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Abstract:

The main objectives of the bachelor thesis Transcriptional control of pluripotency in Arabidopsis were to elucidate the function of *ETHYLENE RESPONSIVE FACTORs* transcription factors and their role in the modulation of pluripotency in the plant. Pluripotency is a privileged ability of plants and is essential for their survival. Pluripotent cells are found in specialized tissues called meristems, and their control is, therefore, the main site of pluripotency regulation - and thus of regeneration and organogenesis.

The response to plant hormones is an intensively studied topic and is far from being elucidated. The role of *ETHYLENE RESPONSIVE FACTORs* is another part of the complex regulatory pathways in response to cytokinins, auxins and ethylene. This work focuses on the family of transcription factor VIII containing *ETHYLENE RESPONSIVE FACTORs* and *ENHANCER OF SHOOT REGENERATION*.

To elucidate the role of this family of repressors, phenotypic analyses of intact plants and in vitro explants as well as histological analysis of expression in transgenic plants were performed.

ETHYLENE RESPONSIVE FACTORs of family VIII were found to have a significant effect on the ability to regenerate the aerial part of the plant, which they affect negatively, whereas they positively influence the developmental processes of these tissues. In histological analysis, the expression of transcriptional repressors was shown to be dependent on the age of the tissue and to be organ-specific.

These findings point to other potential areas for the study of pluripotency in plants. The role of *ETHYLENE RESPONSIVE FACTORs* may be an important part of our knowledge of plant life, development, and ability to adapt to external conditions.

Keywords: pluripotency, meristems, ERF, transcription factors, phytohormones, organogenesis, regeneration

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Abstrakt:

Hlavními cíli bakalářské práce Transkripční kontrola pluripotence u *Arabidopsis* bylo objasnění funkce *ETHYLENE RESPONSIVE FACTORs* transkripčních faktorů a jejich role při modulaci pluripotence v rostlině. Pluripotence je výsadní schopností rostlin a je nezbytná pro jejich přežití. Pluripotentní buňky se nachází ve specializovaných tkáních zvaných meristémy, jejichž kontrola je tedy i hlavním místem regulace pluripotence – a tedy i regenerace a organogeneze.

Odpověď na rostlinné hormony je intenzivně studované téma a není zdaleka objasněné. Role *ETHYLENE RESPONSIVE FACTORs* je další součástí složitých regulačních drah v odpovědi na cytokininy, auxiny a ethylen. Tato práce je zaměřena na rodinu transkripčních faktorů VIII obsahující *ETHYLENE RESPONSIVE FACTORs* a *ENHANCER OF SHOOT REGENERATION.*

Pro objasnění role této skupiny represorů byly provedeny analýzy fenotypu intaktních rostlin a explantátů *in vitro* a dále histologická analýza exprese v transgenních rostlinách.

Bylo zjištěno, že *ETHYLENE RESPONSIVE FACTORs* rodiny VIII mají významný vliv na schopnost regenerace nadzemní části rostliny, kterou ovlivňují negativně, zatímco positivně ovlivňují vývojové procesy těchto pletiv. V histologické analýze bylo prokázáno, že exprese transkripčních represorů je závislá na stáří pletiva a je orgánově specifická.

Tato zjištění poukazují na další možné oblasti pro studium pluripotence v rostlinách. Role *ETHYLENE RESPONSIVE FACTORs* může být významnou součástí znalostí o životě rostlin, jejich vývoje a schopnosti přizpůsobení se vnějším podmínkám.

Klíčová slova: pluripotence, meristém, transkripční faktory, ERF, fytohormony, organogeneze, regenerace

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I declare here that this Bachelor's thesis „Transcriptional Control of Pluripotency“ was written solely by me. All sources and literature are listed at the end of the thesis in chapter “References”.

6th May, 2022 …………………….

