-2, iku2-4/iku2-4;ikl-1/ikl-1, iku2-4/iku2-4;ikl-2/ikl-2*were analyzed. D.f. – degree of freedom, m.s. – mean square. * significant at 0.05 level, ** significant at 0.01 level, *** significant at 0.001 level**

The analysis indicates that seeds from individual plants of Col-0, *iku2-4/iku2-4, ikl-1/ikl-1, ikl-2/ikl-2, iku2-4/iku2-4;ikl-2/ikl-2* genotypes were statistically different from each other. Only seed sizes from individual *iku2-4/iku2-4;ikl-1/ikl-1* plants did not differ amongst themselves statistically (P>0.05).

Next, the Tukey test was applied to confirm the differences between the seed sizes of individual plants within the lines (Table 3.1.2).

Lines									
Plant no		**Col-0**			***iku2-4***			***ikl-1***	
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	316	0.11213	0.01537	400	0.09103	0.01859	441	0.08851	0.02448
2	317	0.10889	0.02041	361	0.09145	0.02066	429	0.08060	0.02796
3	323	0.11208	0.0157	347	0.09274	0.01503	439	0.08074	0.0249
4	427	0.19593	0.01683	329	0.08851	0.01454	382	0.08527	0.02571
$LSD_{0.05}$		0.002307			0.002432			0.003410	
		ikl-2			***iku2-4/ikl-1***			***iku2-4/ikl-2***	
Plant no	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	408	0.09443	0.02095	620	0.08743	0.02356	694	0.08354	0.029179
2	395	0.09884	0.01689	594	0.08700	0.02377	623	0.08674	0.029329
3	312	0.09867	0.01555	605	0.08825	0.02332	663	0.08252	0.028079
4	367	0.10629	0.01891	553	0.08561	0.02358	419	0.08202	0.025536
$LSD_{0.05}$		0.002522			0.002868			0.002972	

Table 3.1. 2 **The differences between the means of the seed size (in mm^2) derived from individual plants within tested genotypes**

Genotypes tested: Col-0, *iku2-4/iku2-4, ikl-1/ikl-1, ikl-2/ikl-2, iku2-4/iku2-4;ikl-1/ikl-1, iku2-4/iku2-4;ikl-2/ikl-2*. S.d.:standard deviation. n=number of seeds

The results showed that within the Columbia genotype the highest mean of dry seed sizes was 0.19593mm^2 ±0.01683 and the smallest 0.10889mm^2 ±0.02041. In *iku2-4/iku2-4* plants the smallest mean of dry seed sizes was 0.08851mm^2±0.01454 and the highest 0.09274mm^2±0.01503. *Ikl-1/ikl-1* produced seeds that were of smallest mean size 0.08060mm^2±0.02796, and 0.08851mm^2±0.02448 the highest. The smallest mean size of *ikl-2/ikl-2* seeds was 0.09443mm^2±0.02095 and 0.09884mm^2±0.01689 was the highest. Double *iku2-4/iku2-4;ikl-1/ikl-1* plants produced seeds that did not show significant variation in seed size, the smallest mean was of 0.08561mm^2±0.02358and 0.08825mm^2±0.02332 the highest. In *iku2-4/iku2-4;ikl-2/ikl-2* the smallest seeds mean was 0.08202mm^2±0.025536 and the highest, 0.08674mm^2±0.029329.

To further investigate the effects of *ikl* mutation on the variability of seed area, one-way ANOVA was carried out on pooled seeds derived from Col-0, *iku2-4/iku2-4*, *ikl-1/ikl-1*, *ikl-2/ikl-2*, *iku2-4/iku2-4;ikl-1/ikl-1* and *iku2-4/iku2-4;ikl-2/ikl-2* plants (Fig. 3.1.7). The least significant difference (LSD) of seed area was calculated, and on this basis groups that were not significantly different from each other were determined (Table 3.1.3).

Lines	**n**	**Mean (mm^2)**	**Median**
Col-0	1383	$0.10946^{a} \pm 0.00046$	0.0003017
iku2-4	1437	$0.09082^{b} \pm 0.00046$	0.0003091
ikl-1	1691	$0.08375^{c} \pm 0.00063$	0.0006765
ikl-2	1482	$0.09943^{d} \pm 0.00048$	0.0003554
iku2-4/ikl-1	1972	$0.08712^{e} \pm 0.00048$	0.0005561
iku2-4/ikl-2	2399	$0.08380^{c} \pm 0.00048$	0.0007995
$LSD_{0.05}$		0.001309	

Table 3.1. 3 **Analysis of seed size in wild type, *iku2-4* and *ikl* plants**
Lines: Col-0, *iku2-4/iku2-4*, *ikl-1/ikl-1*, *ikl-2/ikl-2*, *iku2-4/iku2-4;ikl-1/ikl-1*, *iku2-4/iku2-4;ikl-2/ikl-2*.&: The mean annotated with the same letter symbol are not significantly different from each other ($LSD_{0.05}$, $P<0.001$), ± SEM. n=number of seeds

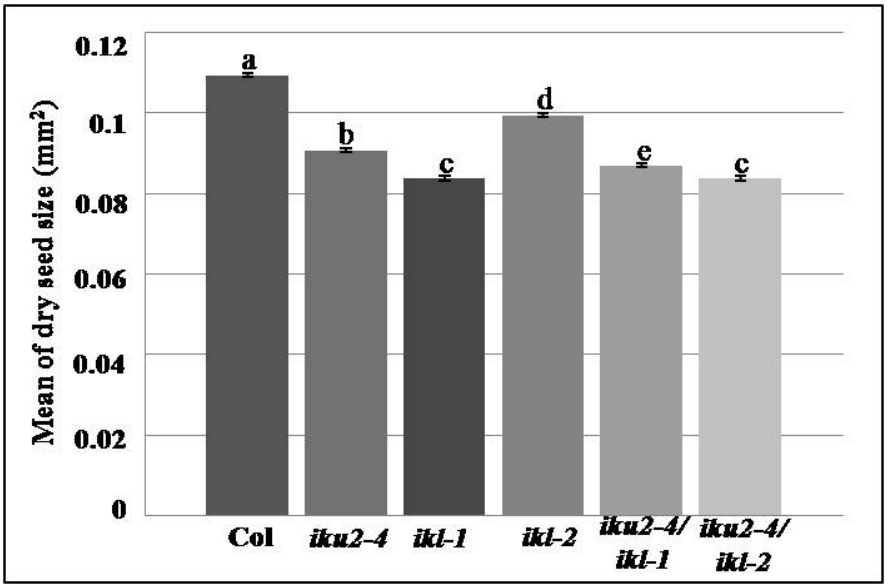

Figure 3.1. 7 **Means of dry mature seeds from *iku2-4* and *ikl* plants**
The bars represent the mean seed size (mm^2) from Col-0, *iku2-4/iku2-4*, *ikl-1/ikl-1*, *ikl-2/ikl-2*, *iku2-4/iku2-4;ikl-1/ikl-1* and *iku2-4/iku2-4;ikl-2/ikl-2* plants. The means annotated with the same letter symbol are not statistically different from each other. Error bars: SEM.

ANOVA analysis of pooled seeds showed that the mean sizes of single *ikl-1/ikl-1* and *ikl-2/ikl-2* mutants (0.08375mm^2 ±0.00063and 0.09943mm^2 ±0.00048, respectively) were significantly different from the mean size of wild type Columbia seeds (0.10946mm^2 ±0.00046) and *iku2-4/iku2-4* seeds (0.09082mm^2±0.00046). Double *iku2-4/iku2-4;ikl-1/ikl-1* and *iku2-4/iku2-4;ikl-2/ikl-2* mutants slightly enhanced the small seed phenotype of *iku2-4/iku2-4* since their mean sizes (0.08712mm^2 ±0.00048 and 0.08380mm^2 ±0.00048 respectively) were significantly different from the mean of *iku2-4/iku2-4* (0.09082mm^2 ±0.00046). (Table 3.1.3)

The distribution of seed sizes for all genotypes analysed confirmed the statistical data (Fig. 3.1.8). Distribution of seed sizes in all of the tested groups ranged from 0.04 – 0.14mm^2. The majority of seeds produced by Columbia wild-type plants was 0.12mm^2 (34.5%) in comparison to 0.09mm² (34.3%) in *iku2-4/iku2-4*. Single *ikl-1/ikl-1* mutants produced seeds that were slightly smaller than wild-type Columbia seeds with the highest percentage (25.19%) of seeds being 0.09mm². The pooled population of *ikl-2/ikl-2* seeds showed the highest percentage (28.8%) of seed size that was 0.11mm². The distribution of seed sizes from *iku2-4 /iku2-4;/ikl-1/ikl-1* plants showed that most (31.5%) of the seeds were of 0.09mm^2. The analysis of *iku2-4/iku2-4;ikl-2/ikl-2* double homozygous line showed that the main percentage (25.7%)of seeds produced was 0.08mm².

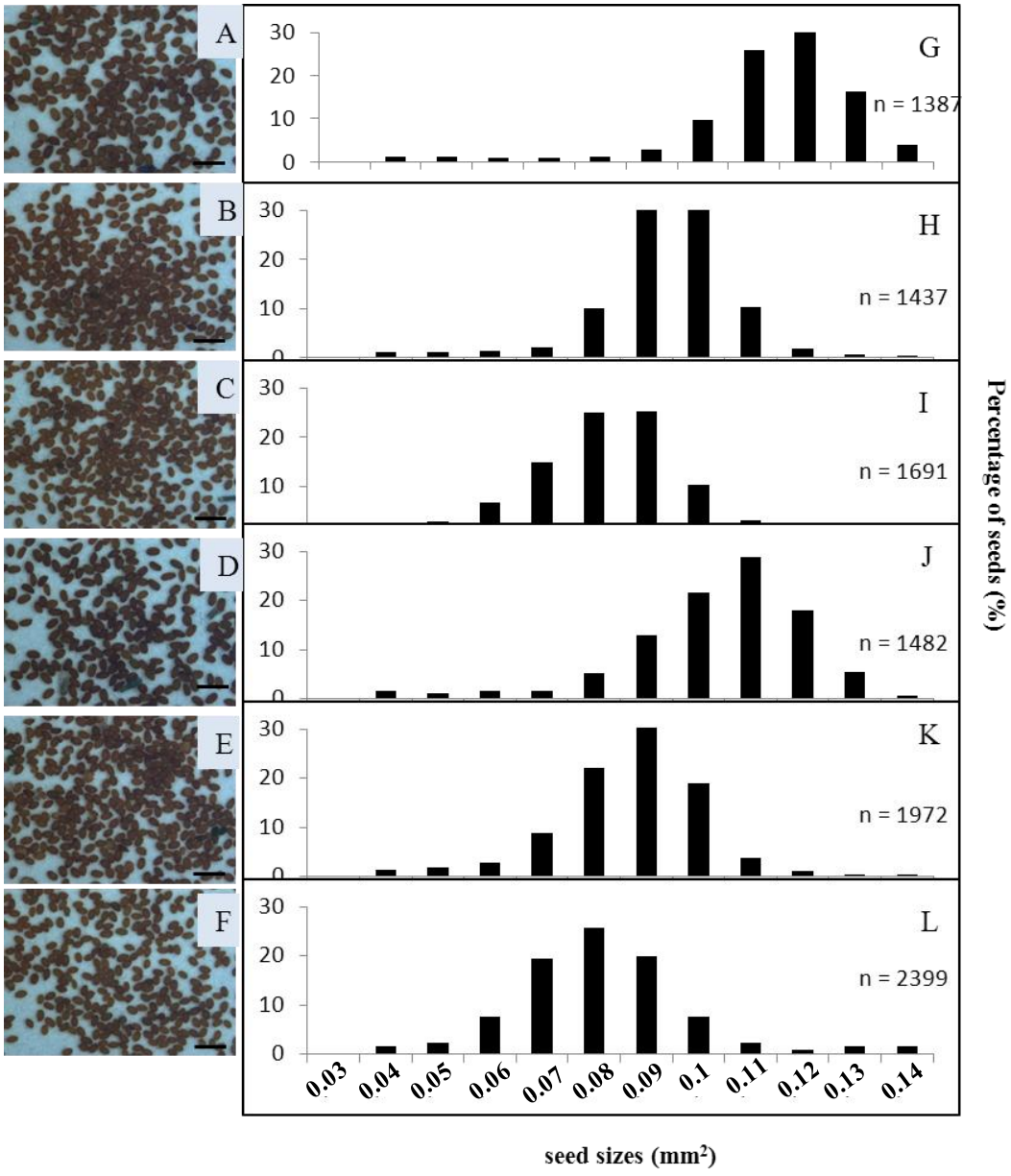

Figure 3.1. 8 **Seed size distribution from *iku2-4* and *ikl* plants**

Left panel: mature dry seeds from (A) Wild-type Columbia-0, (B) *iku2-4/iku2-4* (C) *ikl-1/ikl-1*, (D) *ikl-2/ikl-2* (E) *ikl-1/ikl-1;iku2-4/iku2-4*, (F) *ik2-2/ikl-2*; *iku2-4/iku2-4*. Scale bars:1mm

Right panel: Seed size distribution in progenies from self-fertilised: (G) Wild-type Columbia-0, (H) *iku2-4/iku2-4*, (I) *ikl-1/ikl-1*, (J) *ikl-2/ikl-2*, (K) *ikl-1/ikl-1; iku2-4/iku2-4*, (L) *ikl-2/ikl-2; iku2-4/iku2-4*. X axis represents seed area in mm^2, Y axis the percentage of seeds. n= total number of seeds analysed

Statistical analysis of seed size distribution analysis revealed that progeny seeds from both double mutant plants show a significant enhancement of the *iku2-4* small seed phenotype by *ikl*.

3.1.3 Genetic relationship between *IKU2* and *SERK1, SERK2* function

The *in silico* expression profile of *SERK1* during seed development (Fig.3.1.10) shows that the transcripts of the gene are abundant in the seed coat, endosperm and embryo at pre-globular, globular, heart, linear cotyledon and mature green stages of seed development. *SERK2* expression is confined to the initial pre – globular stages of embryo development, in the chalazal seed coat and embryo proper (Fig.3.1.9). At the globular stage of seed development, *SERK2* is detectable in most of the tissue compartments of the seed: the chalazal and general seed coat, embryo proper, suspensor and peripheral endosperm. At the heart stage of embryo development, *SERK2* expression remains in the chalazal seed coat, embryo proper, micropylar and peripheral endosperm. During the cotyledon stage of embryo development the transcripts of *SERK2* remain detectable in the endosperm, embryo and the seed coat. In the mature green seed, transcripts are detected in the embryo and peripheral endosperm only. Collectively these data reveal that there is an overlap in expression between *IKU2* and *SERK1* in micropylar endosperm at the globular stage of embryo development. *SERK2* at this stage is present in embryo proper and seed coat, therefore does not overlap with *IKU2* at this stage.

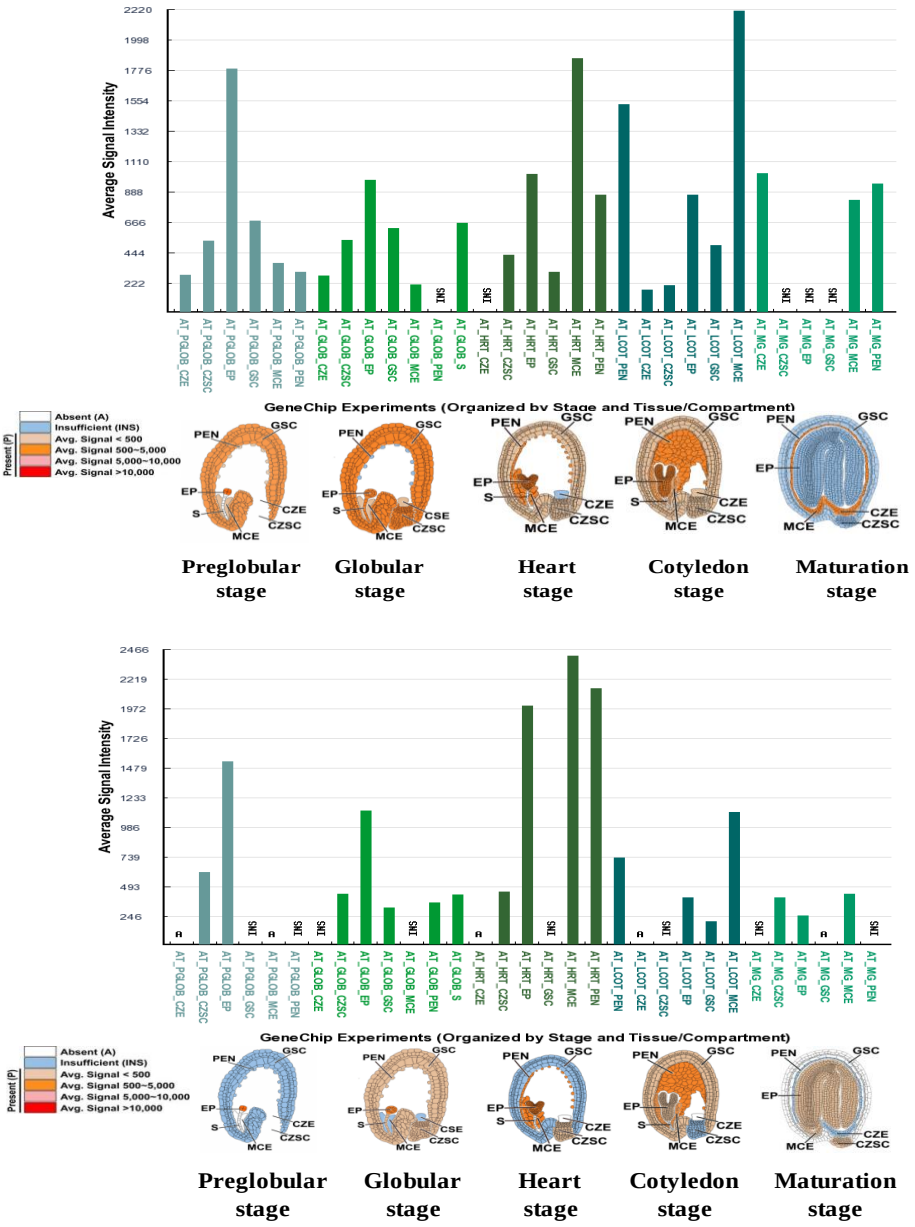

Figure 3.1. 9 ***In silico* expression profile of *SERK1* and *SERK2***

Top panel: *In silico* expression profile of *SERK1*, bottom panel showing the expression profile of *SERK2*.

Bar charts showing average signal intensities of *SERK1* and *SERK2* for all seed compartments: CZE, chalazal endosperm; CZSC, chalazal seed coat; EP, embryo proper; GSC, general seed coat; MCE, micropylar endosperm; PEN, peripheral endosperm. Abbreviation: A, Absent; INS, insufficient. The developmental stages: PGLOB, pre-globular, GLOB, globular, HRT, heart; LCOT, linear cotyledon; MG, mature green.

The images below represent accumulation of the mRNA in different tissue compartments at various stages of seed development. Colours correspond to the different average signal intensity of biological replicates

To test genetically the relationship between *IKU2* and *SERK1* and *SERK2*, triple *iku2-4/iku2-4;serk1-1/serk1-1;serk2-2/serk2-2* mutants were generated by genetic introgression. In the initial step a PCR based assay was applied to genotype the seedlings from *serk1-1/SERK1-1;SERK2-2/serk2-2* (Fig. 3.1.10). *Serk1-1* locus (salk_044330) was genotyped with serk1.Rev (Fig. 3.1.11 R1) gene specific primer in combination with left T-DNA border tag1 primer. Serk1.For (Fig.3.1.10F1) and serk1.Rev (Fig.3.1.10R1) gene specific primers were used to confirm the disruption of the wild type *SERK1* gene. *Serk2-1* (salk_044330) T-DNA insertion was confirmed with serk2.Rev (Fig. 3.1.10R2) and Lb1*sail combination of primers (Appendix 1).The presence or lack of wild type copy of *SERK2* gene was confirmed with gene specific serk2.For (Fig. 3.1.10F2) and serk2.Rev (Fig,3.1.10 R2) oligonucleotides. The PCRs were carried out with the initial step of denaturation at 94ºC for 1 minute, followed by cycles of denaturation at 94ºC for 30s, annealing at 58ºC for 30s and extension at 72ºC for 1 minute (*serk1-1*) and annealing at 62ºC for 30s and extension at 72ºC for 2 minutes (*serk2-2*).

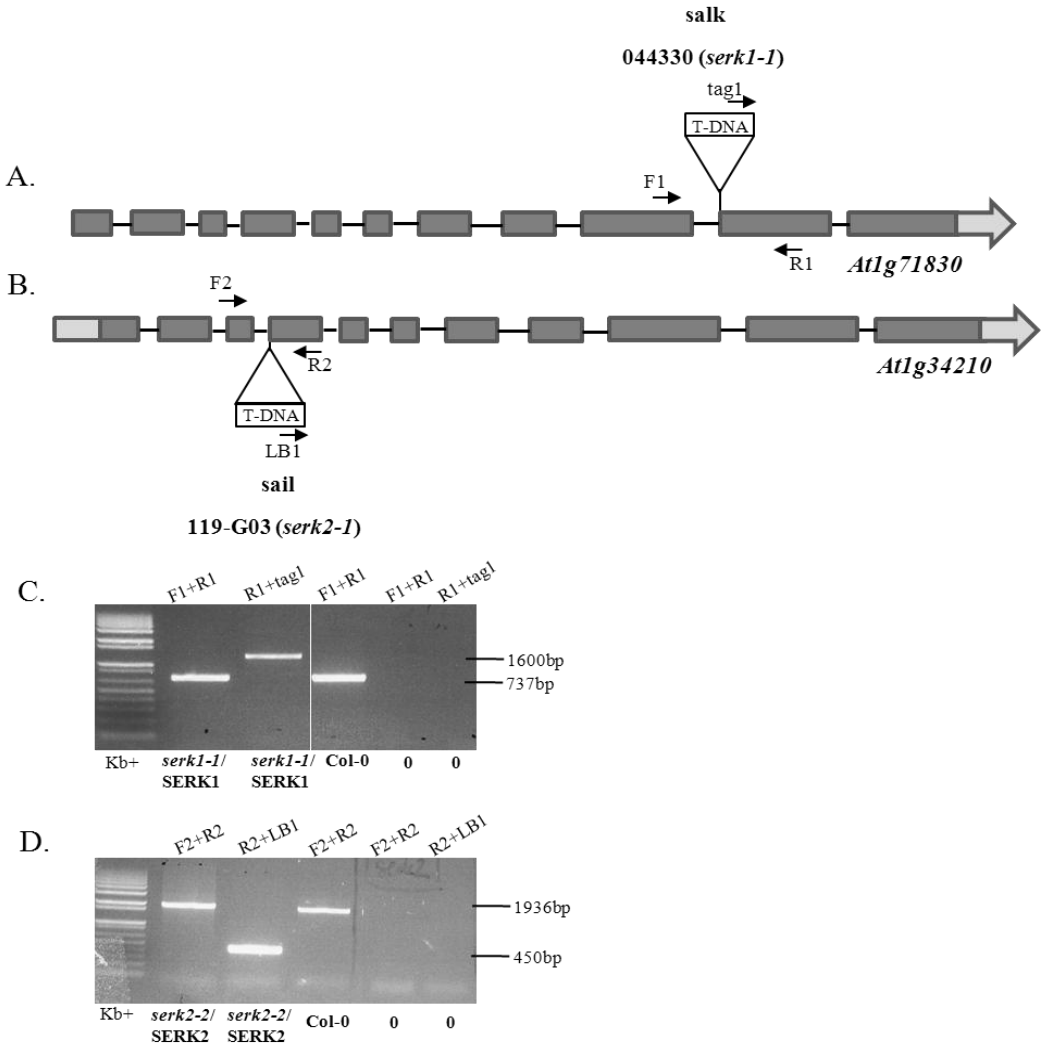

Figure 3.1. 10 **Representation of PCR genotyping of T-DNA insertions in double *serk1/serk2* mutants**

(A) Schematic representation on *SERK1* (*At1g71830)* gene and T-DNA insertion in exon 10. (B) Representation of *SERK2* (*At1g34210*) and the site of T-DNA insertion in exon 4. Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines, and positions for oligonucleotide primers are indicated by arrows. (C) In *serk1-1/SERK1*mutants (salk_044330) gene specific R1 in combination with left border primer (tag1) amplify products of 1600bp. In wild type for the *serk1-1* allele a 737bp PCR product is detected by a gene – specific primer pair (F1+R1). (D) In *serk2-2/SERK2*, insertion of T-DNA was confirmed with gene specific R2 in combination with the LB1 primers that amplify a product of 450bp and the presence of the wild type allele (no T-DNA insertion) with gene specific F2 and R2 primers that amplify products of 1936bp.

In order to generate triple mutant plants, the stigmas of male sterile (*serk1-1/serk1-1;serk2-2/serk2-2*) plants were fertilised with *iku2-4* pollen. The F_1 progeny from the cross was grown to maturity. Because these mutations are recessive, in the F_2 population *serk1-1/serk1-1;SERK2-2/serk2-2;iku2-4/iku2-4* triple mutant plants were identified by PCR. F3 plants were genotyped by PCR and six *iku2-4/serk1-1/serk2-2* male sterile plants were employed for crossing with *serk1-1/serk1-1;SERK2-2/serk2-2;iku2-4/iku2-4* pollen.

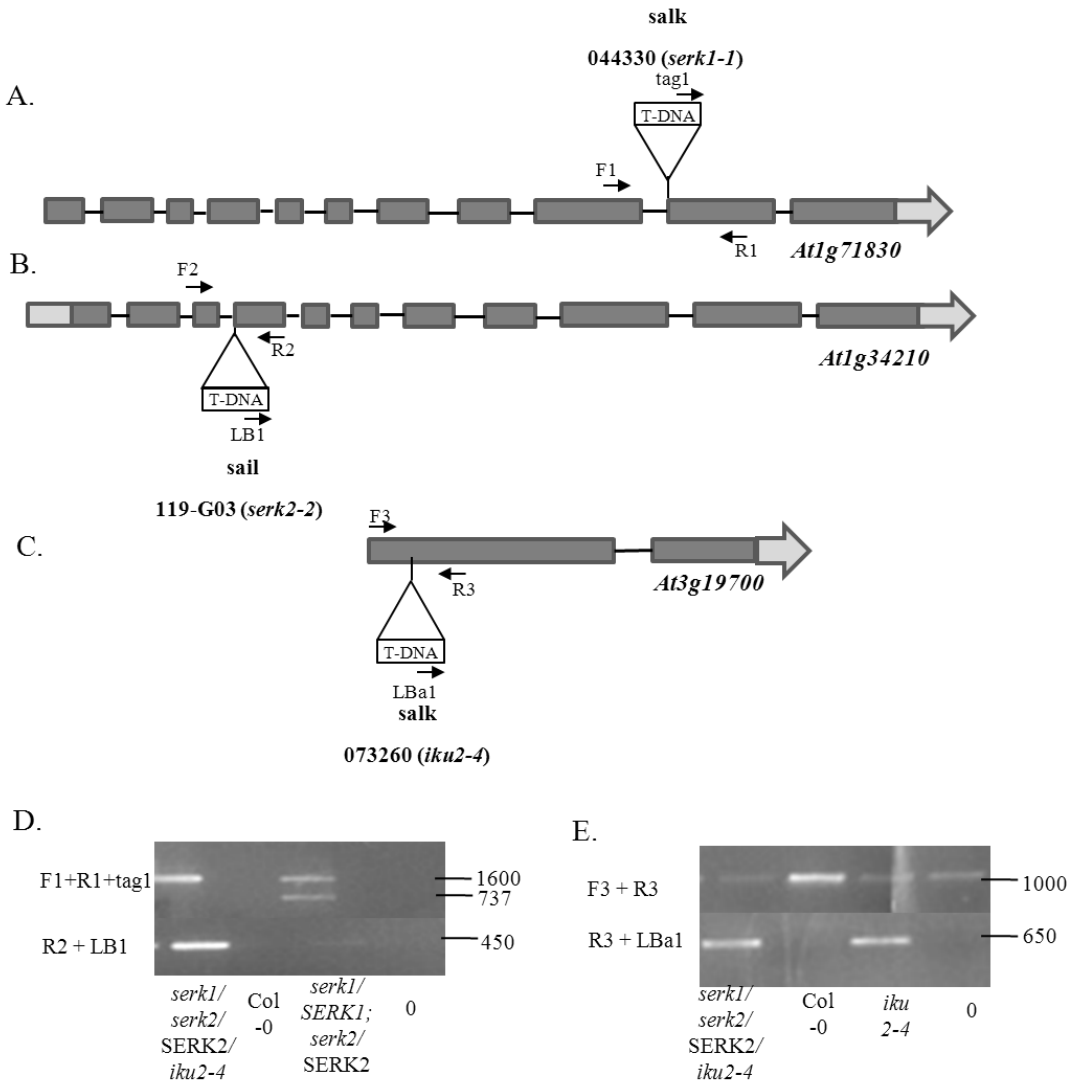

Figure 3.1. 11 **Representation of PCR genotyping of T-DNA insertions in triple *serk1/serk2/iku2* mutants**

(A) Schematic representation of *SERK1* (*At1g71830)* and T-DNA insertion in exon 10. (B) Representation of *SERK2* (*At1g34210*) and the site of T-DNA insertion in exon 4. (C) T-DNA insertion in *IKU2* (*At3g19700*) in exon 1. Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines, and positions for oligonucleotide primers are indicated by arrows. (D) In triple *serk1-1/serk1-1;serk2-2/*SERK2*;iku2-4/iku2-4* mutants (salk_044330, sail_119-G03 and salk_07260 T-DNA insertions respectively) the genetic constitution of *serk1-1* insertion was confirmed with combination of three: F1 + R1 + T-DNA left border (tag1) primers in one PCR reaction, resulting in 1600bp products for the insertion of T-DNA and 737bp products for wild type *SERK1* allele. *serk2-2* T-DNA insertion was confirmed with gene specific R2 in combination with the LB1 primers that amplify a product of 450bp and the presence of the wild type *SERK2* allele, was confirmed by male fertility of the plant (E) Insertion of T-DNA in *At3g19700* (*IKU2*) in the triple *serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4*mutants was confirmed with R3 and Lba1 primer pair (650bp) and the presence or absence of the wild type allele with F3+R3 (1000bp). The full view of gel pictures available in Appendix 2.