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List of symbols and abbreviations

ACC	1-aminocyclopropan-1-carboxy acid
AP2/ERF	APETALA2/Ethylene-responsive factor
ARF	Auxin response factor
AUX1	Auxin resistant 1
AuxRE	Auxin response element
BTP/POZ	BRIC-A-BRAC – TRAMTRACK – BROADCOMPLEX/POX VIRUS and ZINC FINGER domain
CIM	Callus inducing medium
Col	*Arabidopsis thaliana* Columbia ecotype
CUL	CULLIN
dm	Double mutant
GUS	β-glucuronidase reporter gene system
OC	Organizing centra
DREB	Dehydration-Responsive Element Binding-proteins
ESR	*Enhancer of shoot regeneration*
IAA	Indole-3-acetic acid
Laer	*Arabidopsis thaliana* Landsberg erecta ecotype
MATH	MEPRIN and TRAF (TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTOR) HOMOLOGY (MATH) domain
MS	Murashige Skoog medium
PIN	Pin-shaped inflorescence
RAM	Root apical meristem
SAM	Shoot apical meristem
SCF	SKP1, Cullin/CDC53, F-box protein
SIM	Shoot inducing medium
Ws	*Arabidopsis thaliana* Wassilewskija ecotype
-/-	homozygous
+/-	heterozygous

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1 INTRODUCTION

As the need to increase food production grows, higher demands are placed on agriculture to obtain stronger, more pleasant and more productive varieties of crops. The structure of the system enables people to participate in the solution of these matters at different levels. Recently very progressing level is genetics, where scientists obtain basic information about plants as well as develop new methods to improve agricultural issues.

Therefore, I have decided to collect more information on the genetic background and control of plant regeneration. A related topic that is often discussed is pluripotency, the ability of cells to differentiate in any cell type possible in a given plant. Understanding the process may explain many questions about organogenesis, growth, and regeneration, which leads to the possibility of using knowledge gained in future research or practice. For my experiments and research, I have used *Arabidopsis thaliana*, the model organism used by many laboratories worldwide.

2 AIMS OF WORK

1) Obtain background knowledge from literature
    a. Elucidate the role of ERF (ethylene-responsive factor) on pluripotency modulation
    b. Study of ESR (enhancer of shoot regeneration) 1 and 2
2) Acquire skills for conducting research
    a. In vitro tissue culture for assessing shoot regeneration efficiency
    b. Phenotypic analyses of loss-of-function higher-order mutants defective in the transcriptional repressor
3) Data interpretation

3 LITERATURE REVIEW

3.1 *Arabidopsis thaliana*

Wall cress, or mouse-ear cress, is a common dicotyledonous weed from of *Brassicaceae* family with a ground leaf rosette and a 5 – 30 cm high stem (Heynh, 1992). The *Brassicaceae* family is widely used in agriculture, and many of its members play an irreplaceable role in the human diet.

Arabidopsis thaliana itself is not agriculturally significant, yet it has proved its importance in science. There are major characteristics that make *Arabidopsis* a very good model organism for molecular and genetic research. A small genome (114.5 Mb/125 Mb total) folded into 5 chromosomes provides easier orientation. The whole genome was sequenced in 2000 and the genetic and physical maps of the chromosomes were created. Another important feature is a quick life cycle during which is the plant able to produce seeds that can be easily harvested and cultivated on a medium (The Arabidopsis Information Resource, undated).

With the development of molecular biology and genetics, many methods for different experiments were required. Today, using the *Agrobacterium tumefaciens*, gene gun method or CRISPR CAS 9 enables us to create many mutant lines and transgenic plants to study pant metabolism, gene interactions etc. (The Arabidopsis Information Resource, undated).

3.1.1 Morphology

There are approximately 750 natural ecotypes of *Arabidopsis thaliana*, but three are the most popular in many studies. These are the accessions Columbia (Col), Landsberg erecta (Laer) and Wassilewskija (Ws). Although these three are often used as controls in genetic experiments or as background for the insertion of T-DNA, there are genetic differences between these ecotypes. (Passardi et al., 2007). In this study, we used Colombia, so the following text will focus on this specific line.

When compared to Laer and Ws ecotypes, Columbia has bigger and rounder seeds. They also differ in colour, caused by the different amounts of anthocyanins in the seed coat and tannin precursors in the endothelium (Johnson et al., 2002). While Laer has dark brown seeds, Ws is more yellow, and Columbia is somewhere between them (Passardi et al., 2007).