The seed progeny from this cross were expected to show 50% segregation for *iku2-4/iku2-4;serk1-1/serk1-1;serk2-2/serk2-2,* and 50% for *serk1-1/serk1-1;SERK2-2/serk2-2;iku2-4/iku2-4*. As controls, seeds from wild type Columbia and *iku2-4/iku2-4* were grown for analysis. Seeds were allowed to mature and dry seeds were harvested for phenotypic analysis using ImageJ measurement tools and statistical methods (general methods).

First, the variation between the progeny seeds of individual plants within analysed genotypes and crosses was assessed using ANOVA.

	Col-0		***iku2-4***	
Source of variation	d.f.	m.s.	d.f.	m.s.
Plant	2	0.0015131***	2	0.0019664**
Residual	749	0.0002249	2093	0.0004016
	serk1-1/serk2-2/iku2-4 x iku2-4		***serk1/serk2/iku2 x serk1/serk1;serk/SERK2/iku2-4***	
Source of variation	d.f.	m.s.	d.f.	m.s.
Cross	2	0.0021628	5	0.0054882***
Residual	1457	0.0005166	1873	0.0004670

Table 3.1. 4 **Mean squares from analysis of variance (ANOVA)**
Seed sizes from individual plants derived from particular genotypes (Col-0, *iku2-4/iku2-4*), and from crosses *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4* x *iku2-4/iku2-4*, *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4* x *serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4* were analysed. D.f. – degree of freedom, m.s. – mean square. * significant at 0.05 level, ** significant at 0.01 level, *** significant at 0.001 level.

The statistical analysis showed that the seeds derived from individual plants of wild type Columbia and *iku2-4/iku2-4* varied significantly in size. Seeds derived from *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4* x *serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4* crosses also differed significantly in size (Table 3.1.4).

A Tukey test was performed to further analyse the differences between individual plants for each genotype.

Lines						
Plant no		**Col-0**			***iku2-4***	
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	250	0.09613	0.012004691	557	0.08204	0.01929103
2	301	0.09695	0.017765961	539	0.07847	0.018547367
3	201	0.10104	0.013647646	1000	0.07936	0.021164681
$LSD_{0.05}$		0.002400			0.001758	
Cross no	***serk1/serk2/iku2-4 x iku2-4***			***serk1/serk2/iku2-4 x serk1/;serk2/SERK2;iku2-4***		
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	948	0.11401	0.02276784	355	0.11345	0.019453446
2	574	0.11823	0.02376747	389	0.11790	0.021599594
3	513	0.11751	0.021408432	272	0.11700	0.026381166
4	-	-	-	227	0.11162	0.020253464
5	-	-	-	326	0.12372	0.023454486
6	-	-	-	310	0.11477	0.017703372
$LSD_{0.05}$		0.002632			0.003039	

Table 3.1. 5 **The differences between the means of the seed sizes (in mm^2) derived from individual plants for each genotype tested**

Tested genotypes: Col-0, *iku2-4* and progeny seeds from crosses *serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4 x iku2-4/iku2-4* and *serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4 x serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4.* s.d.: standard deviation. n=number of seeds

The results of the Tukey test showed that the highest seed size mean in wild type Columbia was 0.10104mm^2±0.01364 and the lowest was 0.09613mm^2±0.01200. In *iku2-4/iku2-4,* the smallest mean was 0.07847mm^2±0.0185 and the biggest 0.08204mm^2±0.0192. Progeny seeds from *serk1-1/serk1-1;serk2/SERK2;iku2-4/iku2-4* x *serk1-1/serk1-1;serk2-2/SERK2;/iku2-4* crosses shared the smallest mean of 0.11162mm^2±0.0202 and the biggest mean of 0.12372mm^2±0.0234. The crosses between *serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4* x *iku2-4/iku2-4* generated seeds with a lowest mean was of 0.11401mm^2 ±0.0227 and highest mean of 0.11823mm^2 ±0.0237 (Table 3.1.5).

Next, a one-way ANOVA test was carried out to determine the effects of genotypes on seed area variability. The LSD of seed area was calculated, and on

these bases homogeneous groups (not significantly different from each other) were determined.

Lines	n	Mean (mm^2)	Median
Col-0	752	0.098^{a} ±0.00055	0.0997
iku2-4	2096	0.079^{b} ±0.00043	0.0801
serk1/serk2/iku2-4 x iku2-4	1461	0.116^{c} ±0.00059	0.1201
serk1/serk2/iku2-4 x serk1/;serk2/SERK2;iku2-4	1879	0.116^{c} ±0.00050	0.1193
$LSD_{0.05}$		0.001260	

Table 3.1. 6 **Analysis of seed size in wild type, *iku2-4, serk1 and serk2* plants**
Genotypes tested: (Col-0, *iku2-4/iku2-4*) and crosses: *serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4* x *iku2-4/iku2-4, serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4* x *serk1-1*/serk1-1*;serk2/SERK2;iku2-4/iku2-4*. Means annotated with the same letter symbol are not significantly different from each other ($LSD_{0.05}$, P<0.001), ± SEM. n=number of seeds.

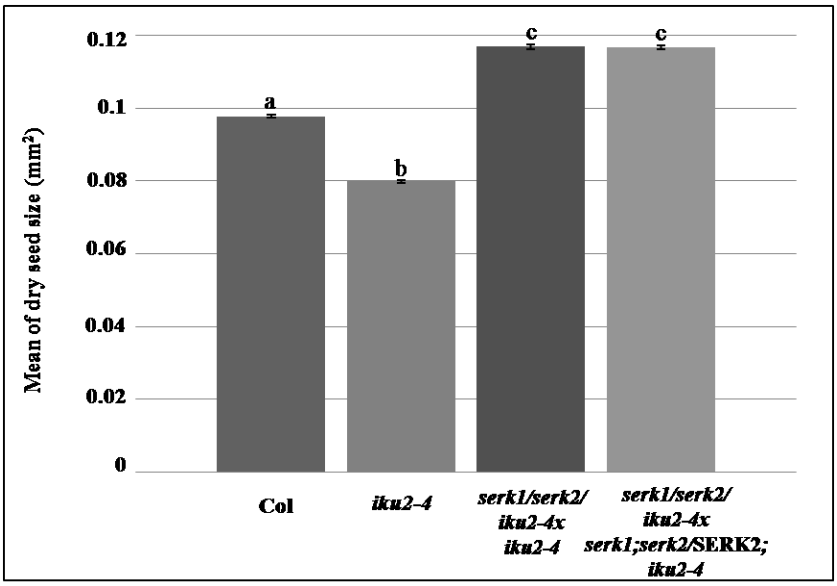

Figure 3.1. 12 **Bar chart showing the means of dry mature seeds from *iku2* and *serk1/serk2* crosses**
The bars represent the mean sizes (mm^2) of seeds from Col-0, *iku2-4/iku2-4*, and progeny seeds from crosses:*serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4* x *iku2-4/iku2-4*, *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4* x *serk1-1*/serk1-1; *serk2/SERK2;iku2-4/iku2-4*. The means annotated with the same letter symbol are not statistically different from each other. Error bars: SEM.

Analysis of the pooled seed sizes indicated that progeny seeds derived from crosses (*serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4* x *iku2-4* and *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4* x *serk1-1*/*serk1-1*;*serk2/SERK2;iku2-4/iku2-4*) did

not significantly differ between themselves (0.116mm^2 ±0.00059 and 0.116mm^2 ±0.00050 respectively). The progeny seeds from both crosses were bigger than control wild type Columbia (0.098mm^2±0.00055) seeds and *iku2-4/iku2-4* seeds (0.079mm^2 ±0.00043) (Fig. 3.1.12, Table 3.1.6). This finding indicates that *serk1-1/serk1-1;serk2-2/serk2-2* suppresses the *iku2-4/iku2-4* phenotype.

The analysis of seed size distribution showed similar patterns with seed sizes ranging from 0.04 – 0.16mm² in progeny seeds from *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4* x *iku2-4/iku2-4*, and *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4 x serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4* crosses. Wild type Columbia seed distribution ranged from 0.03 – 0.13mm² whereas *iku2-4/iku2-4* was 0.06-0.16mm². In progeny seeds from both of the crosses the size of the most common seed size was 0.13mm² (22.24% in *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4 x serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4* progeny and 24.16% in *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4* x *iku2-4/iku2-4* progeny). In wild type Columbia most of the seeds (35%) were of 0.1mm² whereas *iku2-4/iku2-4* produced seeds that were mostly (40%) of 0.09mm^2 (Fig.3.1.13).

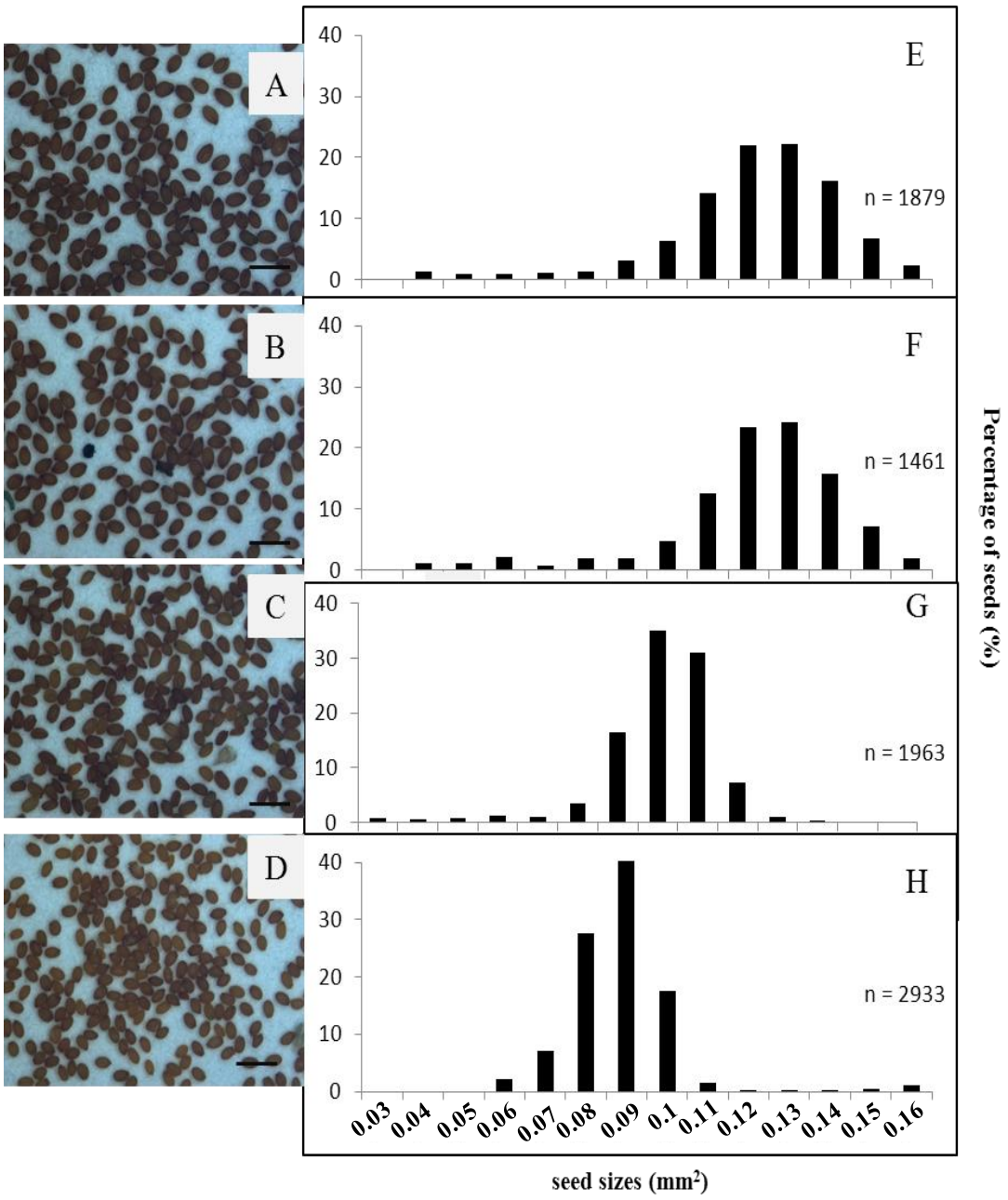

Figure 3.1. 13 **Seed size distribution from wild type, *serk1-1,serk2-2* and *iku2-4* plants**
Left panel: mature dry seeds from the progeny seeds from self-fertilised seeds produced from a cross between (A) *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4* x *serk1-1/serk1-1;serk2-2/SERK2;iku2-4/iku2-4*, (B) *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4 x iku2-4/iku2-4* and progeny seeds from self-fertilised (C) wild type Columbia (D) *iku2-4/iku2-4*. Scale bars: 1mm
Right panel: The seed size distribution in progeny seeds from a cross between (E) *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4 x serk1-1/serk1-1;serk2/SERK2;iku2-4/iku2-4,* (F) *serk1-1/serk1-1;serk2-2/serk2-2;iku2-4/iku2-4* x *iku2-4/iku2-4* and progeny seeds from self-fertilised wild type (G) Columbia seeds, (H) *iku2-4/iku2-4*. X axis represents seed area in mm^2, Y axis the percentage of seeds. n= total number of seeds analysed.

The seed size distributions confirm the previous statistical analysis that, the combination of the *iku2*, *serk1* and *serk2* mutations suppresses the *iku2-4* small-seed phenotype.

3.1.4 Genetic relationship between *IKU2* and *EMS1* function

The *in silico* analysis of *EMS1* (*At5g07280*) gene expression was performed (Fig.3.1.14) using the publicly available Gene Networks in Seed Development website (general methods). mRNA transcripts of *EMS1* are present in the chalazal and general seed coat at the initial, pre – globular stage of embryo development. At the globular stage of embryo development, *EMS1* is expressed in the chalazal and general seed coat as well as in the micropylar endosperm. At the embryo heart stage the expression of the gene is extended to the embryo proper and peripheral endosperm. At the linear cotyledon and mature green embryo stage, *EMS1* transcripts are exclusively present in the endosperm. The overlap in expression of *EMS1* and *IKU2* at early developing seeds further confirms *EMS1* as a potential partner candidate for *IKU2.*

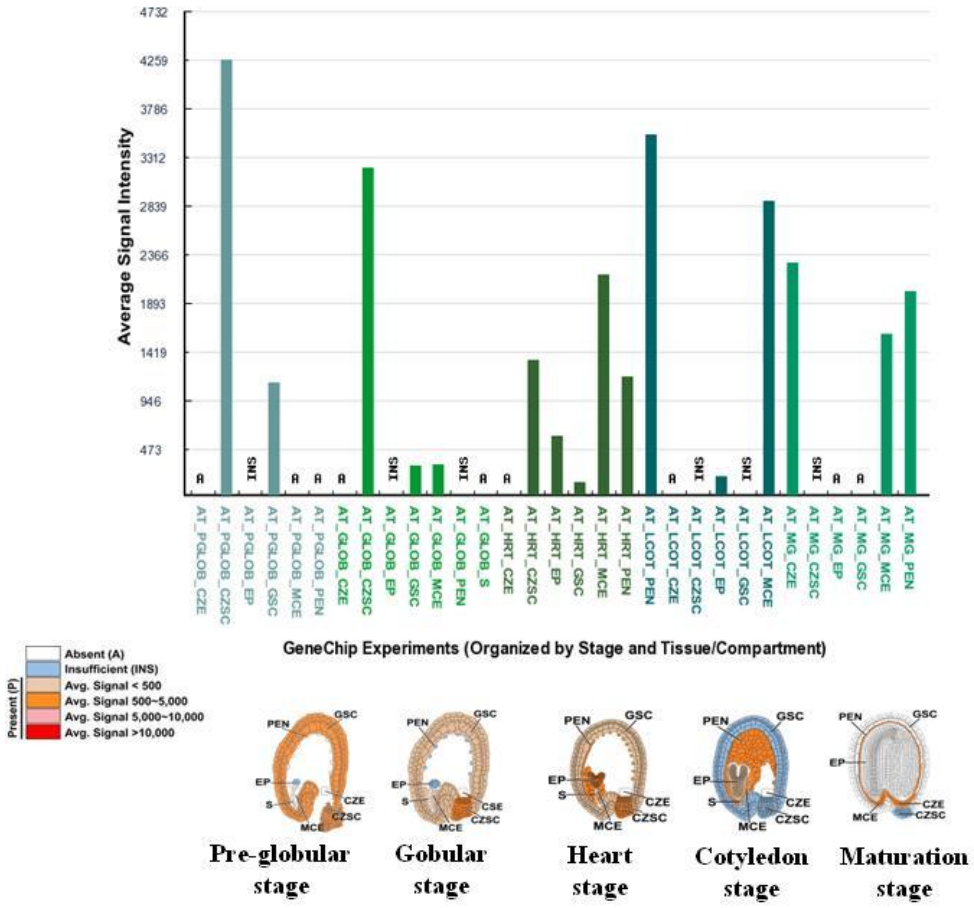

Figure 3.1. 14 ***In silico* expression profile of *EMS1* in developing seeds**
Bar charts showing average signal intensities of *EMS1* for all seed compartments: CZE, chalazal endosperm; CZSC, chalazal seed coat; EP, embryo proper; GSC, general seed coat; MCE, micropylar endosperm; PEN, peripheral endosperm. Abbreviation: A, Absent; INS, insufficient. The developmental stages: PGLOB, pre-globular, GLOB, globular, HRT, heart; LCOT, linear cotyledon; MG, mature green.
The images below represent accumulation of the mRNA in different tissue compartments at various stages of seed development. Colours correspond to the different average signal intensity of biological replicates.

To investigate genetically the relationship between *IKU2* and *EMS* function, double *iku2-4/iku2-4*;*ems1/ems1* mutant plants were generated by manual crossing and self-fertilisation of F_1 plants. Because both mutations are recessive, plant genotypes were confirmed by PCR in the F_2 and *ems1/ems1;iku2-4/iku2-4* and *ems1/EMS1;iku2-4/iku2-4* plants were selected for further analysis. To confirm the presence of the T-DNA insertion in the *EMS1* gene, ems1FLAG2RP (Fig.3.1.15R1)

and LB4 oligonucleotides were used in a PCR reaction. To confirm the disruption of the wild type copy of the gene, a specific ems1FLAG2LP (Fig.3.1.15 F1) primer was used in combination with ems1FLAG2RP (Fig. 3.1.16 R1) (Fig.3.1.15). The PCR reactions were carried out with the initial step of denaturation at 94ºC for 1 minute, followed by cycles of denaturation at 94ºC for 30s, annealing at 58ºC for 30s and extension at 72ºC for 1minute. Full sequences of the nucleotides used in the PCR reactions are listed in Appendix1 and the view of the entire gels in Appendix 2.

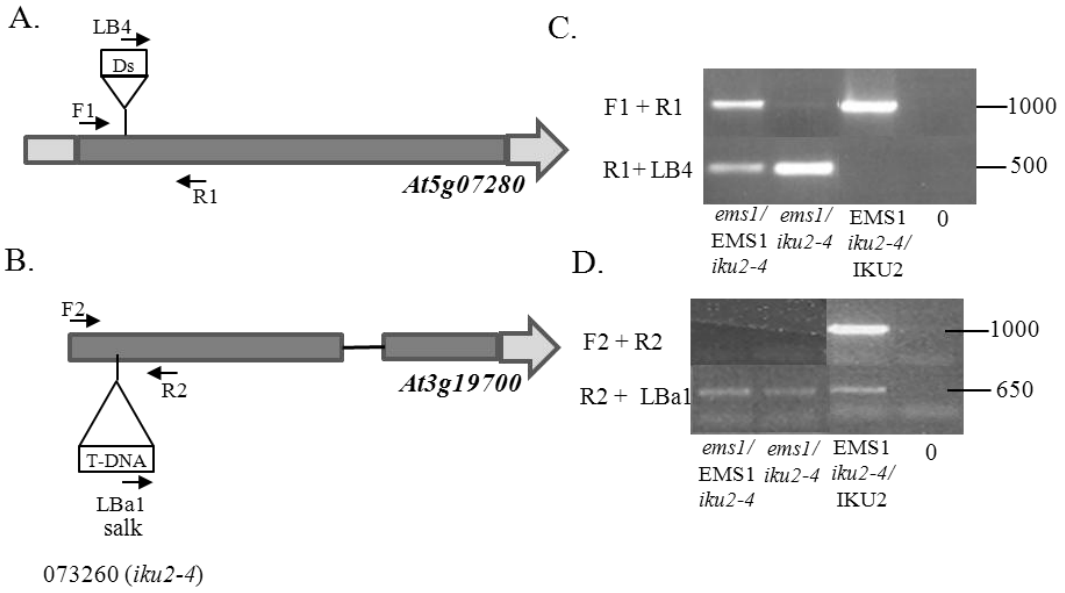

Figure 3.1. 15 **Representation of PCR genotyping of double *ems1/iku2-4* mutants**

(A) Schematic representation of *EMS1* (*At5g07280*) gene and the insertion of Ds element in exon 1. (B) Representation of *IKU2* (*At3g19700*) and T-DNA insertion in exon1. Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines, and positions for oligonucleotide primers are indicated by arrows. (C) In double *ems1*/EMS1;*iku2-4/iku2-4* and*ems1/ems1;iku2-4/iku2-4* mutants the genetic constitution of *ems1* insertion was confirmed with combination of R1 + T-DNA left border (LB4) primers resulting in 500bp products. Wild type allele of *EMS1* was detected with F1+R1 primers producing 1000bp products that were not present in *ems1/ems1* (D) Insertion of T-DNA in *At3g19700* (*IKU2*) gene in the double *ems1*/EMS1;*iku2-4/iku2-4* and *ems1/ems1;iku2-4/iku2-4* mutants was confirmed with R3 and Lba1 primer pair (650bp) and the presence or absence of wild type allele of the gene with F3+R3 (1000bp). The full view of gel pictures available in Appendix 2.

Three male sterile *ems1/ems1;iku2-4/iku2-4* plants were pollinated with *ems1/EMS1;iku2-4/iku2-4* pollen. It was predicted that 50% of the progeny seeds would be *ems1/ems1;iku2-4/iku2-4* and 50% *ems1/EMS1;iku2-4/iku2-4* in the embryo and the endosperm of the seed, whereas the maternal integuments would remain double homozygous *ems1/ems1;iku2-4/iku2-4*. As a control, stigmas of two double homozygous *ems1/ems1;iku2-4/iku2-4* plants were pollinated with *iku2-4* pollen. The progeny seeds from the cross were *ems1/EMS1;iku2-4/iku2-4* in the endosperm and the embryo, while the maternal integument was double homozygous *ems1/ems1;iku2-4/iku2-4*. The seeds from the crosses were collected at maturity for phenotypic analysis of seed sizes using ImageJ and statistical methods (see general methods).

To determine the variation between the progeny seeds of individual plants within analysed genotypes and crosses, ANOVA analysis was employed.

	Col-0		***iku2-4***	
Source of variation	d.f.	m.s.	d.f.	m.s.
Plant	2	0.0010977*	2	0.0009643
Residual	952	0.0003003	1105	0.0003355
	ems1/iku2-4xiku2-4		***ems1/iku2-4xems1/EMS1;iku2-4***	
Source of variation	d.f.	m.s.	d.f.	m.s.
Cross	2	0.1378668***	1	0.0019145
Residual	1296	0.0005374	547	0.0006143

Table 3.1. 7 **Mean squares from analysis of variance (ANOVA)**
Seed areas of individual plants derived from particular genotypes: (Col-0, *iku2-4/iku2-4*) and progeny from crosses: *ems1/ems1;iku2-4/iku2-4 xiku2-4/iku2-4* and *ems1/ems1;iku2-4/iku2-4xems1/EMS1;iku2-4iku2-4* were taken for the analysis. d.f - degree of freedom, m.s. – mean squares. * significant at 0.05 level, ** significant at 0.01 level, *** significant at 0.001 level.

The statistical analysis showed that there were significant differences between the sizes of mature seeds produced by individual plants within wild type Columbia ecotype and progeny seeds generated by *ems1/ems1;iku2-4/iku2-4* x*iku2-4/iku2-4* crosses. The seeds sizes that did not differ significantly were those from individual *iku2-4*/*iku2-4* plants within lines and those from *ems1/ems1;iku2-4/iku2-4* x *ems1/EMS1;iku2-4/iku2-4* crosses (P>0.05) (Table 3.1.7).

To further estimate the differences in seed sizes produced by individual plants and crosses within tested lines a Tukey's test was applied:

Lines						
Plant no		**Col-0**			***iku2-4***	
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	316	0.11213	0.015368	400	0.08851	0.018594
2	317	0.10889	0.020409	361	0.09103	0.020663
3	323	0.11208	0.015699	347	0.09145	0.015026
$LSD_{0.05}$		0.002676			0.002541	
Cross no		***ems1/iku2-4xiku2-4***			***ems1/iku2-4xems1/*EMS1*;iku2-4***	
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	306	0.12502	0.023561	185	0.0824	0.025364
2	479	0.08713	0.023706	364	0.0784	0.024415
3	514	0.10689	0.022379	-	-	-
$LSD_{0.05}$		0.002837			0.00440	

Table 3.1. 8 **The differences between the means of the seed sizes derived from individual plants within tested lines**

Tested genotypes: wild type Columbia (Col-0), *iku2-4/iku2-4* and crosses: *ems1/ems1;iku2-4/iku2-4* x *iku2-4/iku2-4*, *ems1ems1;iku2-4/iku2-4 x ems1/*EMS1*;iku2-4/iku2-4*. s.d.:standarddeviation. n=number of seeds.

Columbia wild type plants produced seeds with the smallest mean of 0.10889mm^2±0.0204085 and the highest of 0.11213mm^2±0.0153676. The cross between *ems1/ems1;iku2-4/iku2-4* x *iku2-4/iku2-4* produced progeny seeds with the smallest mean size of 0.08713mm^2±0.0237056 and 0.12502mm^2±0.0235609 the highest (Table 3.1.8).

Next, one-way ANOVA was carried out to determine the effects of genotypes on seed area variability. The LSD of seed area was calculated, and on this basis homogeneous groups (not significantly different from each other) were determined.

Lines	**n**	**Mean(mm^2)**	**Median**
Col-0	955	0.11104^a ±0.00056	0.11329
iku2-4	1108	0.09025^d ±0.00055	0.08940
ems1/iku2-4xiku2-4	1299	0.10388^b ±0.00075	0.10536
ems1/iku2-4 x ems1/*EMS1*;iku2-4	549	0.07976^c ±0.0010	0.07622
LSD$_{0.05}$		0.001728	

Table 3.1. 9 **Analysis of seed size in wild type, *iku2-4* and *ems1* plants**
Genotypes analysed: Col-0, *iku2-4/iku2-4,* and progeny seeds from crosses: *ems1/ems1;iku2-4/iku2-4* x *iku2-4/iku2-4* and *ems1/ems1;iku2-4/iku2-4* x *ems1/*EMS1*; iku2-4/iku2-4*. The means annotated with the same letter symbol are not significantly different from each other (LSD$_{0.05}$, P<0.001), ± SEM. n=number of seeds.

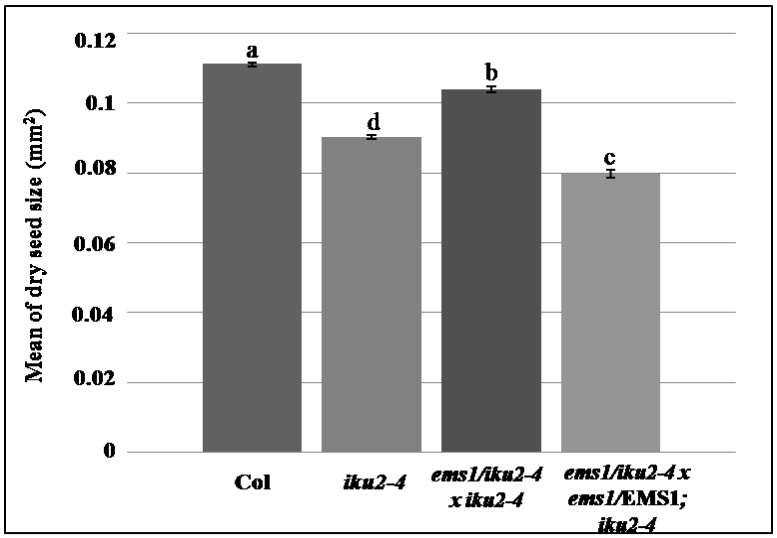

Figure 3.1. 16 **Mean dry seed size analysis**
Bars represent the mean of the seed sizes (mm^2)from Col-0, *iku2-4/iku2-4* and progeny seeds from crosses: *ems1/ems1;iku2-4/iku2-4* x *iku2-4/iku2-4* and *ems1/ems1;iku2-4/iku2-4* x *ems1/*EMS1*;iku2-4/iku2-4*. The means annotated with the same letter symbol are not statistically different from each other. Error bars: SEM.

The ANOVA test indicated that the main effects of genotypes and progeny from the crosses were significant for seed area (P<0.001). From the analysis it can be concluded that the seed size phenotype of progeny derived from *ems1/ems1;iku2-4/iku2-4* x*ems1/*EMS1*;iku2-4/iku2-4* crosses was enhanced in terms of seed size. The seeds were smaller, with a mean size of 0.07976mm^2±0.0010 in comparison to single homozygous *iku2-4/iku2-4* seeds that were of 0.09025mm^2±0.00055 mean size and Columbia wild type (0.11104mm^2 ±0.00056). The progeny seeds from the control *ems1/ems1;iku2-4/iku2-4* x *iku2-4/iku2-4* crosses showed a bigger mean of 0.10388mm^2±0.00075 in comparison to the seeds of analysed *ems1/ems1;iku2-4/iku2-4 x ems1/*EMS1*;iku2-4/iku2-4* crosses and single homozygous *iku2-4/iku2-4* mutants (Table 3.1.9).

The analysis of seed size distribution (Fig.3.1.17) indicated that the seed sizes ranged from 0.04 – 0.16mm^2 for the progeny seeds produced by *ems1/ems1;iku2-*

4/iku2-4 x *ems1/*EMS1*;iku2-4/iku2-4* and *ems1/ems1;iku2-4/iku2-4* x *iku2-4/iku2-4* crosses. Columbia wild type seeds ranged from 0.03 – 0.16mm^2 and *iku2-4/iku2-4* ranged from 0.06 – 0.16mm^2. The distribution of progeny seeds from *ems1/ems1;iku2-4/iku2-4* x *ems1/*EMS1*;iku2-4/iku2-4* crosses showed that the highest percentage (23.31%) of seed sizes of 0.08mm^2 whereas (14.54%) *ems1/ems1;iku2-4/iku2-4 x iku2-4/iku2-4* seeds were 0.12mm^2. Most of the Columbia wild type seeds were 0.1mm^2 (35.04%) whereas (40%) *iku2-4/iku2-4* were 0.09mm^2.

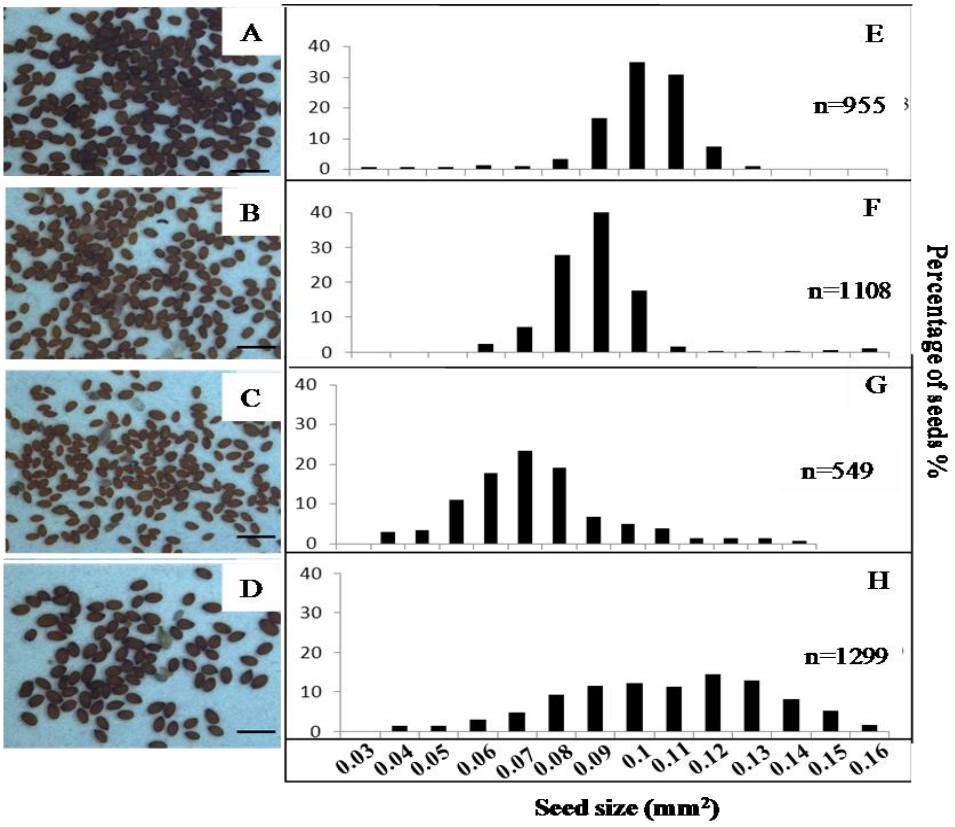

Figure 3.1. 17 **Seed size distribution from wild type and mutant plants**
Left panel: mature dry seeds from self-fertilised (A) wild type Columbia (B) *iku2-4/iku2-4* and progeny from a cross between (C) *ems1/ems1;iku2-4/iku2-4 x ems1/*EMS1*;iku2-4/iku2-4* (D) *ems1/ems1;iku2-4/iku2-4* x *iku2-4/iku2-4*. Scale bars = 1mm.
Right panel: The seed size distribution in mature dry seeds from self-fertilised (A) wild type Columbia (B) *iku2-4/iku2-4* and progeny from a cross between (C) *ems1/ems1;iku2-4/iku2-4 x ems1/*EMS1*;iku2-4/iku2-4* (D) *ems1/ems1;iku2-4/iku2-4* x *iku2-4/iku2-4*. X axis represents seed area in mm^2, Y axis the percentage of seeds. n= total number of seeds analysed.

Collectively these data showed and additive effect of *iku2-4* and *ems1* double mutant which indicates that *IKU2* and *EMS1* act in separate signalling pathways to regulate seed size.

3.1.5 Discussion

One of the aims of this chapter was to elucidate the genetic relationship between *IKU2* and other RLKs involved in plant reproduction. To this aim, the expression pattern of *IKU2* and its close homologue *IKU2-LIKE* (Shiu and Bleecker 2001) was identified at the micropylar and peripheral endosperm, respectively, of the globular embryo stage seeds using bioinformatics analysis. Related receptors often show functional redundancy, for example, mutations in *HAESA* (*HAE*) and its related *HAESA LIKE* (*HSL*) RLK, both expressed in developing flowers (Stenvik et al. 2008) showed an enhancement of dehiscent phenotype in double *hae* mutants when compared to single mutants which indicating that they act redundantly to regulate dehiscence. Other closely related genes that interact with each other belong to *ERECTA*-family. The proper growth and differentiation of the aboveground organs is found to be under control of the three *ERECTA, ERL1* and *ERL2* genes that interact synergistically (Shpak et al. 2004). In this study, phenotypic analysis of *iku2-4/iku2-4;ikl/ikl* mutants showed a significant enhancement of small *iku2-4/iku2-4* seeds phenotype. Therefore, the results indicate that the two related *IKU2* and *IKL* genes are partially redundant. Further experimental approaches to confirm these data would be desirable. For instance, it would be interesting to test whether expression of *IKL* driven by *IKU2* promoter should rescue the small *iku2-4* seed phenotype. This experiment could reveal whether *IKL* is capable of perceiving and transducing the same signal as *IKU2,*

which could help to reveal if the two genes act in parallel or separate pathways to regulate seed development.

RLKs very often form heterodimers with other receptors in order to perceive the corresponding ligand and elicit a downstream response. The selection of a potential candidate receptor that could form a heterodimeric or oligomeric complex with IKU2 is not straightforward due to the abundance of RLKs in the Arabidopsis genome (Shiu and Bleecker 2001), and since only few of them have been assigned a biological function. Most likely, RLKs that interact together localise to the same tissue and developmental stage of the plant (Nodine et al. 2007, Fletcher et al. 1999, Boisson-Dernier et al. 2009). Out of the receptors known to play a role in reproduction (Miyazaki et al. 2009, Takasaki et al. 2000), only *EMS1/EXS* and *SERK1* and *SERK2* are thought to act in embryogenesis and seed development (Canales et al. 2002, Schmidt et al. 1997). Intriguingly, the analysis of expression pattern of *IKU2* and *SERK1* showed that they are both expressed at the micropylar endosperm of the globular embryo. Phenotypic analysis of progeny seeds from *serk1/serk2/iku2-4* x *iku2-4* and *serk1/serk2/iku2-4* x *serk1/;serk2*/SERK2;*iku2-4* crosses showed an increase in seed sizes compared to wild type Columbia. This effect could be caused by a positive maternal effect of *SERK1* and *SERK2* on endosperm and embryo growth. Such a sphorophytic maternal effect has already been demonstrated in seed development (Schruff et al. 2006). Studies of various reciprocal crosses showed strong maternal effect indicating that seed size is affected by the phenotype of the mother plant, maternal genotype of the seeds and the interactions between the mother and the progeny (Alonso-Blanco et al. 1999). An example of a maternal control of seed development is represented by the *APETALA2* transcription factor gene. *ap2* mutant plants produce heavier seeds only when the

maternal plant was homozygous for the mutation. Also, a *da1-1* mutation in a gene encoding a predicted ubiquitin receptor (Li et al. 2008) produces seeds that are only heavier when the mutation is carried by the mother plant which suggests that this type of maternal effect is common in Arabidopsis. Other genes that act sporophytically include *AUXIN RESPONSE FACTOR 2/MEGAINTEGUMENTA (ARF2/MNT)* which encodes a transcription factor that binds to auxin-responsive elements found in the promoters of auxin-regulated genes (Schruff et al. 2006). Mutants for the defective *ARF* genes display enlarged organs (including integuments) with extra cells (Schruff et al. 2006). *ttg2* mutants also show a maternal sporophytic effect on seed size with the differentiation defects observed in mutants endothelial tissues of the inner integuments resulting in reduced endosperm and seed size (Johnson et al. 2002). To further investigate a possible maternal effect of *SERK1* and *SERK2* on final seed size, fertilisation of *serk1/serk2/iku2* with wild type pollen should be carried out. In addition, a control cross between a wild type maternal plant with a triple mutant *serk1/serk2/iku2* pollen would be important in reciprocal crosses, but this plant is male sterile. To finally assess the interaction between *SERK1/SERK2* and *IKU2*, it would be necessary to test how a combination of *serk1/serk2* double mutants affects seed development. To this aim, the progeny seeds from self-fertilised *serk1/serk1/serk2*/SERK2 should be analysed phenotypically together with the progeny seeds derived from the proposed crosses. Finally, an alternative hypothesis to explain the maternal effect of *serk1/serk2* in seed size could be that in male sterile plant the seeds are not produced so there is an increase of the translocation of nutrients to developing seeds generated via cross pollination. This hypothesis could be tested by using fertile plants segregating for *serk1*, *serk2* and

iku2. However, it will be difficult to determine the genetic composition of each seed generated using this approach.

The enhancement of the *iku2-4/iku2-4* small seed phenotype in conjunction with a second candidate receptor, *EMS1* (Zhao et al. 2002), suggests that *IKU2* and *EMS1* act in two separate or parallel signalling pathways to regulate seed size and development. Interestingly, the enhancement is only visible in the progeny seeds from *ems1/ems1;iku2-4/iku2-4 x ems1/*EMS1*;iku2-4/iku2-4* cross where 50% of the seeds had no functional *EMS1* and *IKU2* transcripts present. In control *ems1/ems1;iku2-4/iku2-4 x iku2-4/iku2-4* crosses, where the embryo and endosperm carry a wild type functional copy of *EMS1*, the seeds are significantly bigger. This suggests that the genetic interaction between *IKU2* and *EMS1* takes place at the embryo-endosperm interface of the seed to coordinate the development of both organs during early seed development.

Chapter 3.2

Genetic analysis of *IKU2* and *ERECTA*

3.2.1 Characterisation of loss-of-function *IKU2* mutants in Landsberg *erecta* and Columbia background

The best characterised RLK in plants is ERECTA (ER) which regulates many aspects of plant development and stress responses (van Zanten et al. 2009). The phenotypic analysis of three alleles for *IKU2* in two different ecotypes of *A.thaliana,* Columbia and Landsberg *erecta,* show great differences in the seed size in loss-of-function mutant *iku2-1* (L*er*) when compared to *iku2-4* (Col-0). Since the Landsberg *erecta* ecotype carries a mutation in the RLK ERECTA (Alonso-Blanco et al. 1999) the potential overlapping function between *IKU2* and *ERECTA* was hypothesised (Fig.3.2.1A). In the L*er* background, the loss of function *iku2-1* mutation is caused by a guanine to adenine nucleotide (G-A) substitution which introduces a stop codon in the extracellular LRR region of the gene. Another allele of *IKU* in L*er, iku2-3,* is an amino acid substitution mutation and causes an arginine to lysine change (R935K) in the kinase domain of the IKU2 protein (Luo et al. 2005). *Iku2-4,* obtained from collaborator (Ming Luo, CSIRO) represents a loss of function mutant in the Col-0 background and is caused by T-DNA insertion in the third leucine rich repeat (LRR) of the gene. The size of the dry progeny seeds from *iku2-4* (Col-0) plants ranged from 0.04 to 0.13mm^2 with the highest percentage (38.6%) of them at 0.1mm^2. In wild type Col-0, seed distributions ranged from 0.04 to 0.14mm^2 with 32% being 0.13mm^2 (Fig.3.2.1C). The distribution of progeny seeds from *iku2-1* (L*er*) ranged from 0.04-0.14mm^2 with a peak at 0.07mm^2 (28.7%) when compared to 0.04-0.14mm^2 in wild type L*er* seeds with highest percentage (24.4%) of seeds of 0.11mm^2. The *iku2-3* (L*er*) mutation represent the strongest allele of *IKU2* with seed sizes ranging between 0.04 and 0.14mm^2 and 33.2% of the seeds that are of 0.06mm^2 (Fig.3.2.1C). The enhancement of the small seed

phenotype observed in *iku2-3* and *iku2-1* when compared to *iku2-4* could be due to other genetic factors present in L*er* genetic background of the ecotype. Alternatively, the phenotypic enhancement observed in L*er* could be due to the presence of the loss-of-function *erecta* mutation introduced into Landsberg accession (Redei 1962). Interestingly, *ERECTA* encodes a LRR RLK which affects many aspects of plant growth and development (Torii et al. 1996). The reduction of seed size in the loss-of-function *iku2-1* (L*er*) mutants when compared to loss-of-function *iku2-4* (Col-0) mutants indicates that *IKU*2 and *ERECTA* might act via parallel genetic pathways to regulate seed development.

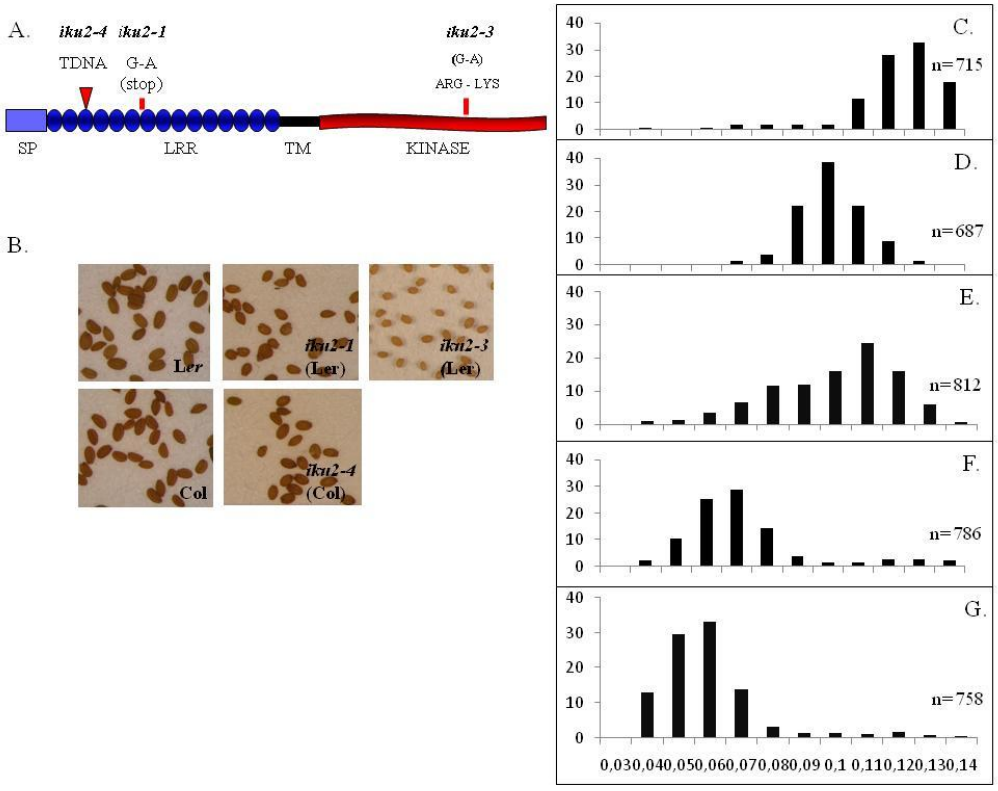

Figure 3.2. 1 **Schematic representation of the different domains identified in IKU2 protein, available mutations and seed phenotypes and size distributions**
(A) SP: Signal peptide (blue box), LRR: leucine Rich Repeat (16 leucine repeats shown as blue ovals); TM: transmembrane domain; KINASE; kinase domain (shown as red tape). Different mutant loci available: *iku2-4* is a T-DNA insertion in the third LRR, *iku2-1* is G-*A* nucleotide substitution that causes premature stop codon in the sixth LRR, and *iku2-3* is a G-A substitution that causes an arginine to lysine substitution in the kinase domain (B) Dry seeds from L*er*, *iku2-1* (L*er*), *iku2-3* (L*er*), Col-0, *iku2-4* (Col-0) (C) Distribution of dry seed sizes from wild type Columbia, (D) *iku2-4/iku2-4* (Col-0) , (E) wild type Landsberg *erecta* (L*er*), (F) *iku2-1/iku2-1* (L*er*); (G) *iku2-3/iku2-3* (L*er*).

3.2.2 Genetic relationship between *IKU2* and *ERECTA* (Col-0) function

The expression profiles of the two genes were analysed *in silico*. At the pre-globular stage of embryo development, the expression profile of *ERECTA* is restricted to the chalazal seed coat and embryo proper (Fig.3.2.2). During the globular stages of embryo growth, *ERECTA* expression continues to be detectable in the chalazal seed coat, and is also found in the embryo proper and suspensor. At the heart stage of embryo development, *ERECTA* transcripts are abundant in the embryo proper but at lower levels in the chalazal seed coat and micropylar endosperm. At the linear cotyledon stage of embryo development, *ERECTA* is highly expressed in embryo proper but with lower intensity in both the chalazal and general seed coat. At the mature green embryo stage, *ERECTA* expression remains high in embryo and at lower levels in the micropylar and peripheral endosperm (Fig.3.2.2).

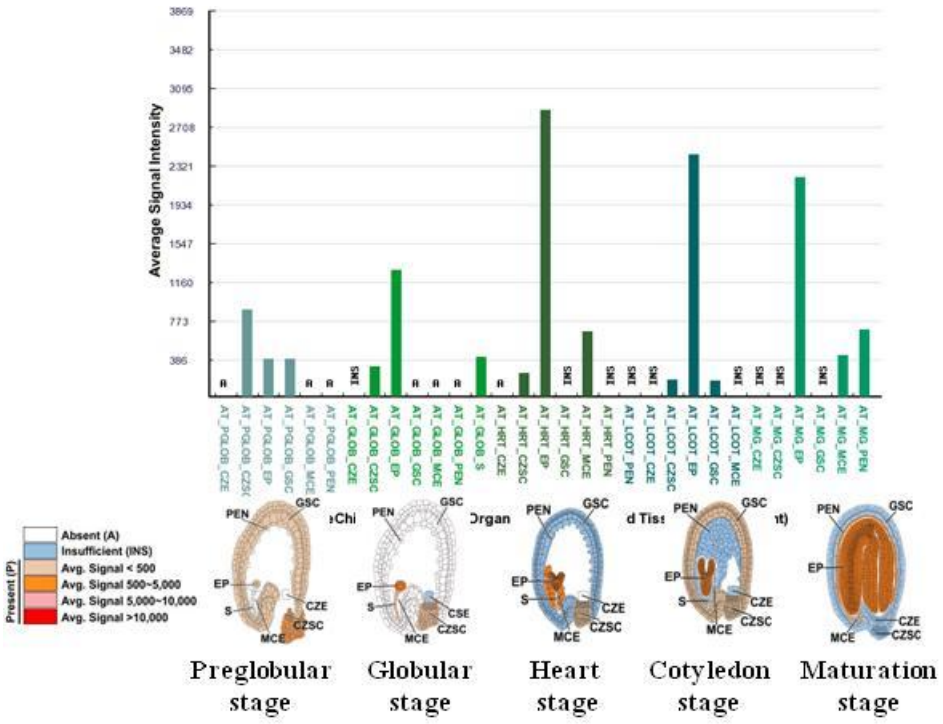

Figure 3.2. 2 ***In silico* expression profile of *ERECTA***

Bar chart showing average signal intensities of *ERECTA* for all seed compartments: CZE, chalazal endosperm; CZSC, chalazal seed coat; EP, embryo proper; GSC, general seed coat; MCE, micropylar endosperm; PEN, peripheral endosperm. Abbreviation: A, Absent; INS, insufficient. The developmental stages: PGLOB, pre-globular, GLOB, globular, HRT, heart; LCOT, linear cotyledon; MG, mature green.

The images below: accumulation of the mRNA in different tissue compartments at different stages of seed development: pre-globular, globular, heart, cotyledon and maturation. Colours correspond to the different average signal intensities of biological replicates.

By contrast, the expression of *IKU2* is restricted to the micropylar endosperm at the globular stage of embryo development (Fig.3.1.5). Collectively, the *in silico* analysis of gene expression revealed that, although not in the same seed compartments, *ERECTA* and *IKU2* are both temporarily expressed at the globular stage of embryo development.

In order to test genetically the relationship between *IKU2* and *ERECTA* in controlling growth and development of the endosperm and surrounding integuments, double loss-of-function *iku2-4* and *erecta105* mutants were generated in Columbia genetic background. This let to exclude the possible genetic background effects on

the variability of seed size in *iku2* mutant alleles. The clearly visible, short pedicel and stunted plant stature cause by the *erecta* mutation enabled the phenotypic selection of *er105*/*er105* plants that were used in manual pollinations with *iku2-4/iku2-4* plants. The F_1 progeny was grown to generate the F_2 generation. Because both mutants are recessive, to enable phenotypic analysis of double mutants, F_2 plants were genotyped by PCR (Fig. 3.2.3) in order to identify *iku2-4/iku2-4;er105/er105* mutants. Plants showing typical stunted stature (*erecta* phenotype) were genotyped for homozygous *iku2-4/iku2-4* insertion. The *er105* mutation was confirmed by using primer pair Erg2248 (Fig. 3.2.3 R2) and er105rc (Fig. 3.2.3) and the absence of the wild type allele was confirmed by primer pair Erg2248 and Erg3016rc (Fig. 3.2.2) (Appendix 1). PCR reactions were carried out with an initial denaturation step of 94°C for 1 minute followed by cycles of denaturation at 94°C for 30 s, annealing at a 58°C for 30s extension at 72°C for 1 minute.

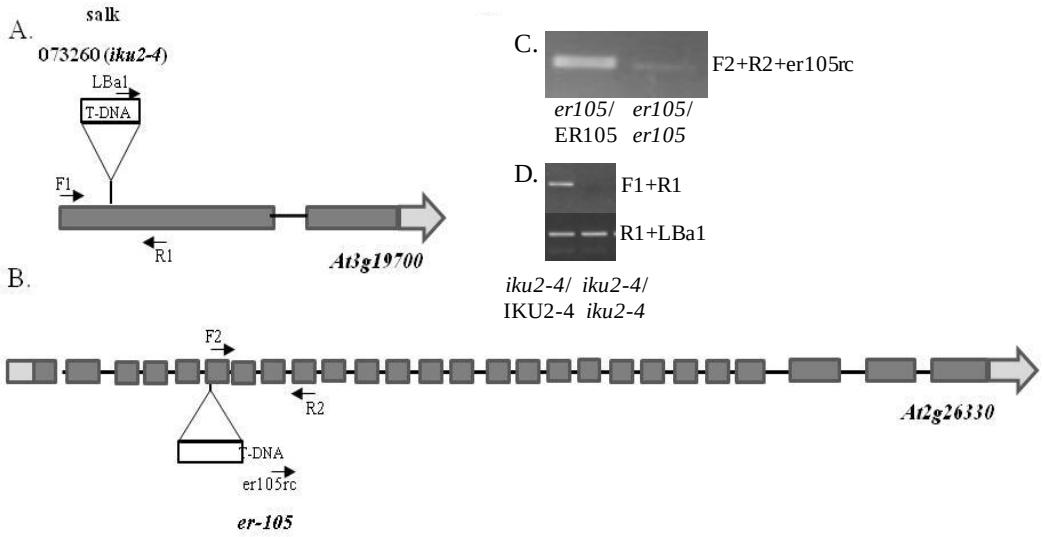

Figure 3.2. 3 **Representation of PCR genotyping of double *er/iku2* mutants**
Schematic representation of (A) *IKU2* (*At3g19700*) and T-DNA inserted in exon 1. (B) *ERECTA* (*At2g26330*) with T-DNA insertion in exon 6. Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines, and positions for oligonucleotide primers are indicated by arrows (C) In double *er105/er105;iku2-4/iku2-4* mutants the genetic constitution of *er105* insertion was confirmed with combination of F2 + R2 + T-DNA left border (er105rc) primer pair (~650bp products). Insertion of T-DNA in *At3g19700* (*IKU2*) gene in the double mutants was confirmed with R1 and Lba1 primer pair (650bp) and the absence of wild type allele of the gene with F1+R1 (1000bp).

Wild-type Columbia, *iku2-4/iku2-4*, *er105/er105* and *iku2-4/iku2-4;er105/er105* plants were grown to generate F_3 seed progenies for further analysis. Mature seeds were collected from eight individuals for each of the four selected genotypes. For each genotype, seed sizes were measured from individual plants and compared using statistical methods in order to initially assess the size variation between seed progeny from sibling plants.

	Col-0		*iku2-4*	
Source of variation	d.f.	m.s.	d.f.	m.s.
Plant	7	0.0013980***	6	0.0028356***
Residual	1953	0.0002416	3755	0.0003626
	er105		***iku2-4/er105***	
Source of variation	d.f.	m.s.	d.f.	m.s.
Plant	7	0.0039204***	7	0.0095479***
Residual	2691	0.0003172	2828	0.0001810

Table 3.2. 1 **Mean squares from analysis of variance (ANOVA)**
Seed areas of individual plants derived from particular genotypes: Col-0, *iku2-4/iku2-4*, *iku2-4/iku2-4;er105/er105* were analyzed. D.f. – degree of freedom, m.s. – mean square. * significant at 0.05 level, ** significant at 0.01 level, *** significant at 0.001 level

The ANOVA test indicated that individual plants within Col-0, *er105/er105*, *iku2-4/iku2-4* and *iku2-4/iku2-4;er105/er105* genotypes differed significantly in seed size (P>0.05) (Table 3.2.2). To further asses the differences between the seed sizes of individual plants the Tukey test was applied.

Lines						
Plant no		**Col-0**			***iku2-4***	
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	249	0.09628	0.011793	557	0.082	0.019291
2	301	0.09707	0.017368	539	0.078	0.018547
3	202	0.10065	0.014709	1000	0.079	0.021165
4	187	0.09528	0.016456	488	0.082	0.018716
5	230	0.09251	0.015305	461	0.085	0.016083
6	226	0.09364	0.018021	290	0.082	0.017084
7	285	0.09423	0.014424	428	0.082	0.019234
8	351	0.09684	0.015099	-	-	-
$LSD_{0.05}$		0.002301			0.001670	
		er105			***iku2-4/er105***	
Plant no	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	300	0.09260	0.015618	381	0.07463	0.014722
2	369	0.08899	0.017496	246	0.07634	0.014638
3	430	0.09315	0.018106	370	0.07052	0.012353
4	267	0.09177	0.016559	323	0.06941	0.013247
5	354	0.08981	0.017163	397	0.06122	0.012591
6	331	0.08675	0.01976	306	0.07414	0.014788
7	312	0.08652	0.019382	435	0.07229	0.011887
8	337	0.08345	0.018469	378	0.07697	0.013824
$LSD_{0.05}$		0.002382			0.001789	

Table 3.2. 2 **Differences between the means of the seed sizes derived from individual plants within tested lines**

Genotypes analysed: wild type Col-0, *iku2-4/iku2-4*, *er105/er105*, *iku2-4/iku2-4;er105/er105*. s.d. - standard deviation. n=number of seeds.