Roots differ in length, although it also depends on the growth conditions. It appears, that Col has a slightly longer main root (around 18 mm) after 7 days long incubation in a vertical position. Root hairs in Col grow from the above elongation area (Schiefelbein and Somerville, 1990) and its length decreases from hypocotyl to calyptra. (Passardi et al., 2007).

According to Koornneef et al. (1991), the number of leaves in a rosette is an indicator of flowering. In Col, there were quite large rosettes comprising 10 leaves after 30 days of growth and compared to WS and Laer, Col began to develop flowers later (Passardi et al., 2007).

There are more types of trichomes in *Arabidopsis*, on sepals and stem, there are simple unbranched trichomes (Luo and Oppenheimer, 1999), the juvenile leaves have only a few trichomes on the adaxial side (Xu et al., 2019) and adult leave's trichomes have three or more branches and have a higher density (Passardi et al., 2007).

Arabidopsis has a typical *Brassicaceae* flower: 4 green sepals, 4 white petals, 6 stamens and 2 carpels fused (Meyerowitz et al., 1991). After pollination, it develops into the silique with two valves separated by a false septum, for 2-3 weeks, the silique elongates rapidly and the seeds mature (Meinke et al., 1998).

The development of higher plants is characterized by the production of phytomers (Fig. 1). Phytomers consist of two functionally different structures: one unit generates lateral organs and the other generates cauline (stem) structures. During *Arabidopsis's* transition from the vegetative phase to the reproductive phase of life, major changes in the plant body occur. In the vegetative phase, only rosette leaves and no internodes, but after the transition to flowering, axillary flowering shoots – paraclades appear with the following production of flowers. (Pouteau et al., 2010)

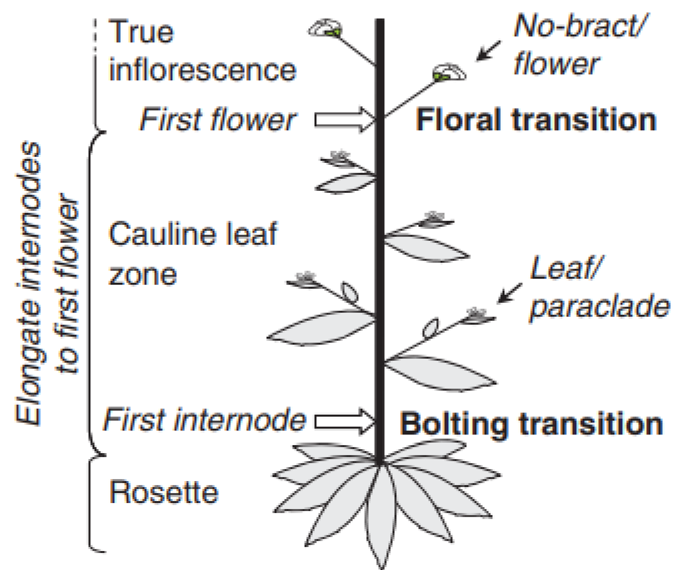

Figure 1 **Characteristics of flowering plant morphology** Rosette leaves are separated by non-elongate internodes. Above rosette, first elongate internode defines the bolting transition. The cauline leaf zone is characterized by presence of paraclades in the axils of the cauline leaf. True inflorescence (floral transition) is situated above the cauline leaf zone and the flowers are not subtended by bracts. Adapted from (Pouteau et al., 2010)

After receiving a signal, some plants change phyllotaxy at the beginning of flowering. *Arabidopsis* has both vegetative and inflorescence phyllotaxy in the spiral pattern. During the studies of genes involved in plant regeneration, some changes in phyllotaxy pattern may appear (Barton, 2010)

According to the aims of the work, I have studied morphology in the higher-order ERF (ethylene-responsive factor) mutants and compared the number of phytomers, bract leaves, the overall morphology of the flower and the pattern of phyllotaxy with the wild type *Arabidopsis thaliana* Col-0 (see results).