Tukey test results revealed that plants within a Columbia genotype produced seeds with the smallest mean size of 0.09251mm^2 ±0.016456 and the highest 0.10065mm^2 ±0.014709. In *iku2-4/iku2-4* the smallest mean of seed size was 0.078mm^2±0.018547 and the highest 0.085mm^2 ±0.016083. *Er105/er105* plants produced seeds that were between 0.08345mm^2 ±0.018469 (the smallest) and 0.09315mm^2 ±0.018106 (the largest). *iku2-4/iku2-4;er105/er105* plants generated seeds with means of: 0.06122mm^2 ±0.012591 (smallest) and 0.07697mm^2 ±0.013824 (the largest) (Table 3.2.3).

A secondary analysis was performed by comparing pooled measurements of seed sizes for each genotype.

Mutant	n	Mean (mm^2)	Standard deviation	Coefficient of variation	Range
Col-0	1961	0.0959	0.0157	16.37	0.1593
er105	2700	0.0926	0.0098	10.58	0.0787
iku2-4	3783	0.0845	0.0161	19.04	0.1184
iku2-4/er105	2836	0.0717	0.0143	19.94	0.1327

Table 3.2. 3 **Statistical analysis of Columbia wild type seeds and mutants *er105/er105, iku2-4/iku2-4, er105/er105;iku2-4/iku2-4***

Basic statistical analyses, such as mean, standard deviation and the coefficient of variation were calculated for all genotypes. The highest standard deviation was found in *iku2-4/iku2-4* seeds, while the lowest was in *iku2-4/iku2-4;er105/er105* mutants. According to the coefficient of variation (V %) the highest degree of statistical dispersion was in the group of *iku2-4/iku2-4;er105/er105* double homozygous seeds and the lowest in *er105*/*er105* seeds. The latter seeds were the most uniform in size (Table 3.2.3).

Source of variation	Degree of freedom (df)	Sum of squares (SS)	Mean square	F- test	p-level
Mutants	3	0.85407	0.284690	1381.99***	<0.001
Experimental error	9779	2.01156	0.000206		
Total	9782	2.86563			

Table 3.2. 4 **Analysis of variance (ANOVA**)

The Columbia wild type seeds and mutants *er105/er105, iku2-4/iku2-4, er105/er105;iku2-4/iku2-4*taken for the analysis of variance test. The degree of freedom, sum of squares, mean square, F-test and p-level are shown in the table.

In the ANOVA the F-test was used to compare the overall differences between the seed sizes of different mutants (Col-0, *iku2-4/iku2-4, er105/er105, iku2-4/iku2-4;/er105/er105*). The value of the F- test was 1389.99***. The results of the ANOVA test indicate highly significant differences (at least 0.001 level) between mutant seed sizes. To compare the means of mutants the Newman-Keuls multiple range test was used (Zar. JH., 1984) (Table 3.2.5).

Comparison	**Difference**	**SE**	**q**	**$q_{0.001;9779}$p**	**Conclusion**
Col-0 vs *er105*	0.0033	0.0004532	7.282	>4.654	Difference is significant at least p=0.001
Col-0 vs *iku2-4*	0.0114	0.0004185	27.240	>5.063	Difference is significant at least p=0.001
Col-0 vs *iku2-4 /er105*	0.0242	0.0004214	57.428	>5.309	Difference is significant at least p=0.001
er105 vs iku2-4	0.0081	0.0004131	19.608	>4.654	Difference is significant at least p=0.001
er105*vs *iku2-4 /er105	0.0209	0.0004160	50.240	>5.063	Difference is significant at least p=0.001
iku2-4*vs *iku2-4 /er105	0.0128	0.0003780	33.862	>4.654	Difference is significant at least p=0.001

Table 3.2. 5 **The Newman-Keuls range test**

The difference for wild type Columbia-0 seed sizes compared with *er105/er105* was 0.0033 therefore the mean size of seeds are significantly higher in Col-0 ($p<0.001$). The mean size of seeds of Col-0 is significantly larger than homozygous *iku2-4/iku2-4* seeds since the difference was 0.0114 ($p<0.001$). The difference in mean seed sizes was also significant between Col-0 and *iku2-4/iku2-4;er105/er105* seeds (0.0242; $p<0.001$). *Er105/er105* seeds were bigger than *iku2-4/iku2-4* and *iku2-4/iku2-4;er105/er105* seeds since the difference was 0.0081 and 0.0209, respectively ($p<0.001$). The mean sizes of *iku2-4/iku2-4* seeds were

significantly higher than double homozygous *iku2-4/iku2-4;er105/er105* and differed by 0.0128 ($p<0.001$) (Table 3.2.5)

The distribution of seed sizes (Fig.3.2.4) confirms the phenotypic differences between *iku2* and *erecta* mutants. Wild type Columbia plants produced seeds that ranged from 0.03 – 0.14mm² (n=1963) with the highest percentage of seed size being 0.1mm² (35.04%). Mature seeds produced by *er105/er105* plants ranged from 0.06 - 0.11mm² (n=2051) and 40.61% of seeds were 0.1mm². In comparison, the distribution of *iku2-4/iku2-4* seed sizes (n=2933) ranged from 0.06 – 0.14mm² with 40.32% of seeds being 0.09mm². The *er105/er105;iku2-4/iku2-4* plants produced seeds that ranged between 0.04 and 0.1mm² (n=2863) with the highest percentage (40.1%) of seeds being 0.08mm².

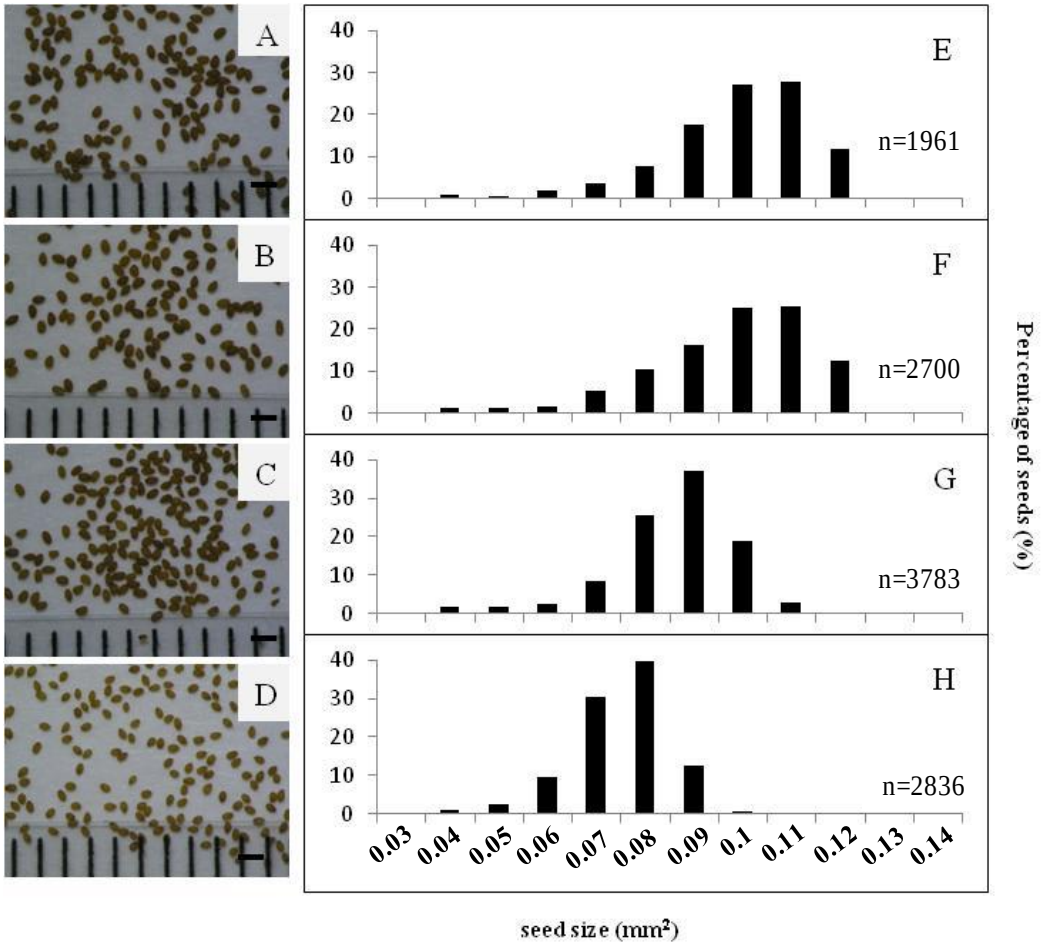

Figure 3.2. 4 **Seed size distribution from wild type and mutant plants**
Left panel: Mature seeds from self-fertilised (A) wild – type Columbia (B) *er105/er105*, (C) *iku2-4/iku2-4*, (D*) iku2-4/iku2-4;er105/er105.* Scale bars:1mm
Right panel: seed size distribution in progeny seeds from self-fertilised (E) wild - type Columbia, (F) *er105/er105* , (G) *iku2-4/iku2-4* and (H) *iku2-4/iku2-4;er105/er105.* X axis represents seed area in mm^2, Y axis the percentage of seeds. n= total number of seeds analysed.

The results of these analyses confirmed that the *iku2* small seed phenotype is enhanced in the presence of *er105/er105.*

In order to assess the effect of the *iku2-4* and *er105* mutations on seed development, laser scanning confocal microscopy was performed in developing seeds (Fig.3.2.5). Seed length, seed width and integument thickness measurements and ANOVA was performed.

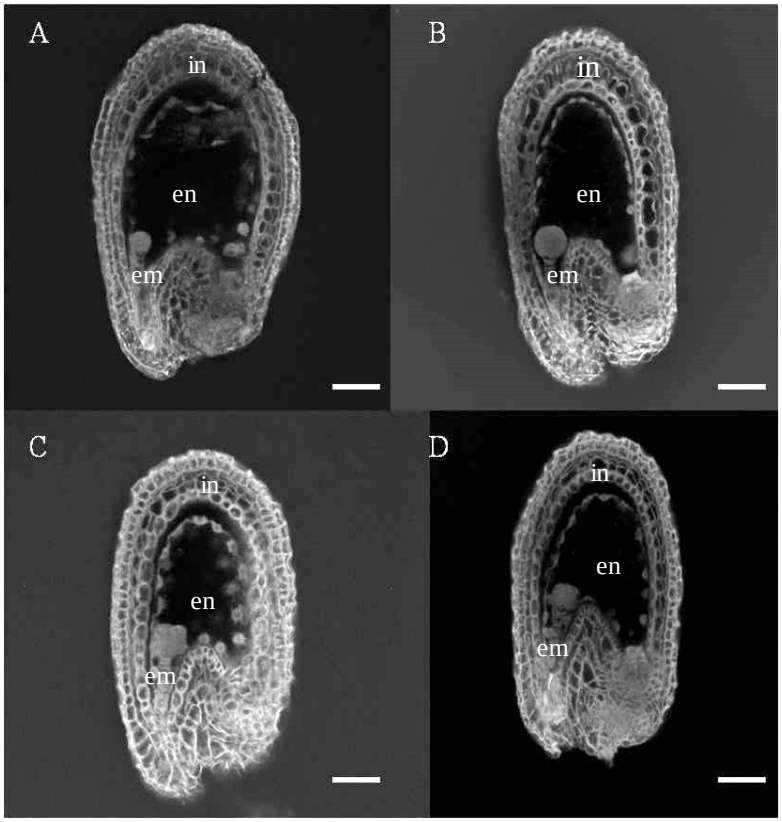

Figure 3.2. 5 **Laser scanning confocal sections of wild type *iku2-4* and *er105* mutant seeds**

Laser scanning confocal sections of seeds at the globular stage of embryo development showing: integuments (in) surrounding the endosperm (en) and the embryo (em). (A) Col-0 (B) *er105/er105* (C) *iku2-4/iku2-4* (D) *iku2-4/iku2-4;er105/er105.* Scale bars represent 50µm.

In wild type Columbia, *er105/er105* and *iku2-4/iku2-4* the mean seed length did not differ statistically at the globular embryo stage ($P<0.001$) (Fig. 3.2.6) and was 304.3µm ±10.3, 304.9µm±6.7 and 316 µm±8.1 respectively. Double homozygous *iku2-4/iku2-4;er105/er105* mutants produced significantly smaller seeds from the other genotypes with a mean of 248.8µm±5.8. (Table 3.2.8). Seed widths showed a similar pattern: Columbia, *er105/er105* and *iku2-4/iku2-4* did not differ significantly ($P<0.001$) and the means were 177.3µm ±5.3, 175.3µm±3.7 and 168µm±3.3, respectively. Double homozygous *iku2-4/iku2-4;er105/er105* mutant

seeds were significantly smaller, with a mean of 142.9µm±3.1. Interestingly, integument thickness differed significantly (P<0.001) in all of the tested genotypes with the biggest mean being found for *er105/er105* (55.86µm±1.1), followed by wild type Columbia (50.45µm±2.1), *iku2-4/iku2-4* (47.96µm±0.9) and with *iku2-4/iku2-4;er105/er105*(43.44µm±0.5) having the smallest integument thickness.

Together, these findings confirm the data obtained from the analysis of dry mature seeds of single and double *iku2-4/iku2-4* and *er105/er105* mutants. However, in the early developmental analysis, single *er105/er105* and *iku2-4/iku2-4* mutants did not show an enhancement in terms of the seed length and width. An enhancement was only visible in the thickness of the maternal integuments, therefore indicates that *IKU2* and *ER* play overlapping roles in regulating seed integument development in Arabidopsis. Double homozygous *iku2-4/iku2-4;er105/er105* mutant seeds were significantly smaller for all of the measured parameters, which indicates an additive effect of *IKU2* and *ERECTA* to regulate integument thickness as well as the seed size during early seed development. Collectively, these data indicate that *IKU2* and *ERECTA* act in separate pathways to regulate seed size in Arabidopsis.

		Seed length		Seed width		Integument thickness	
Genotype	**n**	**Mean (µm)**	**Median**	**Mean (µm)**	**Median**	**Mean (µm)**	**Median**
Col-0	10	304.3^{a} ±10.3	310.0	177.3^{a} ±5.3	172.0	50.45^{a} ±2.1	52.35
er105	23	304.9^{a} ±6.7	302.8	175.3^{a} ±3.7	169.1	55.86^{b} ±1.1	55.60
iku2-4	10	316^{a} ±8.1	322.1	168^{a} ±3.3	167.5	47.96^{c} ±0.9	47.18
iku2-4/er105	30	248.8^{b} ±5.8	254.7	142.9^{b} ±3.1	138.1	43.44^{d} ±0.5	43.77
$LSD_{0.05}$		15.86		8.51		2.34	

Table 3.2. 6 **Analysis of variance (ANOVA)**

Confocal scan laser microscopy images were used to determine the seed length, width and integument thickness of seeds at globular stage of development from different genotypes: Col-0 , *er105/er105*, *iku2-4/iku2-4*,*iku2-4/iku2-4;er105/er105*. The means annotated with the same letter symbol are not significantly different from each other ($LSD_{0.05}$, $P<0.001$), ± SEM. n=number of samples tested.

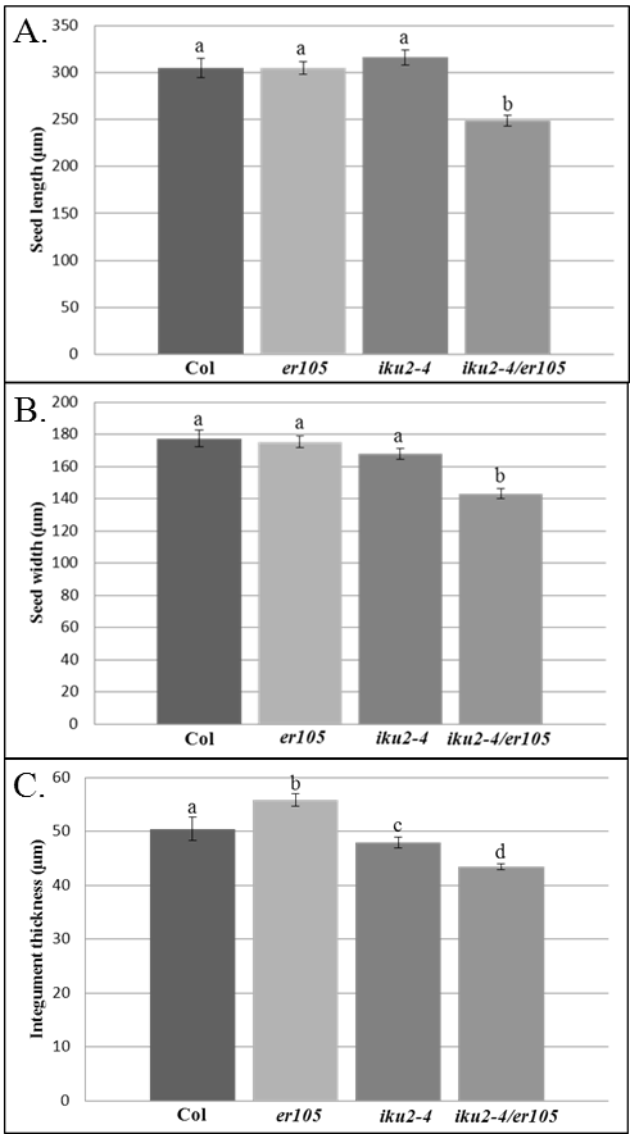

Figure 3.2. 6 **Mean sizes of mutant seeds at the globular stage of development**
Bars represent mean size of (A) seed length, (B) seed width and (C) integument thickness in µm in wild type Columbia: Col-0, *er105/er105*, *iku2-4/iku2-4*, *iku2-4/iku2-4;er105/er105* seeds. Genotypes annotated with the same letter symbol are not statistically different from each other. Error bars: SEM

To genetically dissect in which part of the seed *IKU2* and *ERECTA* genetically interact, seeds from crosses between *er105/er105;IKU2-4/iku2-4* x *er105/er105;iku2-4/iku2-4* plants and *ER105/er105;iku2-4/iku2-4* x *er105/er105;iku2-4/iku2-4* plants were examined (Fig.3.2.7). This approach would reveal the role of both sporophytic and gametophytic seed components, by uncoupling *IKU2* and *ER* function on integument, embryo and endosperm development. The initial analysis determined the variation between the mature seeds progeny derived from these crosses using the ANOVA test.

	er105/iku2-4/IKU2-4 x er105/iku2		***ER105/er105/iku2-4* x *er105/iku2-4***	
Source of variation	d.f.	m.s.	d.f.	m.s.
Cross	3	0.17138794***	1	0.07335164***
Residual	1287	0.00004468	706	0.00009769

Table 3.2. 7 **Mean squares from analysis of variance (ANOVA) for seed areas derived from individual crosses**

Seed area of progeny derived from *er105/er105;iku2-4/IKU2-4* x *er105/er105;iku2-4/iku2-4 and ER105/er105;iku2-4/iku2-4* x *er105/er105;iku2-4/iku2-4* crosses were analysed. D.f. – degree of freedom, m.s. – mean square.* significant at 0.05 level, ** significant at 0.01 level, *** significant at 0.001 level.

The seed progenies derived from crosses (*er105/er105;iku2-4/IKU2-4* x *er105/er105;iku2-4/iku2-4* crosses 1-4 and *ER105/er105/iku2-4/iku2-4* x *er105/er105;iku2-4/iku2-4* crosses 1,2) showed statistically significant variation (P>0.05) (Table 3.2.7). Thus Tukey test was used to estimate the differences between the sizes of progeny seeds derived from individual crosses.

Crosses						
Cross no	***er105/iku2-4/IKU2-4 x er105/iku2-4***			***ER105/er105/iku2-4 x er105/iku2-4***		
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	230	0.05853	0.01012	401	0.06858	0.01213
2	162	0.07458	0.00256	306	0.08911	0.00569
3	231	0.08643	0.00551	-	-	-
4	334	0.10931	0.00621	-	-	-
$LSD_{0.05}$		0.000718			0.001472	

Table 3.2. 8 **The differences between the means of the seed sizes produced by individual crosses**

Crosses analysed: *er105/er105;iku2-4*/IKU2-4x *er105/er105;iku2-4/iku2-4*andER105*/er105;iku2-4/iku2-4* x *er105/er105;iku2-4/iku2-4.* s.d.:standard deviation. n=number of seeds.

The cross between *er105/er105;iku2-4*/*IKU2-4* x *er105/er105;iku2-4/iku2-4* produced seeds with the smallest mean size of $0.05853mm^2 \pm 0.01012$ and the highest $0.10931mm^2 \pm 0.00621$. *ER105/er105/iku2-4/iku2-4* x *er105/er105;iku2-4/iku2-4* cross produced seeds that were of $0.06858mm^2 \pm 0.01213$ smallest mean size and $0.08911mm^2 \pm 0,00569$ the highest (Table 3.2.8).

In addition, an ANOVA analysis was performed on pooled measurements taken from the mature progeny seeds derived from both combinations of crosses.

Lines	**n**	**Mean (mm^2)**	**Median**
er105/iku2-4/IKU2-4 x er105/iku2-4	1291	$0.09181^{a} \pm 0.00053$	0.08022
ER105/er105/iku2-4 x er105/iku2-4	707	$0.07748^{b} \pm 0.00058$	0.09801
$LSD_{0.05}$		0.001734	

Table 3.2. 9 **Analysis of seed size in wild type and mutant plants**

Progeny seeds from *er105/er105;iku2-4/IKU2-4 x er105/er105;iku2-4/iku2-4* and *ER105/er105;iku2-4/iku2-4 x er105/er105;iku2-4/iku2-4* crosses were analysed. Means annotated with the same letter symbol are not significantly different from each other. n=number of seeds.

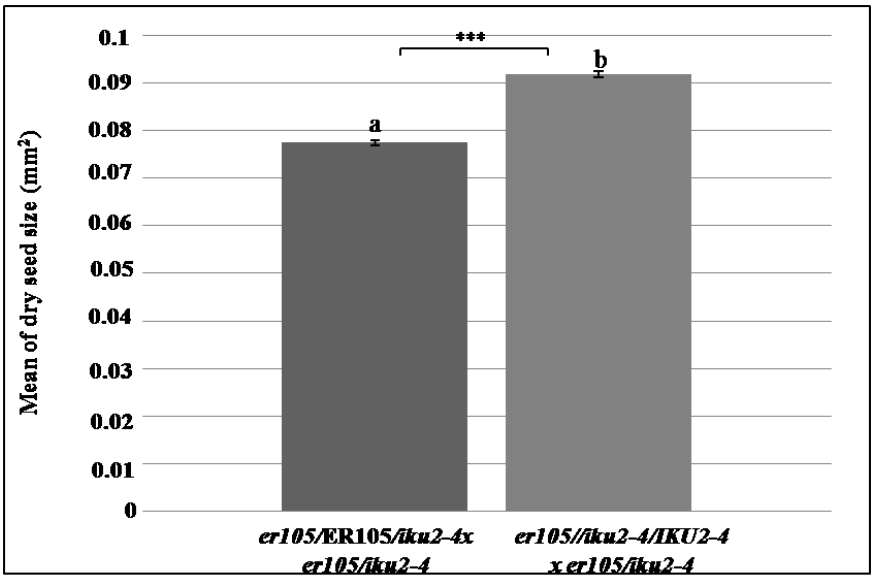

Figure 3.2. 7 **Mean dry seed size analysis** *er105/er105;iku2-4/IKU2-4 x er105/er105;iku2-4/iku2-4* and *ER105/er105;iku2-4/iku2-4 x er105/er105;iku2-4/iku2-4*. The means annotated with the same letter symbol are not statistically different from each other. Error bars: SEM. Asterisks indicate statistical difference between the mean seed sizes.

The analysis revealed that the sizes of progeny seeds differed significantly ($P<0.001$) (Fig.3.2.7). The introgression of *er105/er105;iku2-4/IKU2-4* with *er105/er105;iku2-4/iku2-4* pollen resulted in seeds that the mean area was $0.09181mm^2 \pm 0.00053$ in comparison to *ER105/er105;iku2-4/iku2-4 x er105/er105;iku2-4/iku2-4* crosses that produced significantly smaller seeds with a mean of $0.07748mm^2 \pm 0.00058$ (Table 3.2.11).

To further dissect the differences observed on seed development, the distribution of dry mature seeds from both crosses sizes was also analysed (Fig.3.2.8).

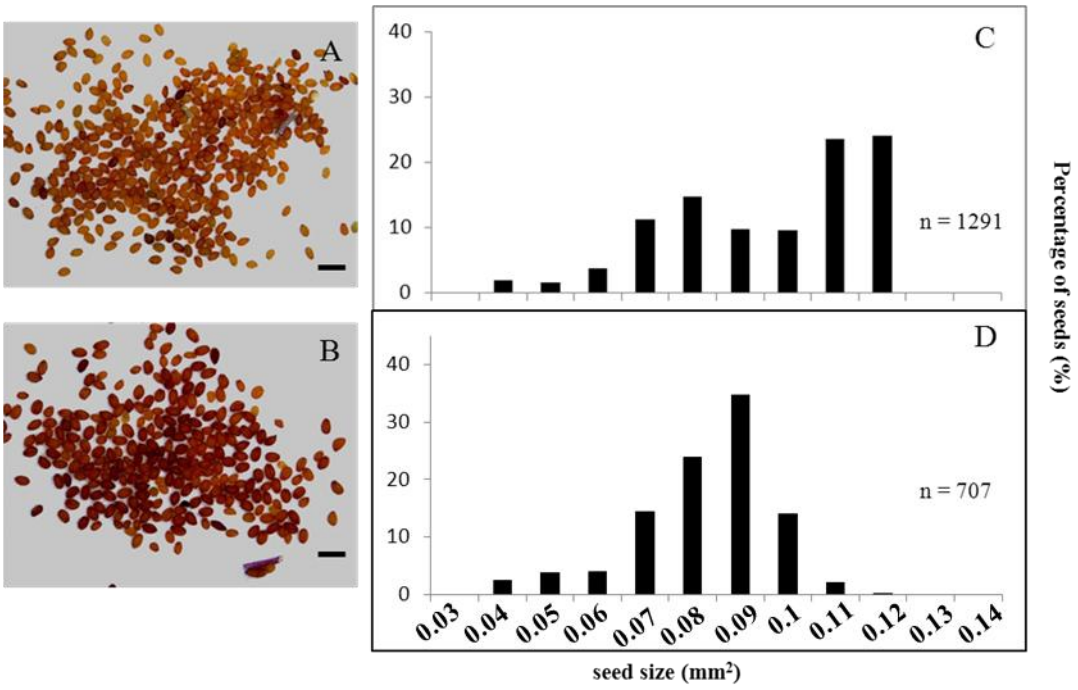

Figure 3.2. 8 **Seed size distribution from *iku2-4* and *er105* crosses**
Left panel: mature progeny seeds from crosses: (A) *er105/er105;iku2-4/IKU2-4* x *er105/er105;iku2-4/iku2-4* (B) *er105/ER105;iku2-4/iku2-4* x *er105/er105;iku2-4/iku2-4*
Right panel: seed size distribution in progeny seeds from crosses (C) *er105/er105;iku2-4/IKU2-4* x *er105/er105;iku2-4/iku2-4* (D) *er105/ER105;iku2-4/iku2-4* x *er105/er105;iku2-4/iku2-4*. Scale bars represent 1mm.

Crosses between *er105/er105;IKU2-4/iku2-4 x er105/er105;iku2-4/iku2-4* plants generated seeds with maternal integuments defective on *ER* only, while 50% the filial tissues carry both mutations. Seeds from this cross (n=1291) displayed the seed size distributions ranging from 0.04 – 0.12mm^2 (Fig. 3.2.8B). Interestingly, a binomial distribution was observed in seed size with one peak (14.7%) representing seeds of 0.08mm^2 and the second (24.08%) being 0.12mm^2. The first group of seeds phenotypically resembled double homozygous *er105/er105;iku2-4/iku2-4* and single homozygous *iku2-4/iku2-4* seeds whereas the second class was similar to *er105/er105* seeds sizes. The enhancement of the small seed phenotype in this cross might indicate that *IKU2* and *ERECTA* regulate the seed size via cross talk between the embryo-endosperm interface within the seed.

In progenies from crosses between ER105/*er105;iku2-4/iku2-4* and *er105/er105;iku2-4/iku2-4* plants, the sporophytic maternal integuments are defective for *IKU2*. The distribution of seeds (n=707) produced from this cross ranged from 0.04 – 0.12mm² with the highest percentage (34.7%) of seeds being 0.09mm^2. Together, the statistical analysis of pooled measurements and the distribution of seed sizes derived from the above crosses confirms that seed size in Arabidopsis is regulated by *IKU2* and *ERECTA* via separate signalling pathways.

3.2.3 *ERECTA* complements the loss-of-function *iku2-1* seed phenotype

To confirm the interaction between *IKU2* and *ERECTA*, *iku2-1*(L*er*) was introgressed genetically into a transgenic Landsberg *erecta* line complemented with an *ERECTA* wild type genomic fragment (L*er*ER) (Masle, Gilmore and Farquhar 2005). Emasculated single homozygous loss-of-function *iku2-1* plants were manually pollinated with L*er*ER pollen and the F_1 progeny was self-pollinated to generate F_2 seed progenies. To identify double homozygous *iku2-1/iku2-1*;L*er*ER/L*er*ER mutants, the F_2 plants were genotyped by PCR amplification (Fig. 3.2.9) and sequenced in order to confirm the single nucleotide change (G to A) present in *iku2-1* mutant allele. For this, the *iku2-1* DNA fragment was amplified with iku2-1For (Fig. 3.2.9 F1) and iku2-1Rev (Fig.3.2.9 R1) primers and a search for the G-A substitution was performed. The PCR conditions in this reaction were: the denaturation step at 94ºC for 1minute followed by 25 cycles of denaturation for 30s, annealing at 58ºC for 30s and elongation at 72ºC for 30s.

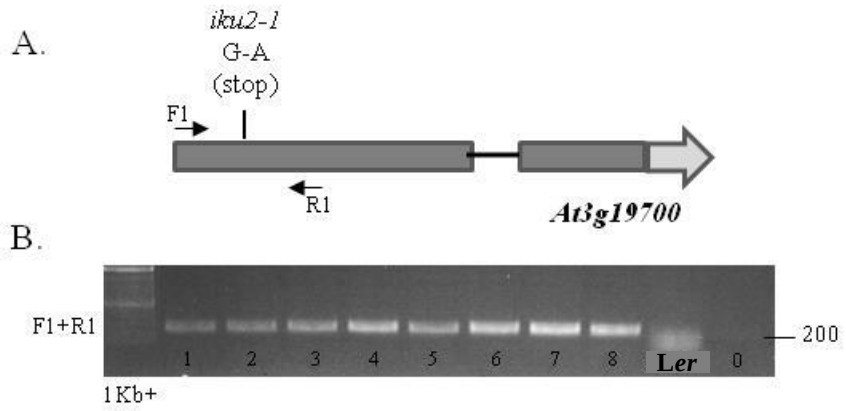

Figure 3.2. 9 **PCR genotyping of *iku2-1***
(A) Schematic representation of *IKU2* (At3g19700) gene and the location of *iku2-1* mutation in exon 1. Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines, and positions for oligonucleotide primers are indicated by arrows (B) F1 and F2 primer pair was used to amplify a 200bp product that includes the G-A nucleotide change resulting in stop codon in *iku2-1* mutant. The cleaned PCR product was sequenced in order to determine the nucleotide present in the sequence. Lane 1-8, individual plants/samples used for genotyping, L*er*: wild type Lansberg *erecta* positive control, 0: negative control.

The identification of homozygous L*er*ER plants was possible due to their clearly visible tall plant phenotype (Masle et al. 2005). The following genotypes were selected for further analysis: L*er*, *iku2-1*/*iku2-1*, *iku2-1*/*iku2-1*;L*er*ER/L*er*ER and L*er*ER. The plants were grown to maturity to enable a phenotypic analysis of dry mature progeny seeds. Mature seeds were collected from individual plants of each particular genotype. The seed sizes were measured for individual plants within each genotype to assess the variation in seed sizes.

	L*er*		**L*er*ER**	
Source of variation	d.f.	m.s.	d.f.	m.s.
Plant	3	0.0040472***	3	0.0232523***
Residual	770	0.0004019	857	0.0005206
	iku2-1		***iku2-1*/L*er*ER**	
Source of variation	d.f.	m.s.	d.f.	m.s.
Plant	3	0.0063181***	7	0.0047821***
Residual	1236	0.0003150	2785	0.0005508

Table 3.2. 10 **Mean squares from analysis of variance (ANOVA**)
Seed areas of individual plants derived from particular genotypes: L*er*, L*er*ER, *iku2-1/iku2-1*, *iku2-1/iku2-1*;L*er*ER/L*er*ER were analysed. d.f. – degree of freedom, m.s. – mean squares. * significant at 0.05 level, ** significant at 0.01 level, *** significant at 0.001 level.

The ANOVA test showed that individual plants of L*er*, L*er*ER, *iku2-1/iku2-1*, *iku2-1/iku2-1*;L*er*ER/L*er*ER produced mature seeds that differed significantly in sizes ($P<0.001$) (Table 3.2.10). To further investigate the mean difference observed in individual plants, a Tukey test was performed on these data.

Lines						
Plant no		**L*er***			**L*er*ER**	
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	231	0.09218	0.02041	209	0.08971	0,02369
2	197	0.10169	0.01545	227	0.11047	0,02021
3	176	0.09419	0.02315	186	0.10156	0,02586
4	167	0.09215	0.02063	247	0.08927	0,02133
$LSD_{0.05}$		0.003662			0.004030	
		iku2-1			***iku2-1*/L*er*ER**	
Plant no	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	370	0.05985	0.0192	214	0.09018	0,02403
2	332	0.05965	0.01968	516	0.09225	0,02335
3	275	0.05843	0.0159	264	0.08844	0,01948
4	260	0.04987	0.01438	269	0.09198	0,02009
5	-	-	-	275	0.09090	0,02246
6	-	-	-	260	0.08166	0,02424
7	-	-	-	469	0.09502	0,02109
8	-	-	-	525	0.08984	0,02827
$LSD_{0.05}$		0.002550			0.002840	

Table 3.2. 11 **The differences between the means of the seed sizes derived from individual plants within tested lines**

Genotypes tested in the analysis: L*er*,L*er*ER, *iku2-1/iku2-1, iku2-1/iku2-1* ;L*er*ER/L*er*ER. s.d.:standard deviation. n=number of seeds.

Wild type Landsberg *erecta* plants produced seeds with the smallest mean size of 0.09215mm^2±0.02063, and 0.10169 mm^2 ±0.01545 the highest. In L*er*ER the smallest mean size was 0.08927mm^2±0.02133 and the highest 0.11047mm^2±0.02021. *Iku2-1 /iku2-1* plants produced seeds that the smallest mean was 0.04987mm^2 ±0.01438 and the highest 0.05985mm^2±0.0192. The mean of dry mature seeds of individual double homozygous *iku2-1/iku2-1*;L*er*ER/L*er*ER plants were 0.08166mm^2±0.02424 (the smallest) and 0.09502mm^2±0.02109 the highest (Table 3.2.11).

Further statistical analysis was performed using ANOVA on pooled measurements on all tested genotypes and statistical differences in the means of each genotype were assessed.

Line	n	Mean (mm^2)	Median
L*er*	772	0.09505^{b} ±0.00073	0.09878
L*er*ER	871	0.09752^{a} ±0.00083	0.09956
iku2-1	1237	0.05739^{d} ±0.00051	0.05400
***iku2-1*/L*er*ER**	2793	0.09060^{c} ±0.00044	0.09332
$LSD_{0.05}$		0.001169	

Table 3.2. 12 Analysis of seed size in wild type, *iku2-1* and L*er*ER plants
Genotypes analysed: L*er*, L*er*ER, *iku2-1/iku2-1*(L*er*), *iku2-1/iku2-1*;L*er*ER/L*er*ER. The means annotated with the same letter symbol are not significantly different from each other ($LSD_{0.05}$, P<0.001), ± SEM.

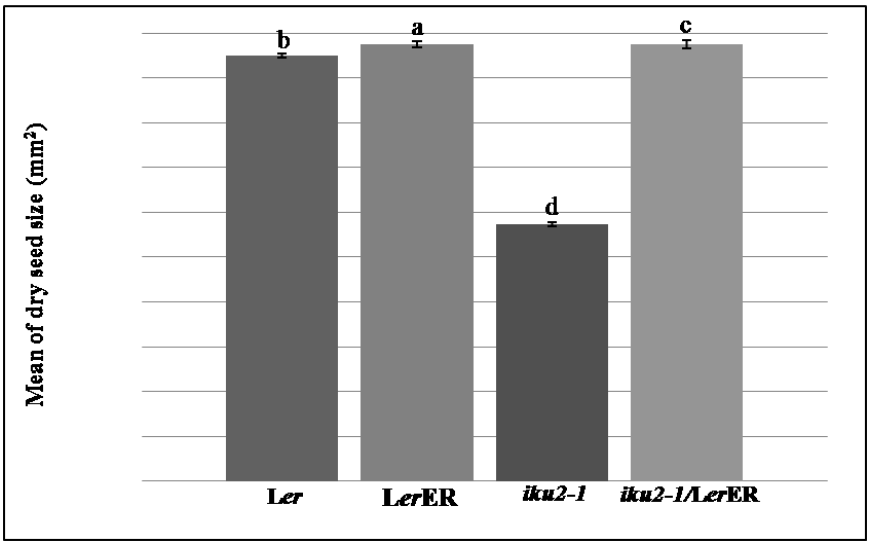

Figure 3.2. 10 **Mean dry seed size analysis**
Bars represent the means of L*er*, L*er*ER/L*er*ER, *iku2-1/iku2-1*, *iku2-1/iku2-1*;L*er*ER/L*er*ER. The means annotated with the same letter symbol are not statistically different from each other. Error bars: SEM.

The results of the ANOVA test indicate that the mean seed sizes of all of the tested genotypes significantly differed amongst themselves ($P<0.001$) (Fig.3.2.10). Interestingly, wild type Landsberg *erecta* seeds were slightly smaller than those generated by L*er*ER ($0.09505mm^2 \pm 0.00073$ compared to $0.09752mm^2 \pm 0.00083$, respectively), confirming the previous data indicating that *ER* plays a role in regulating seed size in Arabidopsis, while *iku2-1/iku2-1* (L*er*) seeds were the smallest in this analysis and showed mean of $0.05739mm^2 \pm 0.00051$. Seeds from iku2-1 plants carrying complementation *ER* transgene (*iku2-1/iku2-1;*L*er*ER/L*er*ER) produced seeds with a mean size $0.09060mm^2 \pm 0.00044$ that was significantly different but still resembled L*er*ER and L*er* seeds (Table 3.2.14). These data clearly show a suppression of the reduced seed size phenotype of *iku2-1*/L*er* by the *ERECTA* transgene (L*er*ER), thus confirming that both *IKU2* and *ERECTA* regulate seed size in Arabidopsis.

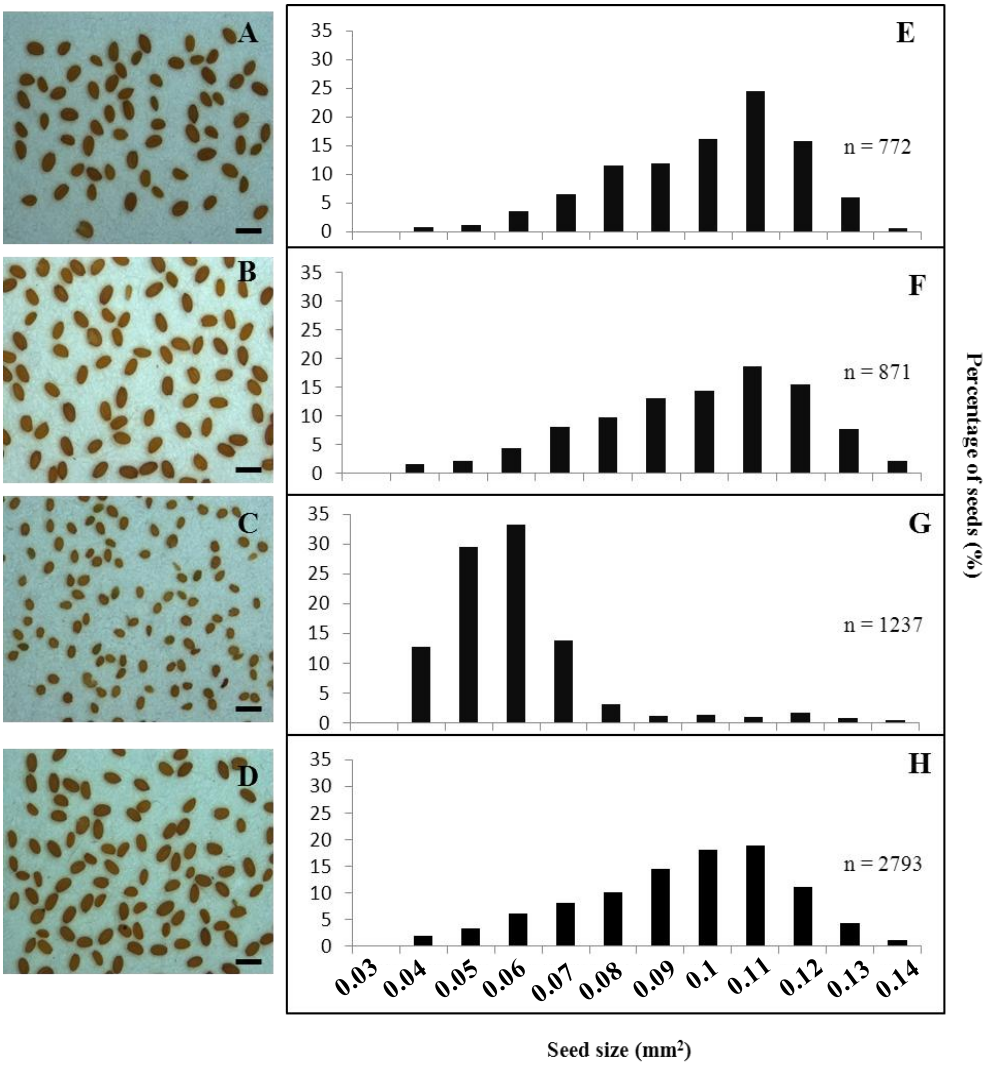

Figure 3.2. 11 **Seed size distribution from L*er*ER and *iku2-1*/LerER seeds**
Left panel: dry mature seeds from self-fertilised (A) wild-type Landsberg *erecta*, (B) L*er*ER/L*er*ER, (C) *iku2-1/iku2-1*(L*er*)(D) *iku2-1/iku2-1*;L*er*ER/L*er*ER. Scale bars: 1mm
Right panel: the distribution of dry mature seed sizes from (E) wild type Landsberg *erecta*, (F) L*er*ER/L*er*ER,(G) *iku2-1/iku2-1*(L*er*), (H) *iku2-1/iku2-1*;L*er*ER/L*er*ER. X axis represents seed area in mm^2, Y axis the percentage of seeds. n= total number of seeds analysed.

The distribution of seeds in all of the tested genotypes ranged from 0.04 and 0.14mm² (Fig.3.2.11). The seeds produced in wild type Landsberg *erecta* plants showed the highest percentage (24.4%) of seeds that were 0.11mm². L*er*ER lines showed a similar pattern of seed size distribution and the highest percentage (18.57%) of seeds was 0.11mm². In *iku2-1*/*iku2-1* plants, the most frequent (33.2%)

seed size was 0.06mm². The highest percentage of progeny seeds produced by double *iku2-1*/*iku2-1*;L*er*ER/L*er*ER was 18.8% representing seeds of 0.11mm². These data further confirm that the naturally occurring *erecta* mutation in Lansberg *erecta* ecotype enhances *iku2-1* small seed phenotype thus confirming that *IKU2* and *ERECTA* regulate seed size in Arabidopsis.

To dissect further the effects of *ERECTA* on the *iku2* mutation laser scanning confocal microscopy analysis was performed on developing seeds at the globular stage of embryo development (Fig.3.2.12).

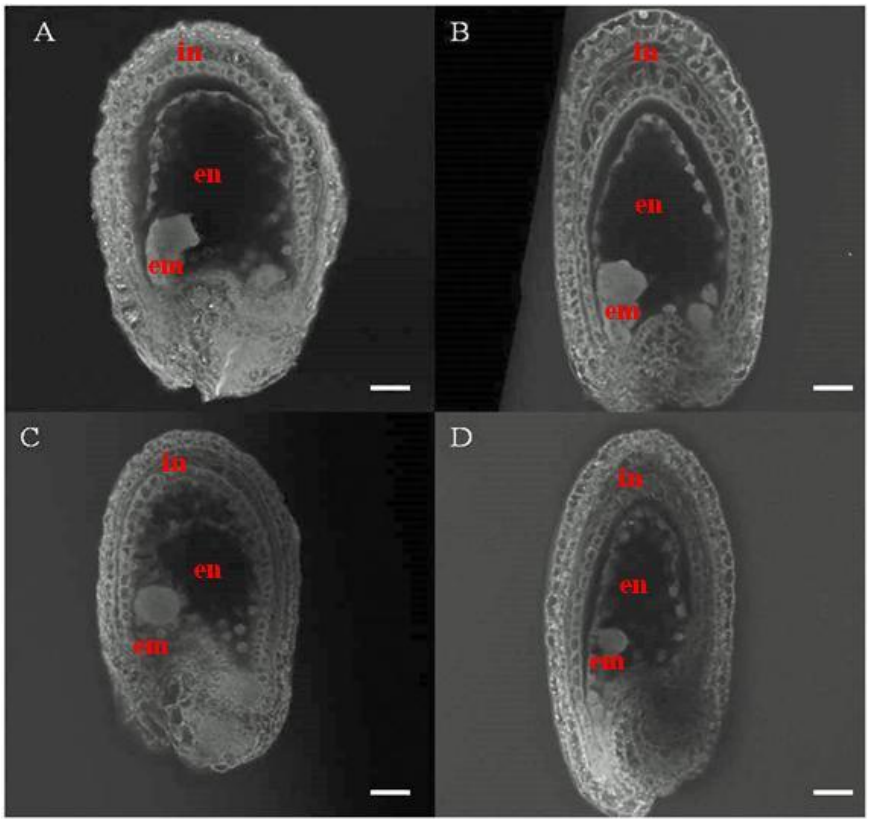

Figure 3.2. 12 **Confocal sections of L*er*ER and *iku2-1*/LerER seeds**
Laser scanning confocal microscopy sections of the seeds at the globular stage of embryo development showing integuments (in) surrounding the endosperm (en) and the embryo (em). (A) L*er* (B) L*er*ER (C) *iku2-1/iku2-1* (D) L*er*ER/L*er*ER;*iku2-1/iku2-1* . Scale bar: 50µm.

Analysis of seeds at globular stage of embryo development included measurements of seed length, width and integument thickness followed by statistical analysis of the obtained data. In terms of the seed length, Landsberg *erecta,* L*er*ER and *iku2-1/iku2-1* differed significantly amongst themselves with the means of 353.7µm±8.9, 417.3µm±4.0, 296.2µm±9.7, respectively. Interestingly, at this stage of development the length of the seeds of complemented L*er*ER/L*er*ER;*iku2-1/iku2-1* (336.2µm±9.9) was not statistically different from L*er* seeds. The width of seeds was significantly different in all of the tested genotypes 231.1µm±6.6 for Landsberg *erecta,* 251.5µm±4.4 for L*er*ER, 166µm±5.4 in *iku2-1/iku2-1*and184.6µm±7.6 in the complemented L*er*ER/L*er*ER;*iku2-1/iku2-1*. In terms of integument thickness, seeds of Landsberg *erecta,* L*er*ER and *iku2-1/iku2-1* differed significantly with means of 59.54µm±2.5, 77.59µm±2.7 and 40.64µm±1.0, respectively. Interestingly, the variability in integument thickness is similar to that of seed length, integument thickness of complemented L*er*ER/L*er*ER;*iku2-1/iku2-1 (*58.44µm±2.8) was not significantly different to that of L*er* integuments.

		Seed length		**Seed width**		**Integument thickness**	
Genotype	n	Mean (µm)	Median	Mean (µm)	Median	Mean (µm)	Median
L*er*	10	353.7^{b} ±8.9	344.8	231.1^{b} ±6.6	232.5	59.54^{b} ±2.5	60.00
L*er*ER	10	417.3^{a} ±4.0	421.0	251.5^{a} ±4.4	248.0	77.59^{a} ±2.7	79.75
iku2-1	10	296.2^{c} ±9.7	290.5	166^{d} ±5.4	168.3	40.64^{c} ±1.0	41.31
***iku2-1*/L*er*ER**	10	336.2^{b} ±9.9	327.5	184.6^{c} ±7.6	180.5	58.44^{b} ±2.8	57.08
$LSD_{0.05}$		23.53		17.78		6.97	

Table 3.2. 13 **Analysis of variance (ANOVA)**

Confocal scan microscopy images were used to determine the seed length, width and integument thickness of seeds at globular stage of development from different genotypes: Landsberg *erecta* (L*er*), Landsberg *ERECTA* (L*er*ER), *iku2-1/iku2-1* and double homozygous *iku2-1/iku2-1;*L*er*ER/L*er*ER seeds (n=number of seeds). The means annotated with the same letter symbol are not significantly different from each other ($LSD_{0.05}$, $P<0.001$), ± SEM.

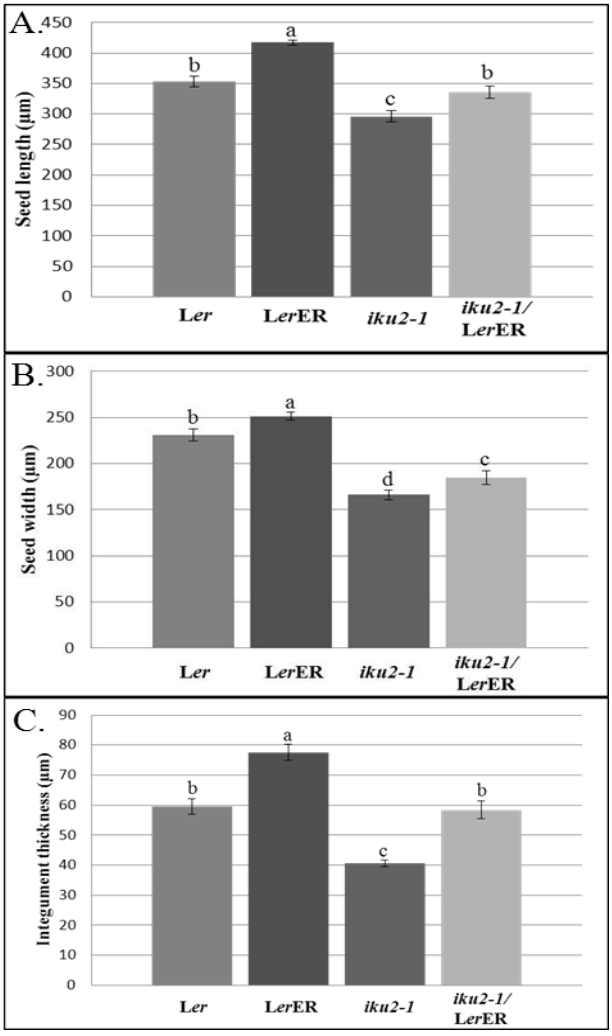

Figure 3.2. 13 **Mean size of mutant seeds at the globular stage of development**
The means of (A) seed length, (B) seed width and (C) integument thickness in µm in wild type Landsberg *erecta* (Col-0), Landsberg *ERECTA* (L*er*ER), *iku2-1* and double homozygous *iku2-1/iku2-1* ;L*er*ER/L*er*ER seeds (n=number of seeds). Genotypes annotated with the same letter symbol are not statistically different from each other. Error bars: SEM

Collectively, despite the limited number of samples analysed, this analysis clearly indicates that *IKU2* function with *ERECTA* via two separate pathways to regulate seed size in Arabidopsis.

3.2.4 Complementation of *iku2-4/iku2-4;er105/er105* with pIKU2:ERECTA$_{myc}$

To investigate whether a functional *ERECTA* in the endosperm was able to complement the seed phenotype observed in *iku2-4/iku2-4;er105/er105* mutants, a construct in which the *ERECTA* coding region was fused to a myc tag and driven by the *IKU2* promoter (pIKU2:ERECTA$_{myc}$) (see general methods). The *myc*-tag is a small peptide that consists of 10 amino acids (EQKLISEEDL) with a predicted size of 1,020 Daltons (Fan et al. 1998). This tag was chosen because of its small size, thus would be unlikely to interfere with the function of ERECTA. Double homozygous *iku2-4/iku2-4;er105/er105* plants were transformed with the pIKU2:ERECTA$_{myc}$ construct and seeds were selected for resistance to hygromycin. Independent primary transformants were selected for analysis and grown to maturity to produce T_1 seeds. T_1 lines were selected based on their hygromycin resistance and self-pollinated. T_2 seed size was measured using ImageJ to assess the ability of the pIKU2:ERECTA$_{myc}$ transgene to partially complement the small seed phenotype observed in double *iku2-4/iku2-4;er105/er105* mutants. Only one line, named $T_2$2, was grown to produce progeny that were used for further statistical analysis, together with Col-0, *iku2-4/iku2-4* and *iku2-4/iku2-4;er105/er105* lines. Seed sizes from individual plants for each line were compared for variation using ANOVA (Table 3.2.14).

	Col-0		***iku2-4***	
Source of variation	d.f.	m.s.	d.f.	m.s.
Plant	3	0.0033724***	3	0.0007280
Residual	1697	0.0001961	1852	0.0003240
	iku2-4/er105		**pIKU2:ERECTA$_{myc}$**	
Source of variation	d.f.	m.s.	d.f.	m.s.
Plant	3	0.0008778	3	0.0036723
Residual	1866	0.0004897	1875	0.0003486***

Table 3.2. 14 **Mean squares from analysis of variance (ANOVA)**
Seed areas of individual plants derived from particular genotypes: Col-0, *iku2-4/iku2-4*, *iku2-4/iku2-4;er105/er105* and pIKU2:ERECTA$_{myc}$ in *iku2-4/iku2-4/er105/er105* background were analysed. D.f. – degree of freedom, m.s. – mean square. * significant at 0.05 level, ** significant at 0.01 level, *** significant at 0.001 level.

Plants of the same genotype were not expected to show significant differences in seed size between them, however the analysis showed that individual plants of wild type Col-0, and transgenic pIKU2:ERECTA$_{myc}$ contained significantly different sized seeds. Only seed sizes from individual *iku2-4/iku2-4* and *iku2-4/iku2-4;er105/er105* plants did not differ significantly in size (P>0.05) (Table 3.2.15). Subsequently, the Tukey's test was applied to test the differences between the seed sizes of individual for each line.

Lines						
Plant no		**Col-0**			***iku2-4***	
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	444	0.10172	0.01248	457	0.08239	0.02024
2	440	0.09799	0.01310	475	0.08067	0.01670
3	434	0.09776	0.01394	457	0.08013	0.01605
4	383	0.10358	0.01652	466	0.08268	0.01874
$LSD_{0.05}$		0.001843			0.002291	
	iku2-4/er105			**pIKU2:ERECTA$_{myc}$**		
Plant no	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	500	0.07864	0.02155	343	0.07399	0.01863
2	448	0.07631	0.02052	370	0.07545	0.01871
3	578	0.07936	0.02345	352	0.07965	0.01689
4	341	0.07750	0.02268	433	0.07461	0.02159
5		-	-	378	0.08091	0.01652
$LSD_{0.05}$		0.002553			0.002489	

Table 3.2. 15 **The differences between the means of the seed sizes (in mm^2) derived from individual plants within tested genotypes**

Genotypes tested: Col-0, *iku2-4/iku2-4, iku2-4/iku2-4;er105/er105,* pIKU2:ERECTA$_{myc}$ in *iku2-4/iku2-4/er105/er105* background. S.d.:standard deviation. n=number of seeds.

The results showed that within the Columbia genotype the highest mean for dry seed sizes was 0.10358mm^2 ±0.01652 and the smallest 0.09776mm^2 ±0.01394. In *iku2-4/iku2-4* and *iku2-4/iku2-4;er105/er105* individual plants produced seeds that were not significantly different from each other. pIKU2:ERECTA$_{myc}$ gave rise to the smallest mean dry seed size of 0.07399mm^2±0.01863 and the highest of 0.08091mm^2 ±0.01652 (Table 3.2.15).

To further investigate the effects of the pIKU2:ERECTA$_{myc}$ construct on the variability of seed area a one-way analysis ANOVA was carried out on pooled seeds from Col-0, *iku2-4/iku2-4* and *iku2-4/iku2-4;er105/er105* and pIKU2:ERECTA$_{myc}$ plants. The LSD of seed area was calculated, and on this basis groups that were not significantly different from each other were determined.

Lines	n	Mean (mm^2)	Median
Col-0	1701	$0.1001^{a} \pm 0.0003$	0.10200
iku2-4	1855	$0.0081^{b} \pm 0.0004$	0.07900
iku2-4/er104	1867	$0.0780^{c} \pm 0.0005$	0.07300
pIKU2:ERECTA$_{myc}$	1533	$0.0768^{c} \pm 0.0004$	0.07600
LSD$_{0.05}$		0.0012	

Table 3.2. 16 **Analysis of seed size in wild type, mutants and pIKU2:ERECTA$_{myc}$ plants**

Genotypes analysed: wild type Columbia, *iku2-4/iku2-4*, *iku2-4/iku2-4/er105/er105* pIKU2:ERECTA$_{myc}$**.** The means annotated with the same letter symbol are not significantly different from each other (LSD$_{0.05}$, P<0.001), ± SEM. n=number of seeds.