3.1.2 Genome

The *Arabidopsis* genome was the first plant genome sequenced. It has caught the eye of many molecular biology laboratories. In 1972 Sparrow et al. proved that the *Arabidopsis* nuclear genome is one of the smallest among the angiosperms. Its genome is only 125 Mb in total (The Arabidopsis genome initiative, 2000)

The nuclear genome is folded into 5 chromosomes (Fig. 2). There are 2 nucleolus organizer regions (NOR), which encode 18S, 5.8S and 25S rRNA genes and so create the nucleolus. They are located on the short arms of chromosomes 2 and 4 near the telomeres (Feldmann et al., 2014). 5S rRNA genes are located near centromeres of chromosomes 4 and 5 (The Arabidopsis genome initiative, 2000)

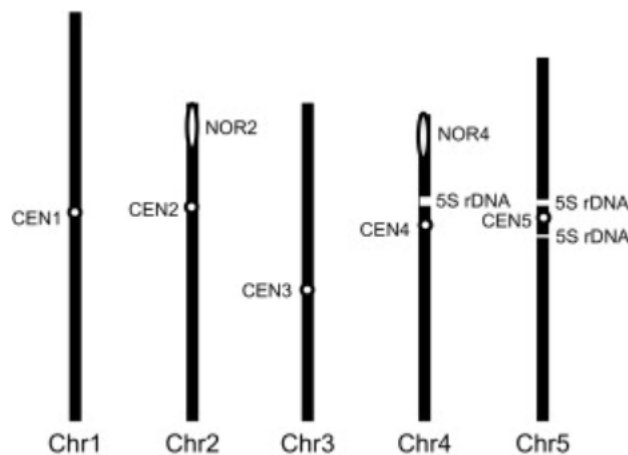

Figure 2 ***Arabidopsis thaliana* chromosomes** The NORs are located near the telomeres of Chr.2 and 4. The 5S rRNA is located near the centromeres of Chr.4 and 5 Adapted from (Feldmann et al., 2014)

3.2 Control of the plant growth

Plants are highly adaptable organisms that can live for many years. Most of them grow constantly and, with their long lives, have developed the ability to replace injured parts of the body. Unlike animals, plants keep small groups of pluripotent cells that provide growth and regeneration throughout their lifetime. To maintain control of body and organ structures, the entire growth and regeneration process is strictly controlled by hormones and other internal signals.

3.2.1 Pluripotency

Throughout evolution, basic cell organisms have developed into an extremely various and complex group of life. Although there are grave differences between all main groups (Animalia, Plantae,

fungi, …), also some shared signs exist in all alive multicellular organisms. Along with the ability to pass the genetic information to the next generation, regeneration of the body is extremely important for organism survival.

Animals can regenerate very well, although each part of their body varies in regeneration ability. The reason is that in an animal body, pluripotent cells occur only in the embryonic state of life and after their body establishment, pluripotent cells do not develop anymore. Compared to animals, plants keep pluripotent cells during their whole lifespan. These cells can proliferate and differentiate into any cell type. Pluripotent cells are located in a specific microenvironment called the stem cell niche (Kubalová, 2019). These occur in the meristem, where all new organs are established, the main meristems are shoot apical meristem (SAM), root apical meristem (RAM) and vasculature meristems (Heidstra and Sabatini, 2014).

In this work, we focused on SAM development. The key role in this process plays the *WUSCHEL* gene, which is the main regulator of the correct SAM development (Laux et al., 1996). As pluripotency and regeneration have to be strictly controlled, there are more factors affecting this process. Between *WUS*-independent regulators, ETHYLENE RESPONSIVE FACTORs (ERF) start to catch the eye of scientists. In this study, we tried to elucidate the effect of ERF on the ability of the SAM regeneration.

3.2.2 Transcriptional control

In different stages of life, various genes are expressed on different levels. Some of the genes are essential for the development of an embryo but are not needed in later phases. This is why every gene has to be regulated in some way. One of the most important ways is regulation on the transcription level.

Transcription can be regulated by the availability of genetic information, transcription initiation, process or end of the transcription. The availability of the information localized in the nucleus is affected by the arrangement of chromatin and DNA methylation.

Chromatin appears as condensed heterochromatin and transcriptionally active decondensed euchromatin. The condensation is highly affected by histone acetylation, which prevents condensation and promotes the bonding of transcription factors. This strategy is catalysed by two enzymes, acetyltransferases and deacetylases.