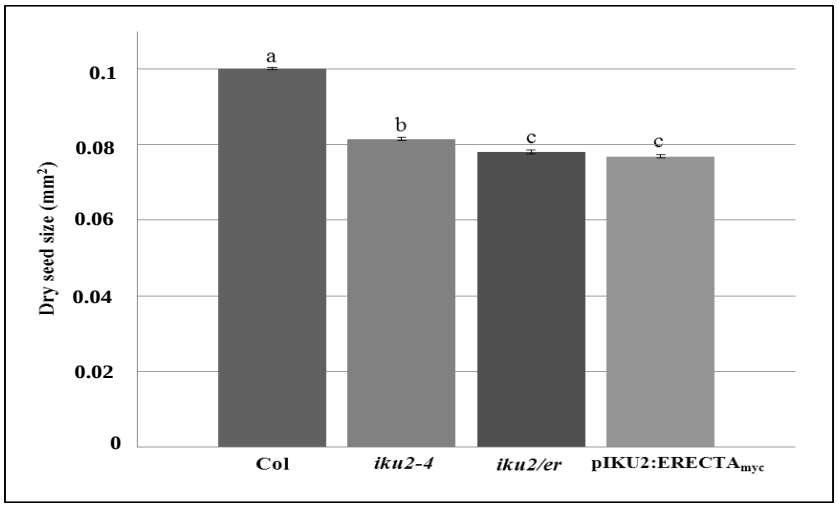

Figure 3.2. 14 **The means of dry mature seeds of pIKU2:ERECTA$_{myc}$**

The bars represent the means of seed sizes from Col-0, *iku2-4/iku2-4*, *iku2-4/iku2-4;er105/er105*, pIKU2:ERECTA$_{myc}$ in *iku2-4/iku2-4/er105/er105* background. The means annotated with the same letter symbol are not statistically different from each other. Error bars: SEM.

ANOVA testing of pooled seeds from pIKU2:ERECTA$_{myc}$;*iku2-4/iku2-4/er105/er105* line confirmed that this construct was not able to rescue the double *iku2-4/iku2-4/er105/er105* small seed phenotype ($0.0768mm^2 \pm 0.0004$ and $0.0780mm^2 \pm 0.0005$ respectively). However, as expected, there were significant differences between the mean size of the wild type Columbia seeds ($0.1001mm^2 \pm 0.0003$) and *iku2-4/iku2-4* seeds ($0.0081mm^2 \pm 0.0004$) which were both

significantly bigger than double *iku2-4/iku2-4;er105/er105* and pIKU2:ERECTA$_{myc}$ seeds (Table 3.2.18).

Analysis of seed size distribution of dry mature seeds confirmed the previous statistical analysis.

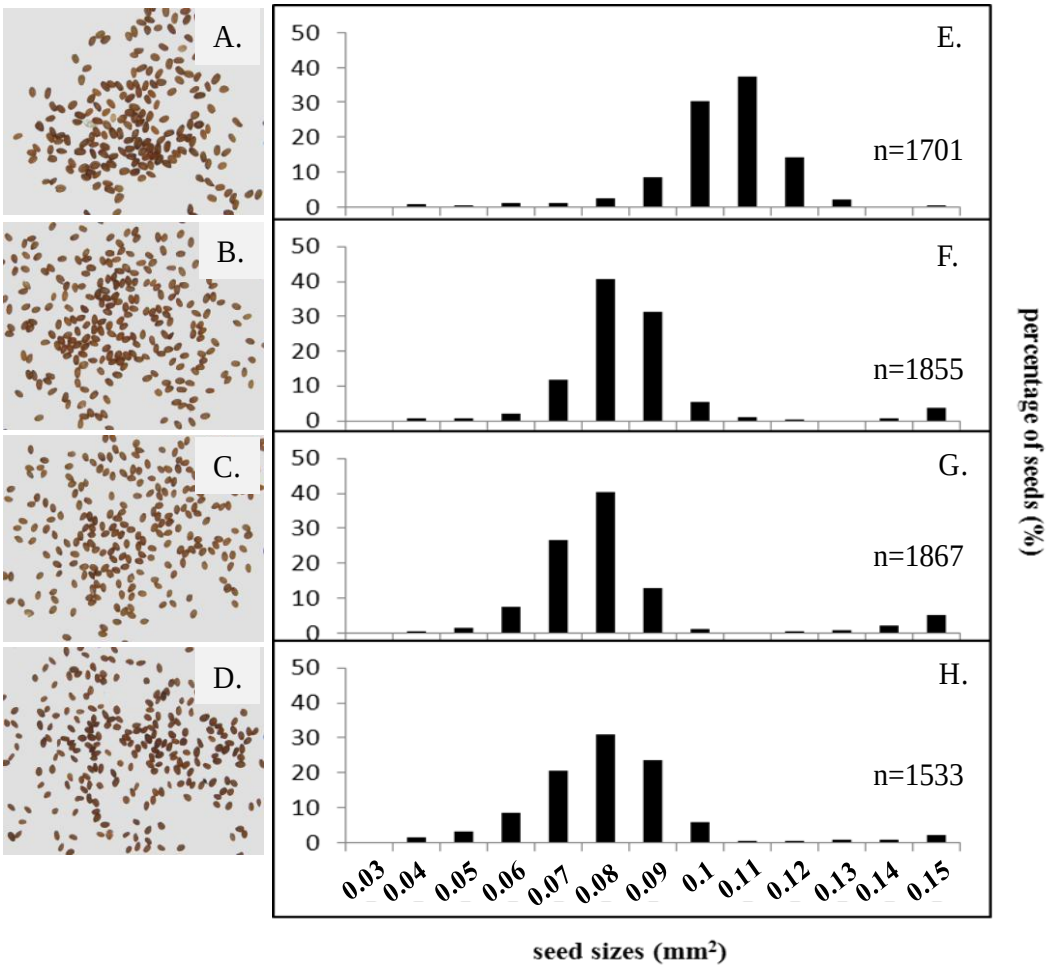

Figure 3.2. 15 **Seed size distribution from pIKU2:ERECTA$_{myc}$ transgenic line**
Left panel: dry mature seeds from self-fertilised seeds from (A) Columbia wild type seeds (B) *iku2-4/iku2-4* (C) *iku2-4/iku2-4;er105/er105* (D) pIKU2:ERECTA$_{myc}$
Right panel: seed size distribution in progeny seeds from Columbia wild type seeds (E) *iku2-4/iku2-4* (F) *iku2-4/iku2-4;er105/er105* (G) pIKU2:ERECTA$_{myc}$ (H)

All of the analysed lines (Col-0, *iku2-4/iku2-4;er105/er105*, pIKU2:ERECTA$_{myc}$ in *iku2-4/iku2-4/er105/er105* background) showed a seed size distribution of 0.04-0.16mm^2, apart from *iku2-4/iku2-4* mutants that ranged between 0.05-0.16mm^2. In Col-0 the highest percentage of seeds (37.4%) was 0.11mm^2 (Fig.3.2.9). In *iku2-4/iku2-4* the highest percentage of seeds (40.7%) was 0.08mm^2. Similarly, most of the mature seeds produced by the transgenic pIKU2:ERECTA$_{myc}$ line and *iku2-4/iku2-4;er105/er105* were 0.08mm^2 (30.8% and 40.1%, respectively).

These data indicate that the pIKU2:ERECTA$_{myc}$ does not complement the *iku2-4/iku2-4;er105/er105* small seed phenotype. Lack of complementation of double *iku2-4/iku2-4;er105/er105* small mutant seeds with pIKU2:ERECTA$_{myc}$ could mean that the construct is not functional or too weakly expressed. Alternative explanation could be that *ERECTA* is required in different seed compartment or developmental stage of endosperm development to regulate seed size. Since *IKU2* is expressed early in the development of the seed at globular embryo stage it would be valuable to test if pIKU2:ERECTA$_{myc}$ is expressed at early stages of seed development and the integument phenotype is alleviated in the transgenic line.

3.2.5 Discussion

Previous genetic studies have shown that *ERECTA* and *IKU2* have retained significant redundancy in various aspects of plant and seed development (Luo et al. 2005, Shpak et al. 2004, Garcia et al. 2005). The analysis of loss-of-function *IKU2* alleles, *iku2-4 and iku2-1*, in Columbia and Landsberg *erecta* backgrounds respectively, led to the hypothesis that *ER* could interact with *IKU2* to regulate seed size. *In silico* transcriptional expression analysis revealed that *ER* and *IKU2* are both expressed in seeds at globular stages of embryo development. However, this does not exclude a possible shared function between *IKU2* and *ER* in early seed development.

A good example of genetic interactions between RLKs, comes from three brassinosteroid receptors: *BRI1, BRL2* and *BRL3* (Cano-Delgado et al. 2004). The major receptor *BRI1* is broadly expressed in Arabidopsis plant whereas *BRL2* and *BRL3* are restricted to the vasculature. These receptors function redundantly to regulate provascular cell growth and differentiation but do not overlap transcriptionally (Cano-Delgado et al. 2004). Similarly, *CLV2* shows a functional overlap with *BAM1* and *BAM2* in the centre of meristem. *BAM1* and *BAM2* transcriptional expression is undetectable at this part of meristem but triple *bam1/bam2/clv2* mutants strongly enhance the *clv2* meristem phenotype (Deyoung and Clark 2008). Intriguingly, *ER* is broadly expressed in several plant tissues (Shpak et al. 2004) and in developing seeds (Fig. 3.2.1), thus potentially could interact with multiple receptors (van Zanten et al. 2009).

Although *IKU2* and *ER* expression in the endosperm differed spatially, the *er-105* significantly enhanced the small seed phenotype found in *iku2-4*. Consistently, complementation of *iku2-1* with *ER* suppressed its small seed phenotype. Together, these results clearly confirm that *IKU2* and *ER* are functionally redundant and act via separate pathways to ensure proper seed size regulation. The analysis of dry mature progeny seeds from *ER105*/*er105;iku2-4/iku2-4* x *er105/er105;iku2-4/iku2-4* and *er105/er105*;*IKU2-4/iku2-4 x er105/er105;iku2-4/iku2-4* crosses indicates that *IKU2* and *ER* regulate seed size via the endosperm-embryo interface within the seed. This finding, together with the micropylar endosperm specific *IKU2* expression data indicates that both *ER* and *IKU2* orchestrate seed development via an endosperm-embryo interaction. It is likely that *ER* plays a role in early seed development in the embryo since the expression of *ER* in the micropylar endosperm (pIKU2:ERECTA$_{myc}$) did not rescue the small seed

phenotype of double *iku2-4/er105*. Other RLKs that are known to play a role in cell-cell communication regulating embryogenesis are *RPK1/TOAD2* (Nodine et al. 2007) and *ACR4/ALE2* (Lau et al. 2010, Tanaka et al. 2007) affecting cell fate specification and patterning. Close examination of *er105/er105;iku2-4/iku2-4* globular embryos did not show any visible abnormalities in embryo patterning therefore *ER* and *IKU2* may regulate the balance between cell proliferation and division in the endosperm (Shpak et al. 2004, Luo et al. 2005). Seed length and width is affected in *iku2-4/er105* double mutants. Interestingly, single *iku2-4* and *er105* mutants show defects on integument development (Cano-Delgado et al. 2004, Garcia et al. 2005) while double *iku2-4/er105* mutants showed an enhancement of the integument phenotype at the globular stage of embryo development.

Collectively, these data revealed that *IKU2* and *ER* act in two separate pathways in the embryo-endosperm interface which ultimately regulates integument development and seed size.

Chapter 3.3

IKU2 Kinase Activity

3.3.1 Characterisation of IKU2 kinase function

As previously mentioned, *IKU2* encodes an LRR receptor like kinase and the protein structure consists of an extracellular LRR domain, a transmembrane domain and an intracellular kinase domain (Fig.3.3.1 A). The *iku2-3* mutation causes an amino acid substitution (arginine 953 – lysine 953) due to a guanine to adenine nucleotide change in the cytoplasmic kinase domain (Fig.3.3.1 A). The amino acid alignment of selected LRR RLKs (Fig.3.3.1 B) clearly indicates that the mutated amino acid is situated around 200 amino acids distal to the RD kinase active site. In most RLKs the conserved arginine (R) and catalytic aspartate (D) form part of the key activation loop that serves as the kinase activator by enhancing phosphotransferase activity (Afzal et al. 2008). The position of the mutated arginine, which is localised distal to the activation loop in *iku2-3* mutants suggests that the *iku2-3* mutation might not interfere with the important kinase activation site and therefore may not affect protein phosphorylation. To test this hypothesis, the following experiments were performed: the structural modelling of the IKU2-3 kinase domain, a phosphorylation activity assay of the IKU2 kinase domain and phenotypic analysis of seeds carrying a IKU2 transgene lacking the kinase domain.

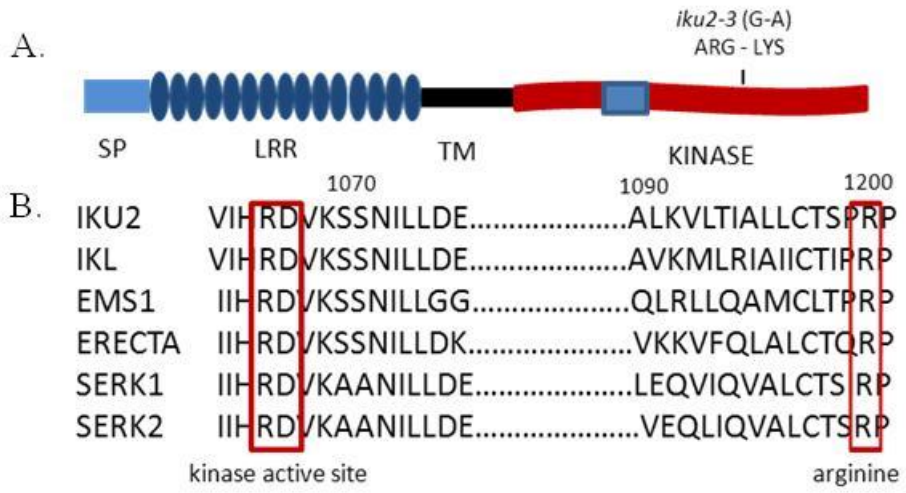

Figure 3.3. 1 **Schematic diagram showing IKU2 protein and amino acid alignment of different LRR RLKs**
(A) SP: Signal peptide (blue box), LRR: leucine Rich Repeat (15 leucine repeats shown as blue ovals); TM: transmembrane domain; KINASE; kinase domain (shown as red tape). The mutant loci available: *iku2-3* is a G-A substitution that causes an arginine to lysine substitution in the kinase domain
(B)amino acid alignment of IKU2, IKU2-LIKE, EMS1, ERECTA, SERK1 and SERK2 kinase domains. Red boxes indicate the RD amino acids as kinase active site and R amino acid mutated in *iku2-3* mutant.

3.3.2 Structural modelling of the IKU2 kinase domain

To gain a better understanding of the effect that the arginine 953 substitution to lysine 953 caused by the *iku2-3* mutation (R953K) has on the structural conformation of the IKU2, the three-dimensional models of the wild type and R953K mutant protein kinase domains were analysed *in silico*. The amino acid sequences of IKU2 and IKU2-3 kinases were compared with SWISS MODEL (http://swissmodel.expasy.org/) (Arnold et al. 2006, Schwede et al. 2003) to obtain a homology based structural model (Fig.3.3.2). Due to the high conservation among the kinase domains of different RLKs, the closest homolog of IKU2 and R953K was IRAK-4 (Wang et al. 2006). Structural analysis of the two models shows that there is no significant conformational change in the structure of the two kinase domains in WT and R953K. (Fig.3.3.2). These data suggest that although *in vivo* the arginine to

lysine amino acid change (*iku2-3*) causes a severe seed phenotype, this amino acid replacement does not affect the intracellular IKU2 structure. Thus it is possible that this mutation interferes with IKU2 function by affecting phosphorylation activity of the kinase active loop or the interaction with a substrate.

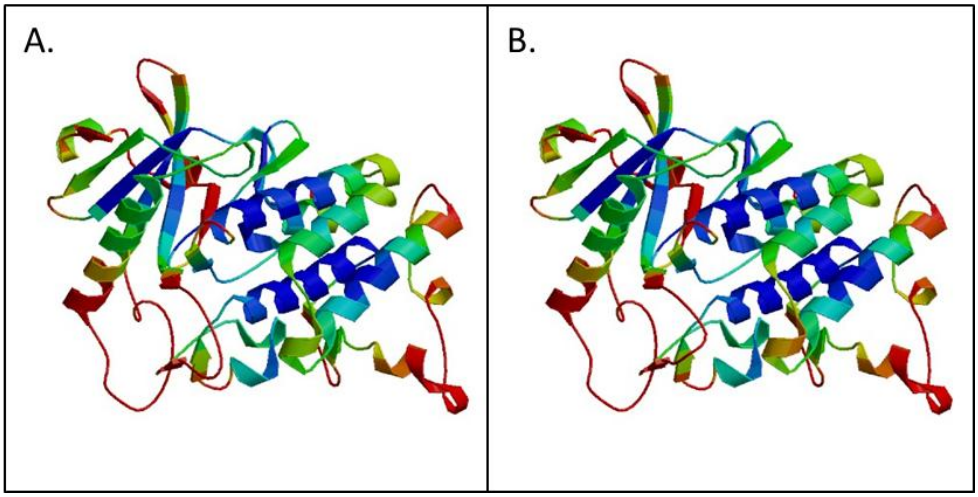

Figure 3.3. 2 **Three dimensional structural model of the kinase domain of IKU2 and R953K mutant**
A. structural three dimensional model of IKU2 kinase domain B. three dimensional model of R953K kinase. Different colours arbitrarily denote distinct secondary structures to aid in visualization.

3.3.3 *In vitro* assay of IKU2 kinase activity

The *in vivo* activation of receptor kinases either in a homo- or a hetero-oligomeric configuration by transphosphorylation is a common feature of receptor like kinases (Tichtinsky et al. 2003). To further investigate if IKU2 is an active kinase and whether the *iku2-3* mutation has an effect on phosphorylation activity, an *in vitro* phosphorylation kinase assay was conducted with recombinant IKU2 kinase domain proteins (Fig.3.3.3).

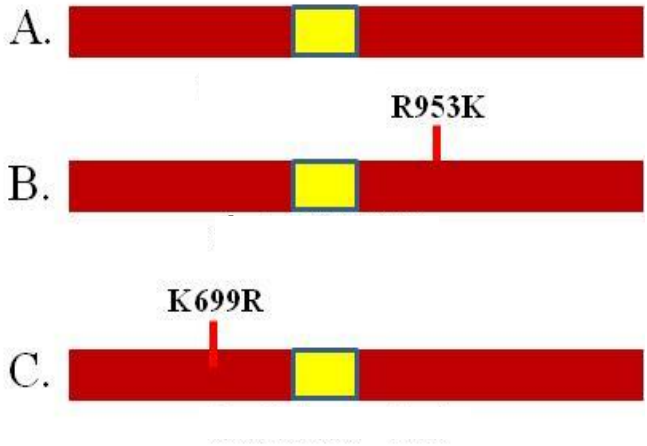

Figure 3.3. 3 **Schematic representation of three recombinant IKU2 kinase domains**
(A) IKU2-WT (B) IKU2-R953K.(C) IKU2-K699R. Red bars indicate kinase domain, yellow boxes indicate RD kinase active site.

To this end, the wild type amino acid IKU2 sequence was obtained for the kinase domain (Fig.3.3.4) by comparing against other known kinases in plants using BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

KIRRDKLNKTVQKKNDWQVSSFRLLNFNEMEIIDEIKSENIIGRGGQGNVYKVSLRSGET
LAVKHIWCPESSHESFRSSTAMLSDGNNRSNNGEFEAEVATLSNIKHINVVKLFCSITCE
DSKLLVYMPNGSLWEQLHERRGEQEIGWRVRQALALGAAKGLEYLHHGLDRPVIHRD
VKSSNILLDEEWRPRIADFGLAKIIQADSVQRDFSAPLVKGTLGYIAPEYAYTTKVNEKS
DVYSFGVVLMELVTGKPETDFGENNDIVMWVWSVSKETNREMMMKLIDTSIEDEYKE
DALKVLTIALLCTDKSPQARPF

Figure 3.3. 4 **Amino acid sequence of IKU2 kinase domain**
The sequence of 316 amino acid residues that constitute the kinase domain of IKU2 from lysine (K636) to phenylalanine (F636). Position of the replaced lysine, K 699 to an arginine, R in IKU2 – inactive kinase (K699R) indicated in red. In IKU2-3, arginine, R 953 mutated into lysine, K in IKU2-3 (R953K) marked in purple. Protein sequence obtained from The Arabidopsis Information Resources (TAIR) (http://www.arabidopsis.org).

Next, the DNA sequence corresponding to the wild type version of the kinase was cloned from Columbia, the IKU2-R953K sequence was cloned from *iku2-3* (L*er*) and the inactive kinase version, IKU2-K699R was generated via PCR mutagenesis (Fig. 3.3.3) which enabled replacement of a lysine residue in the putative ATP-binding site of IKU2 with arginine (K699R) (Fig.3.3.4). To identify active lysine that plays a key role in the phosphorylation activity of the kinase for mutating the amino acid sequence of IKU2 was aligned with Rak1 kinase from tobacco for which the active lysine has been deduced biochemically determined. The fast induction of the recombinant proteins carried out with two different concentrations of IPTG (0.1mM and 1mM) both resulted in the expression of the kinases, clearly visible at 67kDa (Fig.3.3.5 A).

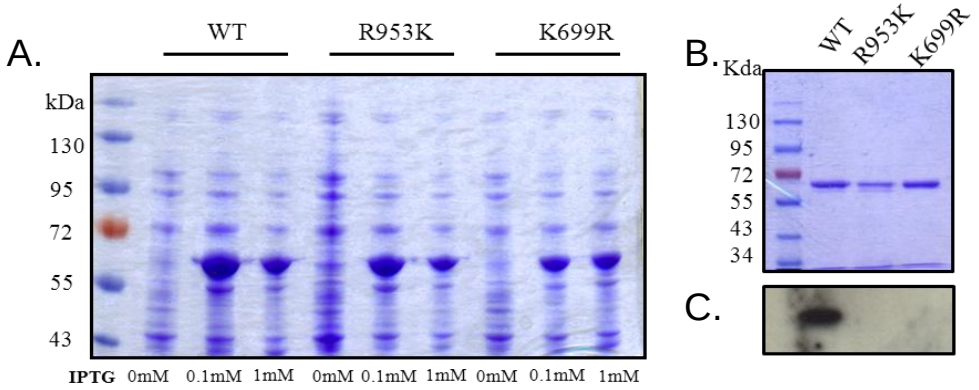

Figure 3.3. 5 **Phosphorylation assay with three different kinase domains of IKU2**
(A) *in vitro* fast induction of the kinase – GST-fused proteins: IKU2-WT, IKU2-R953K, IKU2-K699R inactive kinase. Three different concentrations of IPTG were used in the experiment: 0 as a control, 0.1mM and 1mM. The induced proteins are visible at the size of 67kDa. (B) SDS-PAGE with three different purified proteins: IKU2-WT, IKU2-R953K, IKU2-K699R inactive kinase. The proteins are visible at the size of 67kDa (C) Autoradiograph showing autophosphorylation of the three different kinase domains that is only visible in the IKU2-WT.

To determine the autophosphorylation activity of the recombinant kinases, an *in vitro* phosphorylation assay was performed by incubating the three different

purified proteins (Fig. 3.3.5 B) in the presence of ^{32P}ATP. The incorporation of radioactive phosphorus was only visible for IKU2-WT wild type but for not for IKU2-R953K and IKU2-K699R kinases (Fig.3.3.5 C). These data indicate that *iku2-3* mutation affects the phosphorylation activity of the IKU2.

3.3.4 Phenotypical analysis of seeds carrying an IKU2 lacking the kinase domain

Mutations on the *ERECTA* receptor kinase gene which delete the cytoplasmic kinase domain act in a dominant – negative fashion by interfering with the normal activity of the receptor complex, and have revealed additional insights into ER function during plant development (Shpak et al. 2003). To better understand the importance of the kinase domain for IKU2 function, a truncated version of the IKU2 protein lacking the cytoplasmic kinase domain (pIKU2:IKU2ΔKIN$_{.myc}$) was engineered (see general methods) and introduced into plants lacking a functional IKU2 (*iku2-4*). If the truncated IKU2 further enhances the small seed size phenotype of *iku2-4,* this would indicate that IKU2 receptor functions as a heterodimer with other RLKs to regulate seed size.

pIKU2:IKU2ΔKIN$_{.myc}$ primary transformants were selected for hygromycin resistance and 24 independent lines were selected for further analysis. T_0 plants were grown to maturity and T_1 seeds were harvested and their phenotypes assessed by microscopy. Four independent lines (named $T_1$1, $T_1$4, $T_1$18 and $T_1$20) out of the 24 lines were selected for further analysis. 10 - 12 plants for each line were grown and the presence of the hygromycin resistance gene, and hence construct was determined by PCR (Fig. 3.3.6). The PCR program consisted of denaturation step at 94°C for 2 minutes and initial 25 cycles of 94°C for 30s, annealing at 58°C for 30s and

extension at 72°C for 30s followed by final elongation at 72°C for 5 minutes. The sequences of oligonucleotides used for the analysis (HPTIIFor + HPTIIRev) are available in Appendix1.

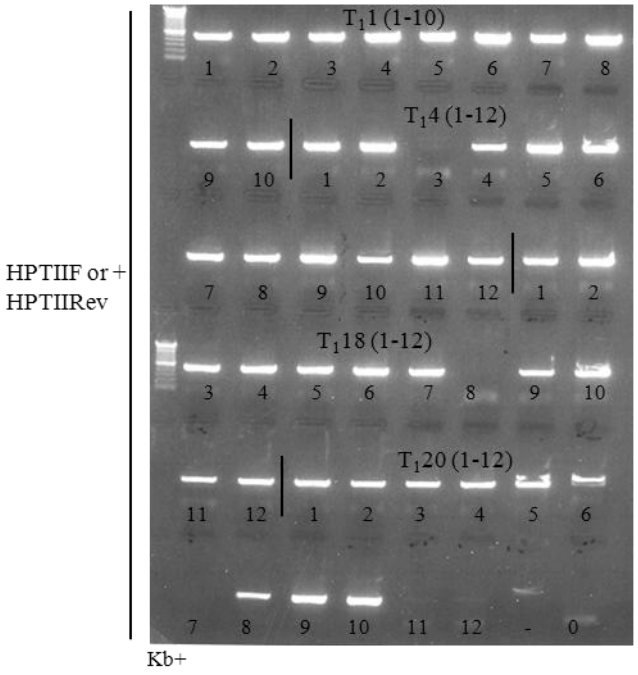

Figure 3.3. 6 **PCR genotyping of hygromycin resistance gene in transgenic pIKU2:IKU2ΔKIN.$_{myc}$ lines.**
$T_1$1, $T_1$4,$T_1$18 and $T_1$20 analysed for the presence of hygromycin resistance gene with HPTIIF and HPTIIRev primer pair. 0: negative contro**l**

T_2 plants were grown to reproductive maturity and the resulting selfed seeds seeds were collected for phenotypic analysis. Three lines were selected for further analysis ($T_2$18.1, $T_2$1.3 and $T_2$4.1) and $T_3$4.1 mature seeds were measured using Image J to allow further statistical analysis in combination with wild type Columbia, *iku2-4/iku2-4*, wild type Landsberg *erecta* and *iku2-3/iku2-3* genotypes as controls.

First, an ANOVA test was used to determine differences between the seed sizes from individual plants within a genotype.

Lines						
	Col-0		***iku2-4***		**L*er***	
Source of variation	d.f.	m.s.	d.f.	m.s.	d.f.	m.s.
Plant	2	0.0005039	2	0.0007301	2	0.0058929***
Residual	765	0.0004339	709	0.0005787	770	0.0004024
	iku2-3		**IKU2ΔKIN**			
Source of variation	d.f.	m.s.	d.f.	m.s.		
Plant	1	0.0000996	2	0.0096454***		
Residual	332	0.0003478	853	0.0003669		

Table 3.3. 1 **Mean squares from analysis of variance (ANOVA)**
Seed areas of individual plants derived from lines: wild type Columbia, *iku2-4/iku2-4*, wild type Landsberg *erecta*, *iku2-3/iku2-3*, IKU2ΔKIN were tested. D.f. – degree of freedom, s.d. – standard deviation. * significant at 0.05 level, ** significant at 0.01 level, *** significant at 0.001 level.

Within tested lines only seed sizes from individual plants derived from wild type Columbia, *iku2-4/iku2-4* and *iku2-3/iku2-3* genotypes did not differ significantly (P>0.05). However, individual plants within wild type Landsberg *erecta* and IKU2ΔKIN lines showed significant differences in seed size (P>0.05) (Table 3.3.1).

Subsequently, Tukey test was applied in order to estimate the differences between seed sizes from plants within each line.