Next to deacetylation, methylation of histone H3 is important for the formation of heterochromatin. Onto methylated lysine of H3, heterochromatin protein 1 (HP1) is bonded, which later enables further binding with deacetylases and other methyltransferases to create so-called closed chromatin.

Another mechanism of chromatin modification is the binding of protein complexes containing Polycomb proteins. They play role in long-term gene silencing and so affect developmental processes (Pavlová, Fischer, 2011).

Transcription factors

The transcription of individual genes is managed by binding transcription factors to regulatory elements. These are short sequences of DNA that are transcribed and are termed *cis*-elements or boxes. Initiation and ending of target gene transcription are characterized by the number and organization of these regulatory elements in and outside the gene promotor area. *Trans*-factors, regulatory proteins, bind to regulatory elements and they can affect the transcription positively or negatively These proteins are encoded by their genes that are subject to other regulations.

The basic *cis*-elements of promoter area is TATA-box localized between 29th and 34th bp before the start of transcription. Its main importance is that it allows DNA-dependent RNA polymerase II to bind to the DNA, which results in the creation of a primary transcript. In the area of -75 to -80 bp, there is another box called CAAT, which enhances the activity of the promotor and other promoter elements. General transcription factors are related to these basic elements. They appear in all cells and recognize every promoter element of genes transcribed by RNA polymerase II.

Genes that are expressed only under certain conditions have specific *cis*-elements, which enable gene transcription under the control of a determined signal. There are many types of special transcription factors and just a small amount of them in a cell is sufficient for the initiation of the expression.

Other regulatory regions called enhancers can be localized up to thousands of base pairs up or downstream of the target gene. Their role is to affect the quantity of transcription, which means that they slow down or speed it up.

Transcriptional factors contain at least two structurally and functionally different motifs. The first one provides the ability to bind to the DNA, and the second secure bonding with other regulatory proteins. Out of 26 000 genes of *Arabidopsis thaliana*, 15 000 encode transcription factors. Binding to the target DNA is enabled by structural motifs that can divide into five main groups by their spatial orientation.

- HTH group – helix-turn-helix binds to DNA as homo- or heterodimers.
- HMG – high mobility group comprises from 3 α-helixes, where two bind to the DNA and create a gap for a different transcription factor, which binds with the third helix of the HMG.
- Zinc finger group – Zn atoms interact with four amino acids of the protein and the peptide chain between them forms a specific finger-like structure and usually does not

bind as dimers. Transcription factors with these motifs often play a role in developmental processes.

- Leucine zipper group (LZ) – usually forms dimers and has a basic character
- The group with homeodomain (HD) – is encoded by genes containing homeobox (a typical sequence of 180 bp coding 60 amino acids chain that folds into the three-helix structure which specifically binds to the DNA). Factors of this group often form complexes with other transcription factors that launch a cascade of more functionally related genes.

There is a unique family of transcription factors in the plant kingdom, in which the homeodomain is immediately followed by a leucine zipper, which enables dimerization. These factors play an important role, especially in differentiation and developmental processes (Pavlová, Fischer, 2011).

3.2.3 Meristems

Plant growth and development of various organs depend on a tissue called meristem. Plants need these meristems not only to develop all the essential parts of the body, such as stem, leaves and roots from one small seed but also to regenerate. The commonly green part of the body growing above ground is initiated from shoot apical meristem (SAM) and roots growing underground come from root apical meristem (Barton, 2010).

Shoot apical meristem (SAM)

The primary meristem located on the tip of the stem is responsible for the longitudinal growth of the plant (Gaillochet and Lohmann, 2015). The organization of the vegetative SAM is quite complex in most dicotyledonous plants. Its shape is triangular and its widest point has around 60 µm. SAM consists of a few hundred pluripotent cells, which are distributed in three layers. Two outermost layers divide parallel to the meristem surface, while the third one divides in all directions. (Barton, 2010)

Furner and Pumfrey (1992) indicated that there are three stem cells per layer in the centre of the meristem, which is called the central zone. When stem cells split, their descendants are pushed to the peripheral region. This is where the cells develop into the primordia, the descendants pushed outward become leaf primordia. Under the central zone is located the so-called rib zone, descendants pushed into this zone generate stem tissues. (Barton, 2010)

There is another small group of cells in the central zone, which controls the maintenance of pluripotency in stem cells. They form the organizing centre (OC), which is characterized by the expression of the transcription factor *WUSCHEL (WUS)* (Mayer et al., 1998). Its product, WUS protein, is involved in a negative-feedback loop. There are three possible pathways of communication between

the organizing centre and the central zone: the WUS is transported symplastically through plasmodesmata from the OC and activates the expression of *CLAVATA3* (*CLV3*) in other stem cells. The CLV3 responds by restricting the *WUS* expression. The second possibility is secretion to the intercellular space, where CLV3 binds to receptor-like kinase CLV1. This process requires cooperation with receptor protein complex CLV2/CORYNE to transport the signal. The signal can be also relayed by the like protein kinase 2, which is the last possible pathway of communication between the organization centre and the central zone to maintain the stem cell activity (Gaillochet and Lohmann, 2015).

Root apical meristem (RAM)

This tissue is located at the top of the root and is responsible for the extension of the root (Gaillochet and Lohmann, 2015). As opposed to SAM the RAM copies the organization from other plant body stem cells, called initial cells. The RAM surrounds the SAMs organizing centre analogue, the quiescent centre (van den Berg et al., 1997). This 'organizer' is formed by four cells characterized by *WUSCHEL-RELATED HOMEOBOX 5 (WOX5)* activity. *WOX5* gene is essential for stem cell fate maintenance (Bennett et al., 2014; Forzani et al., 2014; Sarkar et al., 2007).

When the cells of the quiescent centre are dividing, the daughter stem cells remain in contact. These lately give rise to the different cell lines. As well as in the SAM (Fig. 3), the communication with the further localized cells is underway intercellularly through peptides. While in the SAM, the major genes involved are the *WUS* and *CLV*, the main regulator in RAM stem cells is the CLAVATA3/ESR-RELATED 40 (CLE40) peptide, which acts as a positive regulator of the amount of the protein complex receptor CRINKLY4 (ACR4)/CLV1. This complex restricts the *WOX5* and therefore the entire cycle ends with the balance between the initials and the differentiated columella cells (Gaillochet and Lohmann, 2015).

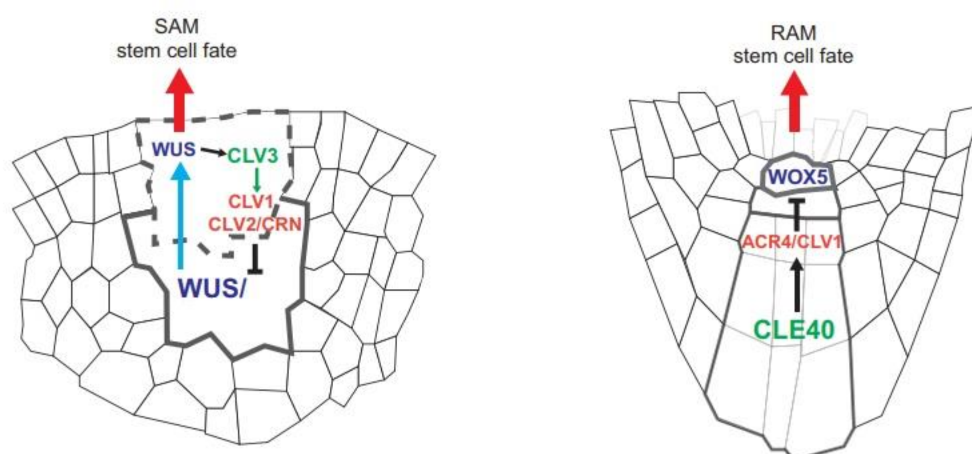

Figure 3 **Organization and control of the SAM and RAM stem cell fate** SAM is controlled mainly by the expression of the *WUSCHEL* gene, which is also involved in the negative-feedback loop: the WUS is symplastically transported (blue arrow) from the OC to the central zone, activates *CLV3* (black arrow), which restricts the *WUS* through the CLV1 or CLV2/CRN. The activity of RAM is regulated through the restriction of the *WOX5* gene expressed only in the quiescent centre by the columella's small peptide CLE40. The communication between the two tissues is provided by ACR/CLV1 receptor. Adapted from (Gaillochet and Lohmann, 2015), edited.