Lines									
Plant no		**Col-0**			***iku2-4***			**L*er***	
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.
1	246	0.09292	0,02049	227	0.08749	0.02344	231	0.09218	0,02041
2	268	0.09147	0,02111	230	0.08505	0.02383	197	0.10169	0,01545
3	254	0.09428	0,02076	255	0.08409	0.0247	345	0.09318	0,02198
$LSD_{0.05}$		0.003600			0.004329			0.002998	
Plant no		***iku2-3***			**IKU2ΔKIN**				
	n	Mean (mm^2)	s.d.	n	Mean (mm^2)	s.d.			
1	155	0.0738	0.01982	270	0.09841	0.01448			
2	179	0.0727	0.01746	305	0.09408	0.01953			
3		-	-	280	0.08690	0.02247			
$LSD_{0.05}$		0.00403			0.003144				

Table 3.3. 2 **The differences between the means of the seed sizes from individual plants**

Tested lines: wild type Columbia, *iku2-4/iku2-4*, wild type Landsberg *erecta*, *iku2-3/ iku2-3*, IKU2ΔKIN., s.d. - standard deviation

The mean size of the smallest wild type Columbia seeds was $0.09147mm^2 \pm 0.02111$ and the biggest $0.09428mm^2 \pm 0.02076$. In *iku2-4/iku2-4* the smallest mean size was $0.08409mm^2 \pm 0.02470$ and the largest $0.08749mm^2 \pm 0.02344$. In Landsberg *erecta* the smallest mean size of the seeds was $0.09218mm^2 \pm 0.02041$ and the highest $0.10169mm^2 \pm 0.01545$. IKU2ΔKIN line produced seeds of the smallest mean of $0.08690mm^2 \pm 0.02247$ and the biggest $0.09841mm^2 \pm 0.01448$. The smallest mean of *iku2-3/iku2-3* seeds was $0.0727mm^2 \pm 0.01746$ and the highest $0.0727mm^2 \pm 0.01746$ (Table 3.3.2).

One-way ANOVA was carried out to determine the effects of each genotype on the variability of pooled seed areas. The least significant difference (LSD) of seed area was calculated, and on this basis homogeneous groups (not significantly different from each other) were determined.

Lines	n	Mean (mm^2)	Median
Col-0	768	$0{,}09288^{a} \pm 0.00075$	0.09331
iku2-4	712	$0.08554^{b} \pm 0.00090$	0.08144
IKU2ΔKIN	856	$0.09312^{d,a} \pm 0.00067$	0.09374
L*er*	774	$0.09505^{d} \pm 0.00073$	0.09885
iku2-3	334	$0.07321^{c} \pm 0.0010$	0.06850
$LSD_{0.05}$		0.001989	

Table 3.3. 3 **Analysis of seed size in wild type, mutant and transgenic plants**
Col-0, *iku2-4/iku2-4* , IKU2ΔKIN, L*er* and *iku2-3/iku2-3*. The means annotated with the same letter symbol are not significantly different from each other ($LSD_{0.05}$, P<0.001), ± SEM. n=number of seeds.

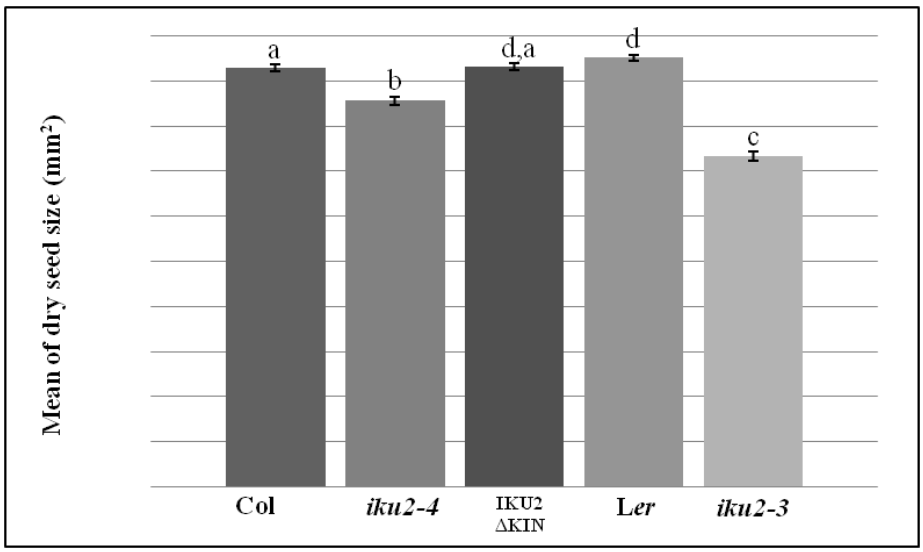

Figure 3.3. 7 **Mean dry seed size analysis**
Bars represent the means of Col-0, *iku2-4/iku2-4*, IKU2ΔKIN line in *iku2-4*, L*er* and *iku2-3/iku2-3*. The means annotated with the same letter symbol are not statistically different from each other. Error bars: SEM.

The results indicated that the most significant effects were observed in seed sizes (P<0.001). Pooled seeds derived from IKU2ΔKIN line ($0.09312mm^2 \pm 0.00067$) were not statistically different from wild type Columbia ($0{,}09288mm^2 \pm 0.00075$) and Landsberg *erecta* seeds ($0.09505mm^2 \pm 0.00073$). Interestingly, IKU2ΔKIN plants did

not exhibit an enhanced small dry seed phenotype when compared to *iku2-4/iku2-4* (0.08554mm^2 ±0.00090) but instead reverted the seed sizes to wild type (Fig.3.3.7).

The distribution of mature dry seed sizes in all of the analysed lines ranged between 0.04 and 0.14mm^2 except for *iku2-4/iku2-4* whose seeds ranged from 0.04, 0.07 to 0.13mm^2. IKU2ΔKIN produced seeds with the highest percentage (29.7%) of 0.1mm^2 seeds, Columbia wild type seed distribution showed the highest percentage (32.8%) of seed sizes that were 0.13mm^2 and *iku2-4/iku2-4* 0.1mm^2 (38.6%). Landsberg *erecta* produced seed range with the highest percentage of 0.11mm^2 seeds (24.4%) and as expected *iku2-3/iku2-3* were the smallest with 35% of seeds that were 0.07mm^2 (Fig. 3.3.8).

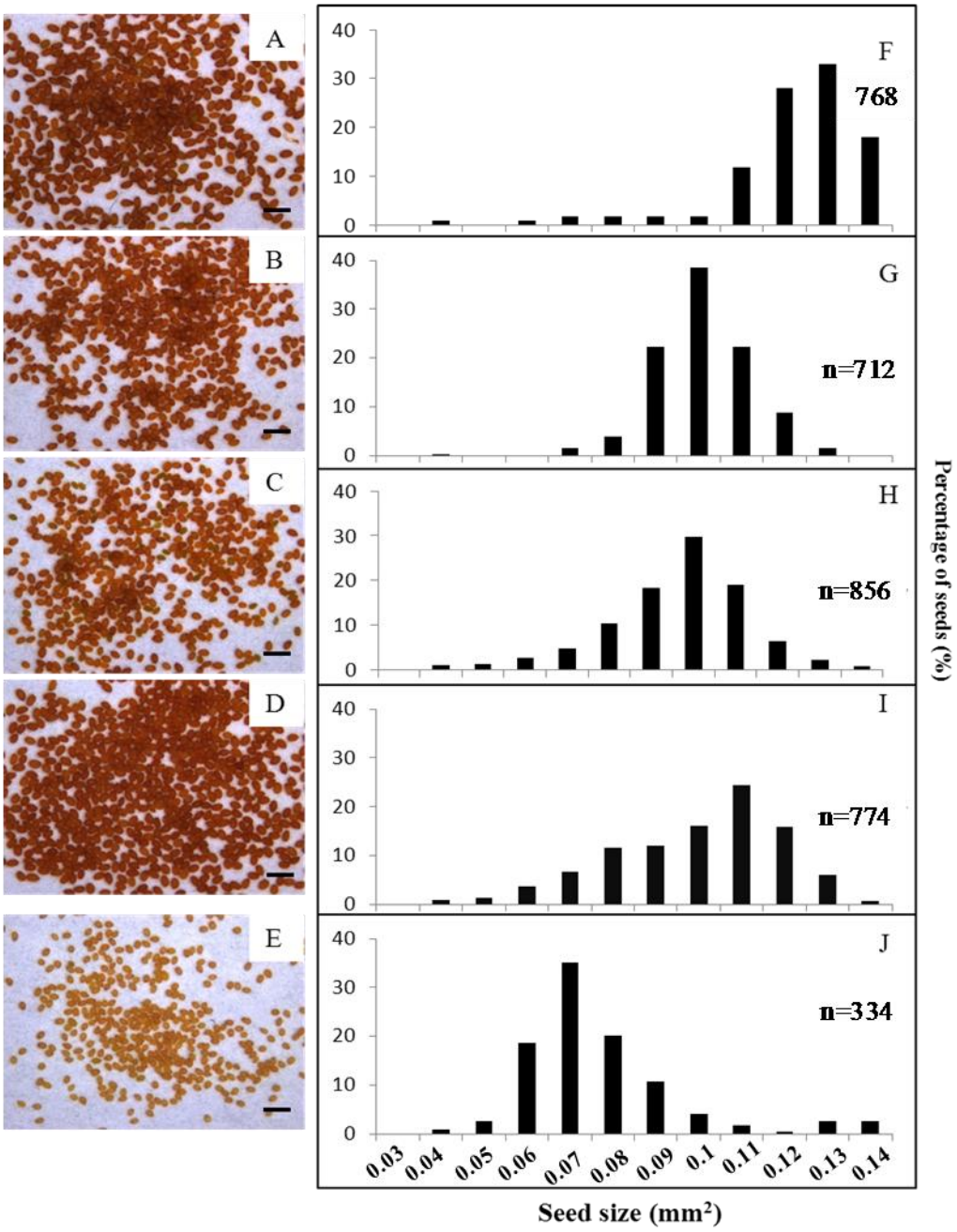

Figure 3.3. 8 **Seed size distribution from wild type and mutant plants**
Left panel: dry mature seeds from self-fertilised (A) wild-type Columbia, (B) *iku2-4/iku2-4* (C) IKU2ΔKIN. in *iku2-4/iku2-4*(D) wild type Landsberg *erecta* (E) *iku2-3/iku2-3*. Scale bars: 1mm
Right panel: the distribution of dry mature seed sizes from (F) wild type Columbia (G) *iku2-4/iku2-4* (H) IKU2ΔKIN. in *iku2-4/iku2-4* (I) wild type Landsberg *erecta* (J) *iku2-3/iku2-3*. X axis represents seed area in mm^2, Y axis the percentage of seeds. n= total number of seeds analysed.

These results confirmed the statistical analysis data, in mature seeds IKU2ΔKIN did not enhance but instead reverted the small seed phenotype observed in *iku2-4*.

In order to further assess the effect of these mutations on early seed development, laser scanning confocal microscopy was performed on seeds at the globular stage of embryo development (Fig. 3.3.9).

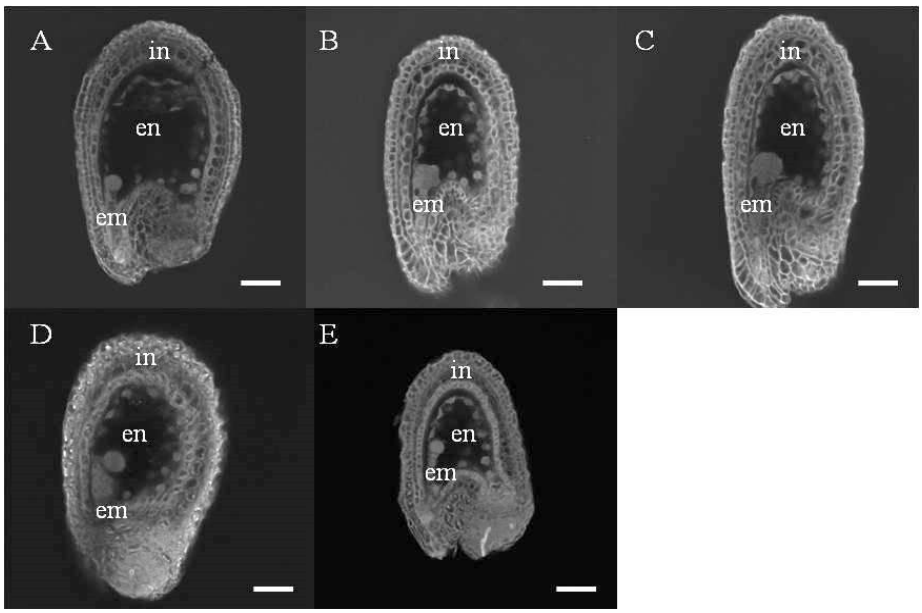

Figure 3.3. 9 **Confocal sections of early developing seeds**
Confocal sections showing the organisation of the seed at the globular embryo stage with the integuments encapsulating the endosperm and the embryo. (A) wild type Columbia-0 (B) *iku2-4/iku2-4* (Col-0) (C) IKU2ΔKIN. (D) wild type Landsberg *erecta* (E) *iku2-3/iku2-3* (L*er*). Scale bar: 50µm.

The parameters measured at this early stage of development included seed length, width and integument length and the statistical analysis of the obtained data. Interestingly, the mean seed lengths of the IKU2ΔKIN seeds (266.2µm±6.5) were significantly smaller than wild type Columbia, Landsberg *erecta* and mutant *iku2-4/iku2-4* seeds (304.9µm±8.9, 353.7µm±8.9 and 307.8µm ±8.2, respectively) but not significantly different from *iku2-3/iku2-3* (281.2µm ±6.9). The mean width of the seeds at the early developmental stages was significantly smaller (145.9µm±5.3) than all of the tested genotypes: wild type Columbia (177.3µm±5.3), *iku2-4/iku2-4* (167.7µm±3.4), L*er* (231.1µm±6.6), *iku2-3/iku2-3* (166.0µm±5.0). Interestingly, the width of *iku2-4/iku2-4* immature seeds was not significantly different from Columbia wild type. In terms of integument thickness, IKU2ΔKIN (43.39µm±0.9) integuments were significantly thinner than wild type Columbia (51.88µm±1.9), but did not differ statistically from *iku2-4/iku2-4*(44.49µm±1.5) and *iku2-3/iku2-3*(43.33µm±1.4) integuments (Fig. 3.3.12).

		Seed length		Seed width		Integument length	
Genotype	n	Mean (µm)	Median	Mean (µm)	Median	Mean (µm)	Median
Col-0	10	304.9^{b} ±8.9	309.5	177.3^{b} ±5.3	172.0	51.88^{b} ±1.9	52.00
iku2-3	10	281.2^{c} ±6.9	279.6	166.0^{b} ±5.0	160.2	43.33^{c} ±1.4	41.99
iku2-4	10	307.8^{b} ±8.2	317.0	167.7^{b} ±3.4	169.5	44.49^{c} ±1.5	43.88
IKU2ΔKIN	12	266.2^{c} ±6.5	262.8	145.9^{c} ±5.3	143.9	43.39^{c} ±0.9	42.98
L*er*	10	353.7^{a} ±8.9	344.8	231.1^{a} ±6.6	232.5	57.32^{a} ±1.8	58.24
$LSD_{0.05}$		20.70		14.15		3.92	

Table 3.3. 4 **Analysis of variance (ANOVA) seeds at the globular stage of development**.

Confocal scan microscopy images were used to determine the seed length, width and integument length of seeds at globular stage of development from different genotypes: wild type Columbia (n=10), *iku2-4 /iku2-4* (n=10), IKU2ΔKIN (n=12) Landsberg *erecta* (n=10), *iku2-3/iku2-3* (n=10). The means annotated with the same letter symbol are not significantly different from each other ($LSD_{0.05}$, $P<0.001$), ± SEM. n=number of seeds

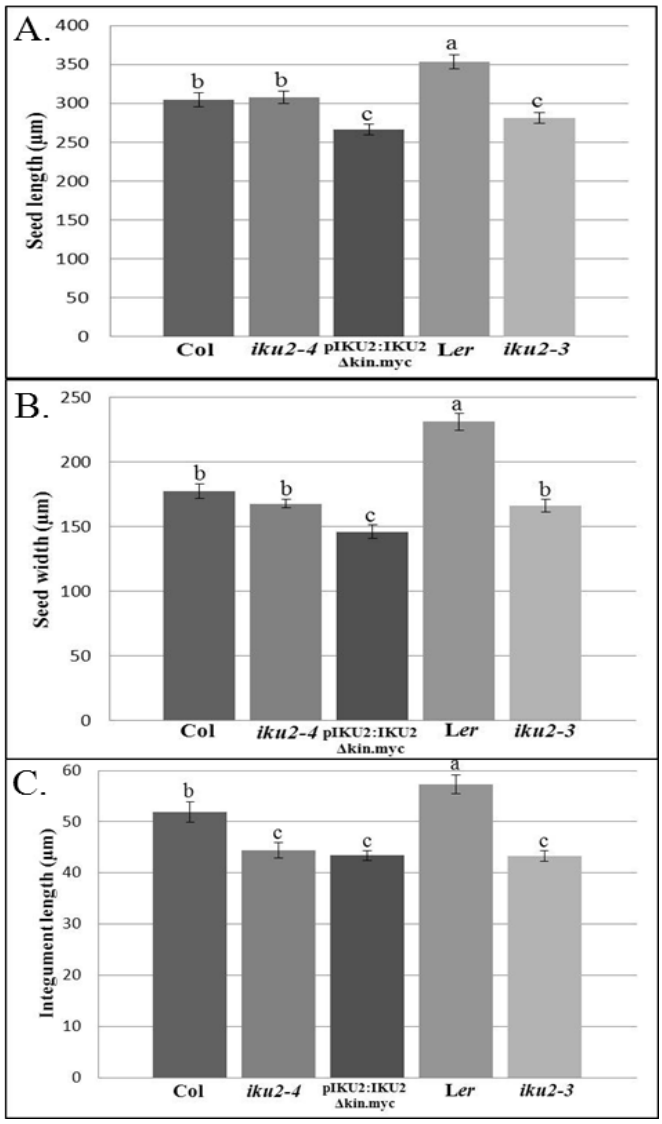

Figure 3.3. 10 **Mean size of seed measurements at the globular stage of embryo development**

Means of (A) seed length, (B) seed width and (C) integument length in µm in wild type Columbia (n=10), *iku2-4/iku2-4*(n=10), IKU2ΔKIN (n=12), Landsberg *erecta* (n=10), *iku2-3/iku2-3* (n=12) seeds. Genotypes annotated with the same letter symbol are not statistically different from each other. Error bars: SEM

Collectively, the results of these analyses indicate that the truncated IKU2 kinase (Col-0 background) has a great effect on early stages of seed development producing a severe seed phenotype, similar to *iku2-3/iku2-3* (L*er*). This observation indicates that the kinase domain is required for IKU2 in both Landsberg *erecta* and Columbia genetic backgrounds. Further, this confirms biochemical analysis showing that the R953K (*iku2-3*) produces inactive kinase which requires IKU2 function. However, unlike in the inactive kinase mutant (*iku2-3*), the mutant lacking the kinase domain did not have a visible phenotype in dry seeds.

3.3.5 Discussion

To better understand the mode of action of IKU2, a structural modelling analysis was performed on wild type IKU2 and R953K mutant kinase domains of IKU2-3. The protein model of the IKU2 kinase domain resembles other typical kinases and confirmed that the *iku2-3* mutation did not significantly affect conformation of the protein. Similar analysis has been conducted in RLK mutants, such as in *strubellig*, where some amino acid substitutions lead to other conformational changes in the structure of the kinase domain. Intriguingly, these s*trubellig* mutants were able to retain their function *in vivo,* and biochemical analysis confirmed that is not an active kinase (Vaddepalli et al. 2011). The *in vitro* autophosphorylation kinase experiment confirmed that *iku2-3* leads to kinase inactivation and thus reveals the importance of this residue in conveying phosphorylation to interacting partners.

The importance of a kinase activity in RLKs has been revealed in studies of other plant receptor proteins. In *CLAVATA1*, which is involved in SAM development and possibly in defence responses (Replogle et al. 2012), the kinase activity is important for function and also ligand binding (Trotochaud et al. 1999). Similarly,

mutation in the kinase domain of FLS inactivates the receptor that becomes flagellin (ligand) insensitive (Gomez-Gomez and Boller 2000). In addition, many alleles of BRI1 with decreased kinase activity show extreme dwarfism and male sterility (Friedrichsen et al. 2000, Vert et al. 2005, Noguchi et al. 1999). Early studies in BR1 cytoplasmic domain (CD) demonstrated that BRI1-CD is a Thr/Ser protein kinase (Oh et al. 2000, Wang et al. 2005a, Wang et al. 2005b). However, a recent study on the *bri1-301* mutant, which has an effect on *BRI1* kinase activity, indicates that the kinase activity is not essential in plant growth and development (Xu et al. 2008). In other RLKs such as SYMRK/NORK, (Stracke et al. 2002, Yoshida and Parniske 2005), SRK (Stahl et al. 1998), and ERECTA (Lease et al. 2001, Shpak et al. 2003) the kinase activity is strictly required for ligand binding and signal transduction.

Many of the characterised receptor like kinases show autophosphorylation in *in vitro* assays, among them ALE2 and ACR4 which play a role in organogenesis and epidermis-related tissues (Tanaka et al. 2007). SERK1-5 are also active kinases, though the activity ranges from high (SERK1, SERK2, SERK3) to low (SERK4, SERK5) (Karlova et al. 2009). In legumes, Nod factor receptors (NFRs) are required for nodulation and play a role in exchange of the signal between the legume host and the microbial microsymbiont. NFR1 and NFR5, show in vitro kinase activity (Broghammer et al. 2012, Madsen et al. 2011). In this chapter the importance of IKU2 kinase activity has also been confirmed by introducing the truncated kinase of IKU2 into null allele (*iku2-4*). Expression of truncated receptor kinases has been used as a powerful tool to reveal *in vivo* function and signal transduction of animal receptor kinases. Overexpression of truncated type II serine/threonine receptor kinases can compete with endogenous receptors for complex formation and therefore acts as a dominant-negative mutant (DNIIR). As the TGF receptors are responsible

for skeletal development, transgenic mice developed progressive skeletal degeneration and stiff and torqued joints (Serra et al. 1997). Receptor tyrosine kinase Flt-1 and Flk-1/KDR plays a pivotal role in angiogenesis in mouse embryo development. Flt-1 tyrosine kinase deficient homozygous mice developed normal vessels and survived which indicates that this receptor without the kinase domain is sufficient for normal embryonic development with normal angiogenesis (Hiratsuka et al. 1998). In Zebrafish, Eph-related tyrosine receptor kinases (RTK) act in establishing spatial patterning of the developing brain. Introduction of a truncated Rtk-1 receptors interferes with the endogenous Rtk-1 and leads to expansion of the eye field into diencephalic territory and loss of diencephalic structures (Xu et al. 1996). In frog, oocyte expression of truncated form of receptor *FIBROBLAST GROWTH FACTOR RECEPTOR* (*FGFR*) affects wild type receptor function (Amaya, Musci and Kirschner 1991). Mutant activin receptor inhibits signalling and affects induction of mesoderm *in vivo* and patterning of the embryonic body plan (Hemmati-Brivanlou and Melton 1992). In *Drosophila*, a truncated epidermal growth factor receptor (DER) was used to demonstrate its importance in differentiation of the non neuronal cone or pigment cells in the eye. *PLATELET-DERIVED GROWTH FACTOR* (*PDGF*) receptor lacking the cytoplasmic domain can inactivate the wild type function of the receptor by forming complexes that do not perform signal transduction (Ueno et al. 1991).

In this study, expression of IKU2 lacking the kinase domain enhanced the early small seed phenotype observed in *iku2-4* (Fig.3.3.10), thus suggesting that *IKU2* may act with other transmembrane proteins to regulate seed size. Interestingly, mature IKU2ΔKIN seeds were indistinguishable from wild type seeds. One possible explanation could be that IKU2ΔKIN defects at early stages of seed development

could be compensated by yet unknown factors at later stages of seed development since IKU2 is expressed only at the early micropylar endosperm (Luo et al. 2005). Alternatively, the deletion of kinase domain could result in a partially functional protein as it was observed in other RLKs (Clark, Williams and Meyerowitz 1997, Wang et al. 1998, Torii 2000). In future, the analysis of these IKU2ΔKIN lines in different genetic backgrounds (wt) could address this caveat. Although this approach was used to uncover the redundancy of the receptors like ERECTA (Shpak et al. 2003) often it causes cosuppression observed in SRK (Conner et al., 1997) and BRI1 (Schumacher and Chory 2000) studies.

Collectively these data indicate that the kinase domain of IKU2 plays a pivotal role in a yet unknown signalling pathway that regulate seed development and thus seed size.

Chapter 4

General discussion

Plant sexual reproduction in angiosperms requires the communication between sporophytic and gametophytic tissues and between the three components of the seed: embryo, endosperm and maternal integuments (Berger 2003). Studies in recent years have revealed that transmembrane receptors and signalling peptides play a pivotal role in different aspects of plant development (Torii 2012). The Arabidopsis genome consists of over 600 receptor like kinase genes (Shiu and Bleecker 2001), however only a few have been functionally characterised. Intriguingly, in rice almost one-third of the detected RLKs are expressed during seed development, highlighting the importance of RLKs in regulating different aspects of seed biology (Gao and Xue 2012). Three receptor like kinases, RPK1, TOAD2 and ACR4, are known to regulate embryo development in Arabidopsis (Nodine et al. 2011, Nodine et al. 2007). However, only one, IKU2, is known to specifically regulate endosperm development in plants (Luo et al. 2005, Garcia et al. 2005, Garcia et al. 2003)

4.1 *IKU2* regulates seed development in Arabidopsis

Some RLKs share high level of functional redundancy with their closest homologs (Shpak et al. 2004, Albrecht et al. 2005, Stenvik et al. 2008). This study has revealed that *IKU2* is redundant with its closest uncharacterised homolog *IKU2-LIKE*. The seeds from *iku2-4/ikl* double mutant plants enhance significantly the small seed phenotype observed in *iku2-4* (Fig.3.1.7), which indicates that the two receptors do not interact with each other biochemically in plasma membrane but regulate seed size via two separate pathways. In addition, this study also found that *ems1*, a RLK known to regulate anther and seed development (Canales et al. 2002),

has an additive effect when combined with *iku2-4*, therefore the two RLKs also regulate seed size via two signalling pathways (Fig.3.1.16).

Detailed analysis of the different *IKU2* mutant alleles isolated in two genetic backgrounds (Columbia and Landsberg *erecta*) suggested that *IKU2* could interact with other genetic factors present in the Lansberg *erecta* background. Because this ecotype is defective in *ERECTA* function, an RLK that regulates many aspects of plant development through the interaction with other RLKs such as *TMM* in stomata development (Shpak et al. 2005), *CLV2* and *CRN* in meristem development (Durbak and Tax 2011), it was hypothesised that *IKU2* could act together with *ERECTA* to regulate seed development. Temporal and spatial expression of these genes in developing seeds indicated that *IKU2* transcripts accumulate exclusively in the micropylar endosperm while *ERECTA* transcripts accumulate primarily in embryo proper. Genetic analysis confirmed that *IKU2* and *ERECTA* regulate seed size via two separate pathways, most probably operating in the endosperm and embryo of the seed (Fig.3.2.4), which subsequently affects integument development and reveals the previously unrecognised role of *ERECTA* in seed development. This finding supports previous studies emphasising the importance of cross talk between the three distinct tissues of the seed (Nowack et al. 2006, Garcia et al. 2005).

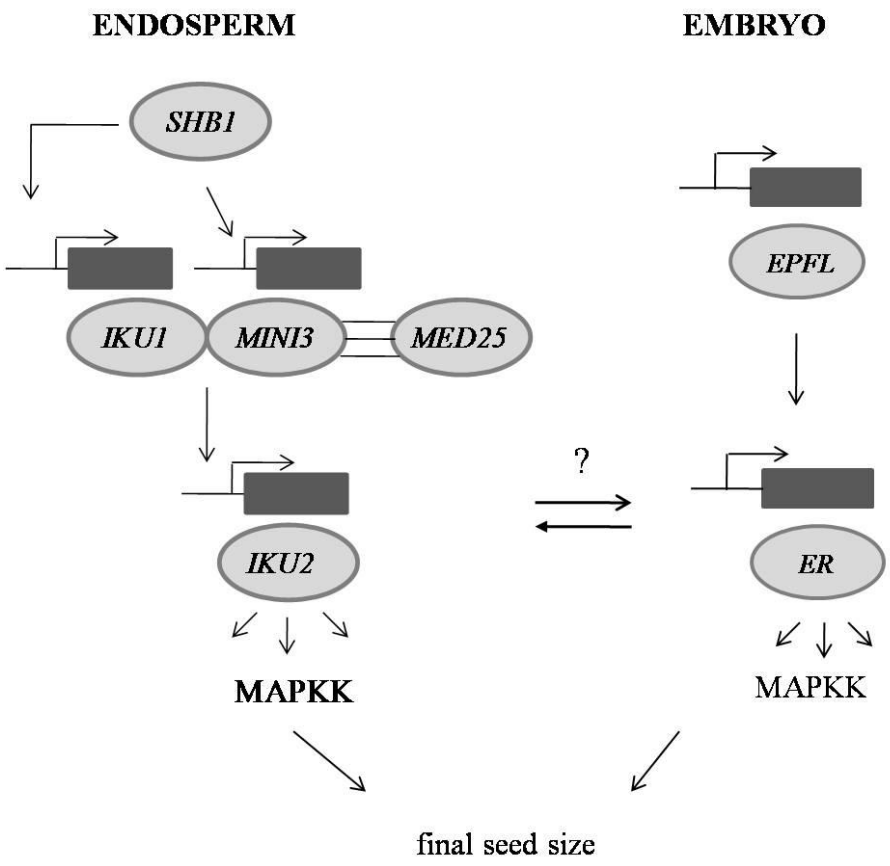

Figure 4. 1 **Diagram of *IKU2* and *ERECTA* signalling pathways in final seed size**

IKU2 and *ERECTA* signalling pathways operating in endosperm and embryo regulate final seed size in Arabidopsis. *SHB1* binds to promoter of *IKU1* and *MINI3* to regulate their transcriptional activity. *IKU1 and MINI3* physically interact to bind IKU2 promoter to regulate its transcriptional activity. MED25 acts as a mediator protein of *MINI3* transcription factor. *IKU2* could activate a *MAPKK* signalling pathway. *EPFLs* secreted peptides activate *ERECTA* and could also activate MAPKK signal transduction. Grey ovals represent the genes, grey boxes coding sequences, lines promoters of the genes. Arrows indicate the pattern of regulation of gene expression in *IKU* and *ERECTA* signalling pathways.