Callus

A callus is a mass of undifferentiated cells, which develops after the damage to the plant body. Most of this tissue is totipotent, which means that one cell can give rise to a whole new plant body (Steward et al., 1958; Nagata and Takebe, 1971). Its pluripotency allows cells to develop into any other cell kind and its fate is mostly controlled by plant hormones, especially cytokinin and auxins (Skoog and Miller, 1957).

Calli develops after the wound or other external stimuli. It means that there may be more types of calli, depending on the location or fate of the cell. It can be classified according to the ratio between differentiation and dedifferentiation, controlled by phytohormones (Skoog and Miller, 1957). Calli cells that show some degree of organ regeneration are termed rooty, shooty, or embryonic callus (Zimmerman, 1993; Frank et al., 2000). These cells also have distinct expression profiles of various genes (Iwase et al., 2011a). The callus with no signs of differentiation is called compact or friable callus (Zimmerman, 1993; Frank et al., 2000).

There are various ways to induce the callus in the plant body. First of all, the stimuli observed were wounds, after the development of molecular biology and biochemistry, Skoog and Miller (1957) identified the formation of callus that depended on phytohormones. The mechanism of wound induction will be described below. Hormonal regulation is the subject of a separate chapter (see Cytokinin and auxin).

WOUND INDUCED DEDIFFERENTIATION1 (WIND1) from APETALA2/ETHYLENE-RESPONSIVE ELEMENT (AP2/ERF) transcription factor family is the main transcription factor in the response to the damage of the plant body. There are four *WIND* genes (*WIND1-4)* and after the wound, their overexpression starts within a few hours. WIND proteins interact with the *Arabidopsis* response regulators (ARR), which are too upregulated after wounding. The ARR are two-component sensor promoters, also induced by cytokinin and dependent on WIND1 (Iwase et al., 2011). Based on these findings, it was dedicated, that *WIND* are the major hormone-independent regulators of cell dedifferentiation and formation of the callus, although the exact regulatory pathway wasn't elucidated yet (Momoko et al., 2013).

3.2.4 Hormonal regulation

The right balance between plant hormones auxin and cytokinin play a crucial role in many developmental processes, such as cell division, dedifferentiation and differentiation, but also response to outer environmental signals or positional information of meristems (Gaillochet and Lohmann, 2015). This chapter describes the contexts between plant hormones, cytokinin, auxin, ethylene and pluripotency.

Cytokinin

As mentioned above, the expression of *WUS* regulates the shoot apical meristem, respectively the stem cells of the OC and neighbouring cell layers. In local transcriptional networks of these domains, the cytokinin plays a crucial role in promoting *WUS* (Buechel et al., 2010; Gordon et al., 2009; Müller and Sheen, 2008; Zürcher et al., 2013).

The cytokinin signalling pathway is involved in the *WUS/CLV* regulation of the SAM. Relations between the regulation of cytokinin production and signalling in the SAM can be explained by the mutual regulation of both genes of these processes (Fig. 4).

The signal is transmitted after the cytokinin binds to the binding domain of the receptor from the histidine kinase family (AHK2, 3 and 4). These receptors are localized in the plasma membrane and are coded by a gene family termed CRE (Pavlová, Fischer, 2011). After the activation, the receptor transfers the phosphoryl group to histidine phosphotransfer proteins (AHP) (Kakimoto, 2003). The signal is further submitted to the type A or B *ARABIDOPSIS RESPONSE REGULATORS* (*ARRs*) (Muller, Sheen, 2007), which compete for the phosphoryl group between each other.