Signal transduction via transmembrane RLKs involves the interaction with hormones or secreted peptide ligands. However, only a few peptide-receptor pairs have been identified to date in plants. For example, in *Arabidopsis* EPFL4 and EPFL6 peptides biochemically interact with ERECTA in vascular tissues to regulate

inflorescence development (Uchida et al. 2012a), in the leaf epidermis EPF2 and EPF1 peptides interact with ERECTA and ERL1 respectively to regulate stomata development (Lee et al. 2012b). Although the interacting ligand of IKU2 remains unknown, the recent identification of a seed specific peptide (CLE8) with a specific role in embryo and endosperm development (Fiume and Fletcher 2012), constitutes a possible candidate.

4.2 IKU2 is an active receptor kinase

In silico structural analysis of R953K IKU2 mutation (*iku2-3*) showed no alteration in conformation of the cytoplasmic kinase domain when compared to wild type (Fig.3.3.2). However, *in vitro* phosphorylation assays revealed that the R953K mutation leads to an inactive form of the IKU2 kinase (Fig.3.3.5). This finding, together with the extreme small seed phenotype observed in R953K mutant *IKU2* and in early seeds carrying a truncated IKU2 lacking the kinase domain (3.3.10), indicate that IKU2 is part of an uncharacterised signalling pathway required for seed development. The importance of the cytoplasmic kinase domain has been also revealed for other RLKs (Clark et al. 1997, Chevalier et al. 2005, Vaddepalli et al. 2011, Wang et al. 1998, Gomez-Gomez and Boller 2000, Friedrichsen et al. 2000). Receptors lacking the intracellular kinase domain often interfere with the function of interacting receptors leading to dominant negative effects (Shpak et al. 2003). For example, CLV2 which lacks a kinase domain forms a dimer with CORYNE to transmit signals that regulate the development of the shoot apical meristem (Muller et al. 2008). Similarly, BAK1 interacts with the receptor BRI1 to mediate brassinosteroid-dependant signalling (Chinchilla et al. 2009, Oh et al. 2010, Kinoshita et al. 2005). Most receptor-like kinases phoshorylate mitogen-activated

protein kinase (MAPK) (Madhani and Fink 1998), a widely-conserved signalling pathway that contributes to the regulation of cell proliferation and differentiation in plants (Bergmann, Lukowitz and Somerville 2004).

The downstream factors regulated by the *IKU2* signalling cascade remain to be characterised. However, molecular analyses of other RLK signalling pathways in plants have revealed that only a small number of transcription factors are directly regulated (Betsuyaku et al. 2011, Wang et al. 2007, Gudesblat et al. 2012, Bergmann et al. 2004). Future experiments should be directed to the identification of the interacting receptor partner of IKU2 as well as downstream targets of the IKU2 signalling pathway.

4.3 Final summary and future perspectives

Collectively, the data generated in this study has revealed that *IKU2* orchestrates proper seed development with other RLKs (*IKL, EMS1, ERECTA*) via separately acting signalling pathways which implies great complexity of communication between the embryo, endosperm and maternal integuments during seed development. Interestingly, *IKU2* and *ERECTA* are required in endosperm and embryo, respectively, to regulate seed size in *Arabidopsis thaliana*. IKU2 is an active LRR receptor-like kinase and loss of kinase activity leads to severe seed phenotypic defects. The next stage of this study would be to identify the potential interacting receptor partners of IKU2 as well as its ligands and the direct targets regulated by the *IKU2* signalling pathway. Furthermore, this study reveals the importance of RLKs in regulating seed size which could be valuable in the future for the genetic improvement of seeds in crop species.

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Appendix 1

Table A.1. 1 List of oligonucleotide sequences

SERK1.FOR	**cgtgacaacagcagtccgtggcaccatcgg**
SERK1.REV	ccctttaatcgaaccatagcac
SERK2.FOR	cggctagtaactgggccgcatagatcc
SERK2.REV	gatctgggaggagaaatttgc
ATIKU2G.FOR	acgtacgtgt tggtggtgaa gcttaggag
ATIKU2G.REV	gatcatgcatgtcctatcgttgcgatg
ATIKU2-LIKE.G.F	agagttgaacttatttacaactcaatgtc
ATIKU2-LIKE.G.R	attggtctatgtcatcacaccaagaatcc
EMS1FLAG2LP	gctttcccttggttgatcttc
EMS1FLAG2RP	gatcagattgcaagatcgagg
ERG2248	aagaagtcatctaaagatgtga
ER-105	agctgactatacccgatactga
ERG3016RC	agaattttcaggtttggaatctgt
AT IKU2-1 LER FOR	ctactatttatcgttcgccttctc
ATIKU2-1 LER. REV	cttccaaggttgatctcgacgac
ATIKU2LIKE.G.F1	gtgatgcctcatcaacagctcaattaa
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SALK_094492R	agaattcgttttcgaacatttgtag
SALK_120595F	gaaaactatacacaacacgaacacg
SALK_120595R	gcagttagagctttacaacaactcc
LB1* SAIL	gccttttcagaaatggataaatagccttg
IKU2 073260 LP	tctttaagaaccgcagctctg
IKU2 073260 RP	tatgctgatgcaaacgtgttg
ATIKU2RT.FOR	atgctccggctactatttatcgtt
ATIKU2RT.REV	gtagtgcaatctatgagttatacaac
LBA1SALK	tggttcacgtagtgggccatcg
CLE8 FOR	atgaaagtgttgaagagagattc
CLE8 REV	tgatctagaacaatggttgatg
IKU2LIKE FOR2	gttactgatcttgctagcgtctc
IKU2LIKEREV3	tgacttggtccctcaatcatcaac
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IKU2.STOP.GW.R	gg accactttgtacaagaaagctgggtttatacaactttagtaatctcatc

IKU2RTFOR1	catagtcatgtgggtttggagcgt
IKU2FOR2	gtcaaagga actcttggtt acattg
IKU2.RT.REV1	cgtaacttgcttctccactgttc
IKU2-3.FOR	act accaaagtga acgagaagag cgac
IKU2-3.REV	gttataggaaggctctatttttcaagcatac
IKU2.NOKIN.MYC	ttacaaatcctcctcagagataagtttctgctcaagatcacgtaactgaataag
73260LP	tctttaagaaccgcagctctg
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ATIKU2LPREV.GW	gg accactttgtacaagaaagctgggtggagagaagaagtggtgacg
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IKU2KIN.MUT.R	ctctccggacaccagatatgtctaacggctagtgtttcaccgct
IKU2.KIN.FOR (XHOI)	ggctcgagagataagacgagataagttgaataaaacg
IKU2.KIN REV (NOTI)	ccgcggccgcttatacaactttagtaatctcatcattagc
ERG2248	aag aag tca tct aaa gat gtg a
ER-105	agctgactatacccgatactga
ERG3016RC	agaattttcaggtttggaatctgt
IKU2_KIN.FOR	cttattcagttacgtgatctt
IKU2.EXI.REV	ctttggtagtatatgcatattcggggcaatgtaaccaagag
IKU2.EXII.FOR	ctcttggttacattgcccccgaatatgcatatactaccaaagtgaacg
IKU2_KIN.REV	ctcttgaatggtcttagaatc
LBA1SALK	tggttcacgtagtgggccatcg
CLE8P.F1	agaactgacaattacagtgaaag
CLE8P.F1.GW	ggacaagtttgtacaaaaaagcaggctgactgttaatgtcactttctaagc
CLE8P.REV	catcgaatctctcttcaacactt
CLE8P.REVGW	ggaccactttgtacaagaaagctgggttttacttttagtttctcatgtacttc
NPTII.FOR20	cagtgacaacgtcgagcacagctg
NOSP.REV	acaagccgttttacgtttggaactg
IKU2LIKEPFOR1	acgttacttaataacaattgtaaac
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CLE8P.F1.GWNEW	ggacaagtttgtacaaaaaagcaggctgacaattacagtgaaagattc
IKU2PKPNIASCI.FOR	ggcgcgccggtaccgcttaggagttaatttgagt
IKU2PBAMHI.ASCI.REV	ggcgcgccggatccgttctctacgtcggaaggat

IKU2.KIN.FOR1 (XHOI)	ggctcgagaagataagacgagataagttgaataaaacg
ER.ATG.FOR	atggctctgtttagagatattgttc
ER.ATG.FOR.GW	ggacaagtttgtacaaaaaagcaggctgctctgtttagagatattgttcttc
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Appendix 2

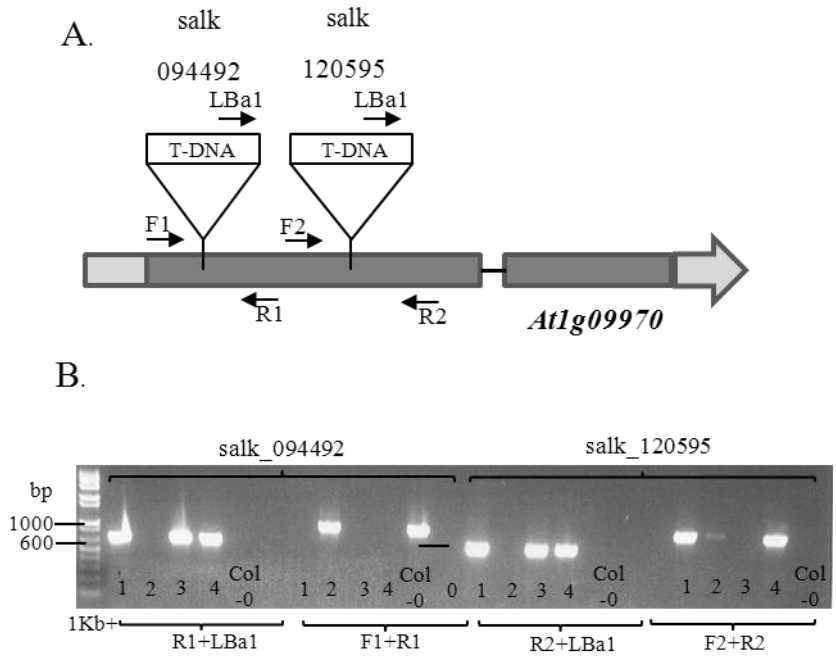
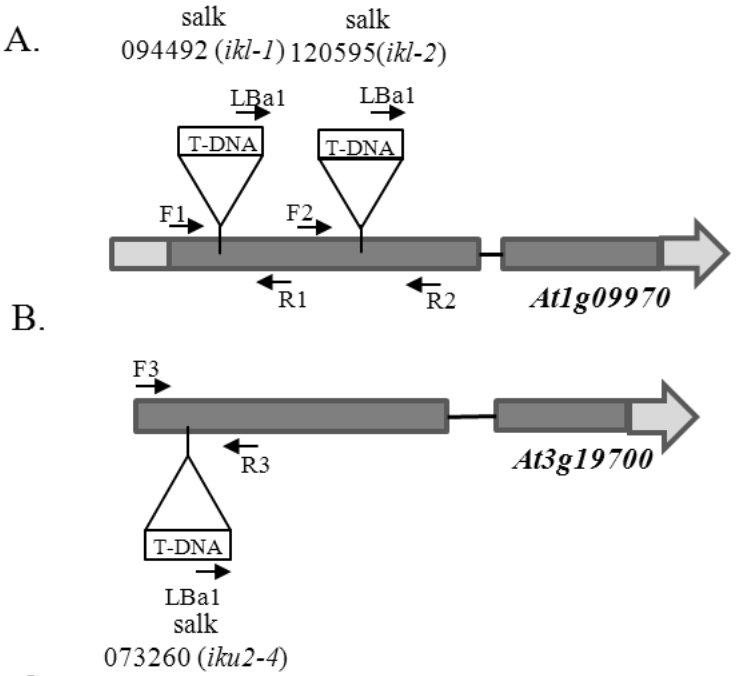
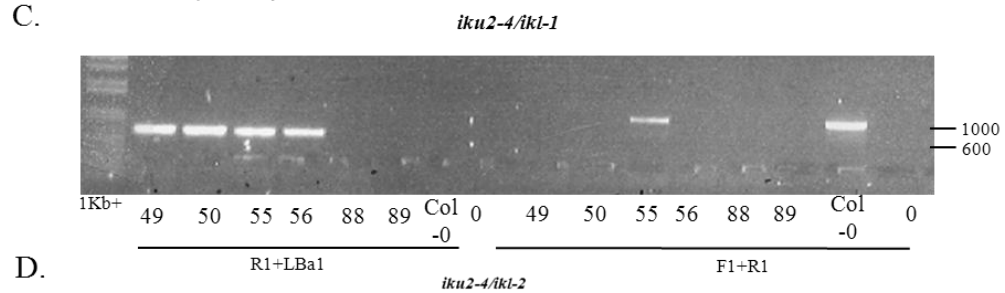
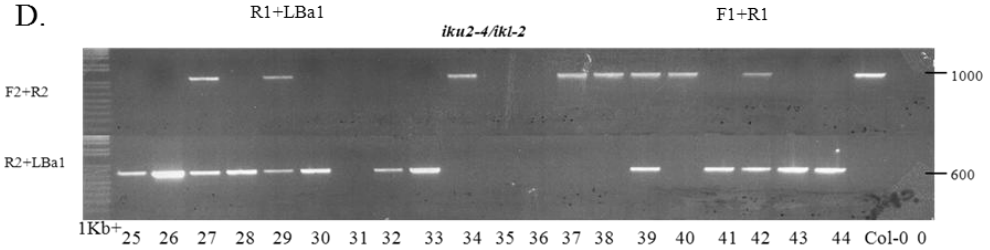


Figure A.2. 1 **PCR genotyping of T-DNA insertions in *iku2/ikl* double mutants**
Schematic representation of T-DNA insertions in *At1g09970* (*IKL*) (A) and *At3g19700* (*IKU2*) (B) genes. Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines and positions for oligonucleotide primers are indicated by arrows. Insertion of T-DNA represented by triangles. (C) In double homozygous (ho) mutant *iku2-4*/*ikl-1* (salk_73260 and salk_094492 respectively) gene specific R1 in combination with left border primer (LBa1) amplify products of 600bp. In wild type (wt) for the *ikl-1* allele a 1000bp PCR product is detected by a gene – specific primer pair (F1+R1), whereas the same product is absent in double homozygous *iku2-4*/*ikl-1*(D)In double homozygous (ho) *iku2-4*/*ikl-2* mutants (salk_07260 and salk_120959 respectively) insertion of T-DNA was confirmed with gene specific R2 in combination with the LBa1 primers that amplify a product of 600bp and the presence of the wild type allele (no T-DNA insertion) with F2 and R2 primers that amplify products of 1000bp. The same product is absent in homozygous (ho) *ikl-2*. E. Insertion of T-DNA in *At3g19700* (*IKU2*) gene in both of the double mutants was confirmed with R3 and Lba1 primer pair (650bp) and the wild type allele of the gene with F3+R3 (1000bp).


A.
salk
094492 (*ikl-1*)
salk
120595(*ikl-2*)
LBa1
LBa1
T-DNA
T-DNA
F1
F2
R1
R2
At1g09970
B.
F3
R3
At3g19700
T-DNA
LBa1
salk
073260 (*iku2-4*)


C.
iku2-4/ikl-1
1000
600
1Kb+
49 50 55 56 88 89 Col -0 0 49 50 55 56 88 89 Col -0 0
R1+LBa1
F1+R1


D.
iku2-4/ikl-2
F2+R2
R2+LBa1
1000
600
1Kb+
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 Col-0 0

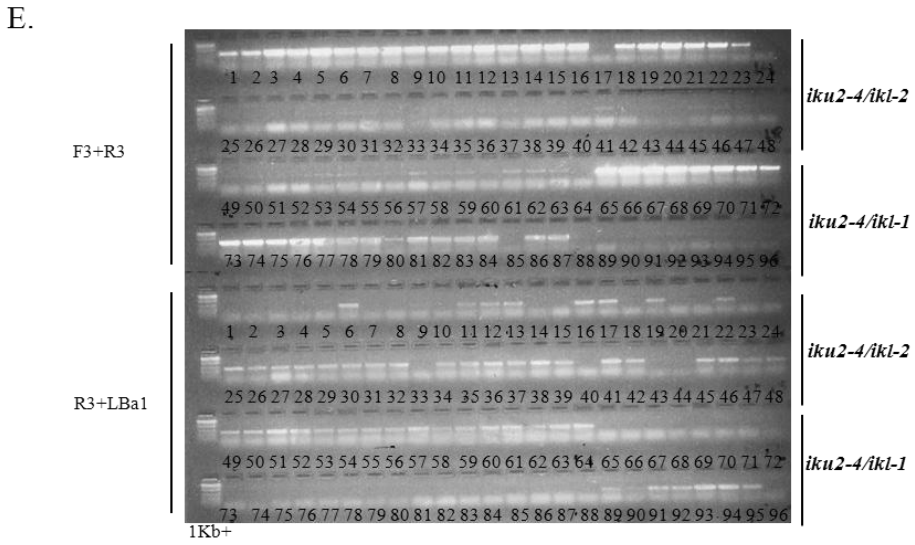

E.
F3+R3
R3+LBa1
iku2-4/ikl-2
iku2-4/ikl-1
iku2-4/ikl-2
iku2-4/ikl-1
1Kb+

Figure A.2. 2 **PCR genotyping of T-DNA insertions in *iku2/ikl* double mutants**
Schematic representation of T-DNA insertions in *At1g09970* (*IKL*) (A) and *At3g19700* (*IKU2*) (B) genes. Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines and positions for oligonucleotide primers are indicated by arrows. Insertion of T-DNA represented by triangles. (C) In double homozygous (ho) mutant *iku2-4*/*ikl-1* (salk_73260 and salk_094492 respectively) gene specific R1 in combination with left border primer (LBa1) amplify products of 600bp. In wild type (wt) for the *ikl-1* allele a 1000bp PCR product is detected by a gene – specific primer pair (F1+R1), whereas the same product is absent in double homozygous *iku2-4*/*ikl-1*(D)In double homozygous (ho) *iku2-4*/*ikl-2* mutants (salk_07260 and salk_120959 respectively) insertion of T-DNA was confirmed with gene specific R2 in combination with the LBa1 primers that amplify a product of 600bp and the presence of the wild type allele (no T-DNA insertion) with F2 and R2 primers that amplify products of 1000bp. The same product is absent in homozygous (ho) *ikl-2*. E. Insertion of T-DNA in *At3g19700* (*IKU2*) gene in both of the double mutants was confirmed with R3 and Lba1 primer pair (650bp) and the wild type allele of the gene with F3+R3 (1000bp).

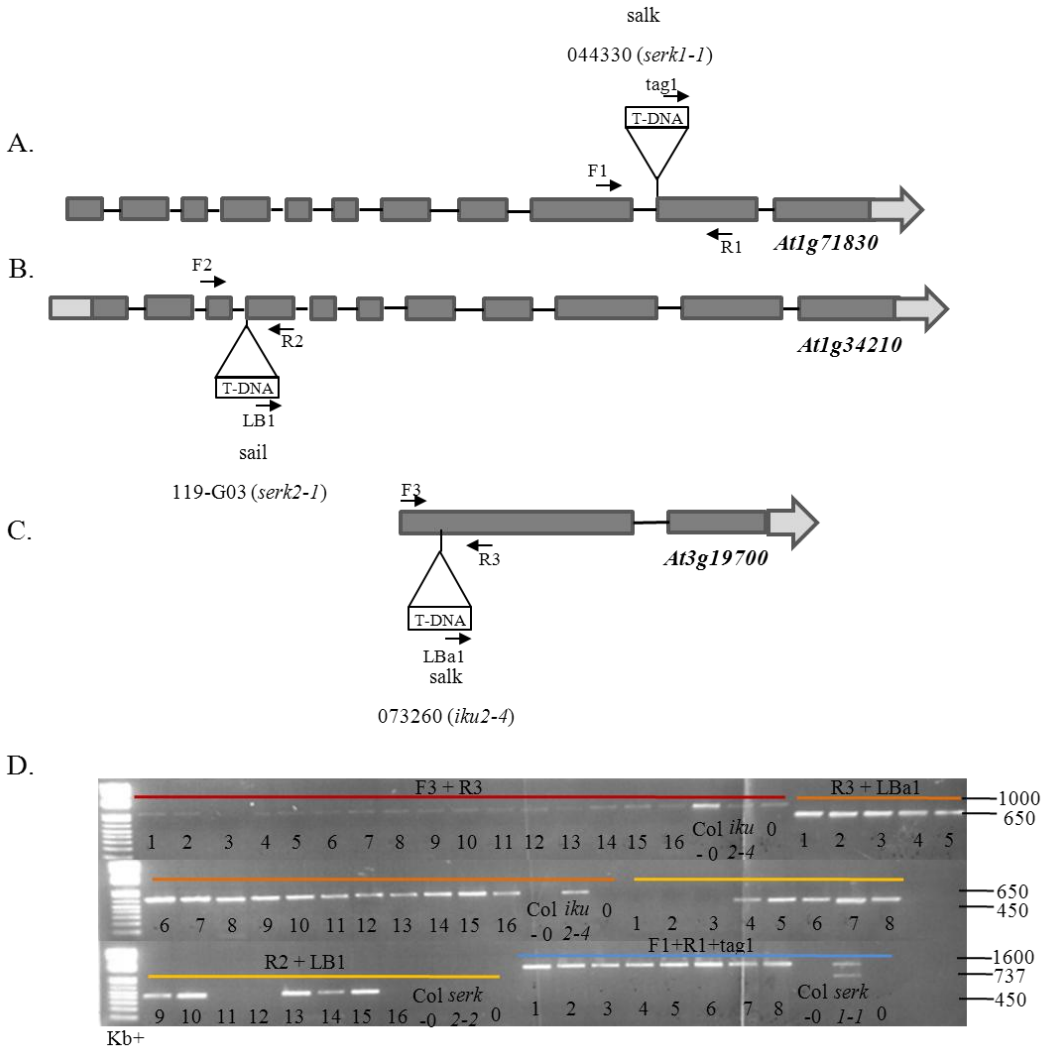


Figure A.2. 3 **Representation of PCR genotyping of T-DNA insertions in triple *serk1/serk2/iku2* mutants**

(A) Schematic representation of T-DNA insertion in *At1g71830 (SERK1)*. T-DNA inserted in exon 10. (B) T-DNA insertion in exon 4 of *At1g34210* (*SERK2*). (C) T-DNA insertion in exon 2 of *At3g19700* (*IKU2*). Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines, and positions for oligonucleotide primers are indicated by arrows. (D) In triple *serk1-1/serk1-1;serk2-2/*SERK2*;iku2-4/iku2-4* mutants (salk_044330, sail_119-G03 and salk_07260 T-DNA insertions respectively) the genetic constitution of *serk1-1* insertion was confirmed with combination of three: F1 + R1 + T-DNA left border (tag1) primers in one PCR reaction in plants 1-8, resulting in 1600bp products for the insertion of T-DNA and 737bp products for wild type allele of *SERK1* that were not present in *serk1-1/serk1-1*. *serk2-2* T-DNA insertion was confirmed with gene specific R2 in combination with the LB1 primers that amplify a product of 450bp and the presence of the wild type *SERK2* allele, although not genotyped must be present since the phenotype of the plant was not male sterile. Insertion of T-DNA in *At3g19700* (*IKU2*) gene in the triple mutants was confirmed with R3 and Lba1 primer pair (650bp) and the presence/loss of wild type allele of the gene with F3+R3 (1000bp).

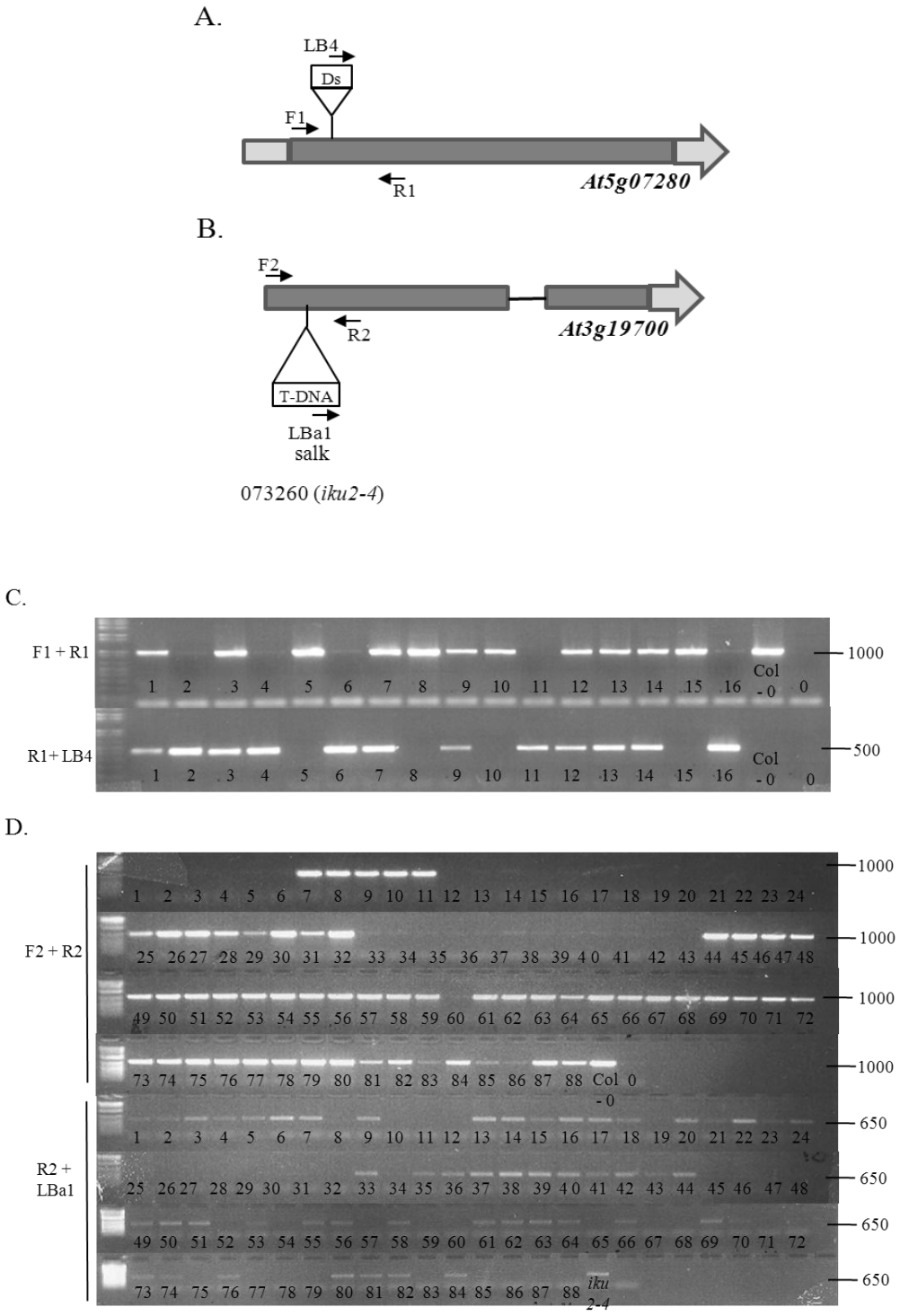


Figure A.2. 4 **PCR genotyping of double *ems1/iku2-4* mutants**

(A) Schematic representation of Ds element insertion site in exon 1 of *At5g07280*. (B) Insertion site of T-DNA in exon 1 of *At3g19700*. Exons are depicted as solid grey bars, UTRs: light grey bars, intron regions as solid lines, and positions for oligonucleotide primers are indicated by arrows. (C)In double *ems1/ems1;iku2-4/iku2-4* mutants the genetic constitution of *ems1* insertion was confirmed with combination of R1 + T-DNA left border (LB4) primers resulting in 500bp products. Wild type allele of *EMS1* was detected with F1+R1 primers producing 1000bp products that were not present in *ems1/ems1*. (D) Insertion of T-DNA in *At3g19700* (*IKU2*) gene in the double mutants was confirmed with R3 and Lba1 primer pair (650bp) and the presence/loss of wild type allele of the gene with F3+R3 (1000bp).