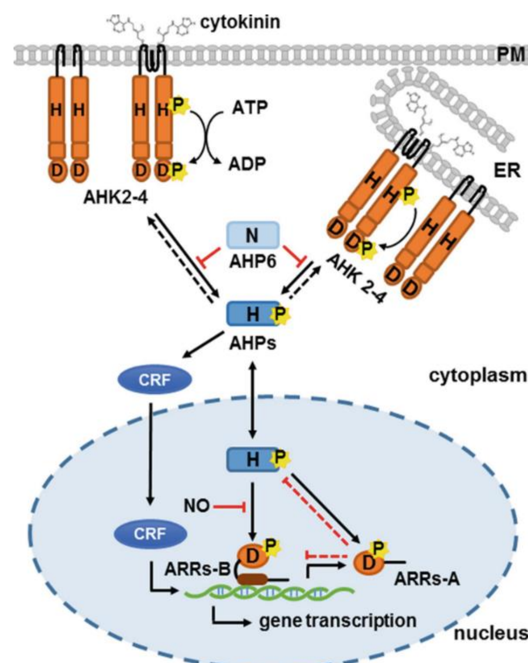

Figure 4 **Cytokinin signalling pathway** The signal is received by histidine kinase receptor (AHK), which transfers the phosphoryl group to the histidine phosphotransfer proteins. This group can be accepted by two types of ARRs acting as positive or negative regulators of the target genes. Adapted from: (Pekarova et al., 2018)

ARRs of type B are transcription factors that can activate the expression of their target genes. These include ARR-A or cytokinin response factor (CRF) (Pavlová, Fischer, 2011). The A-type ARRs act as repressors to the target genes involved in the cytokinin signalling pathway (Muller, Sheen, 2007).

Recent studies have shown that type-A ARRs are repressed by *WUS*, which leads to the idea of the ability to increase cytokinin signalling. Furthermore, increased expression of the A-ARRs can decrease the expression of *WUS.* The signal probably leads through A-ARRs to *CLV*, which is a well-known antagonist of *WUS* (Leibfried et al., 2005, Gordon et al., 2009).

Auxin

Auxin and its polar transport are one of the basic mechanisms of the creation and development of individual organs in plants. Higher concentrations of auxins activate developmental processes and play a major role during embryogenesis (especially its polarization). During postembryonal development, auxin affects cell division and cell extension. Signs of its activity are very variable, showing in the development and also the growth of the plant. Between main functions is stimulation of differentiation of conductive mesh, the activity of cambium, and fruit growth. Auxin inhibits the development of axillary meristems into buds and lateral stems – auxin plays role in apical dominance (in cooperation with cytokinin).

Auxin influx and efflux depend on transport proteins, dissociation of the molecule, and so on pH too. While one of the basic auxin indole-3-acetic acid (IAA) is a weak acid, which can be dissociated in the acidic environment of the cell wall in 50–70 % and in the neutral environment of cytosol, the dissociation is higher. In an undissociated state, IAA is transported by passive diffusion, while dissociated molecule has to be transported actively by symport with two H^+.

This transport is mediated by a transport protein termed AUX1 (auxin resistant 1), which can be localized polarly or evenly based on the function of the cell. From cytosol to the apoplast, IAA^- is transported by protein PIN (pin-shaped inflorescence). Basipetal transport is enabled by basal localization of PIN and distal localization of AUX1. The level of PIN transport is regulated by proteins, that form PIN functional complexes. These complexes are unstable in localization in the plasma membrane and thus can react to different signals by fast change of their localization.

Auxin influences the expression of a few hundred genes on a transcriptional level. It can act as an inhibitor or can stimulate the expression of the target gene. These genes contain so-called *cis*-elements AuxRE (auxin response element) in their promotor. AuxRE can bind with monomers, homo- or heterodimers of corresponding transcription factors ARF (auxin response factor).

The activity of ARF is controlled by a group of small unstable proteins called AUX/IAA. They form homo- and heterodimers, which bind to ARF and inactivate them. AUX/IAA proteins are degraded by complex ubiquitin ligase E3 marked by SCF^{TIR1} (SKP1, Cullin/CDC53, F-box protein), where TIR1 (transport inhibitor response1) determines its specificity to auxin (Fig. 5).

The type of genes regulated this way is called early and it involves genes coding AUX/IAA or gene CDC2, which encodes a basic subunit of cyclin-dependent kinases and so auxin impacts cell

differentiation. Another type is late and these encode transcription factors, that regulate the expression of other genes. Late genes for example involve the gene for the enzyme catalyzing the synthesis of ACC, which is a precursor of phytohormone ethylene (Pavlová, Fischer, 2011).

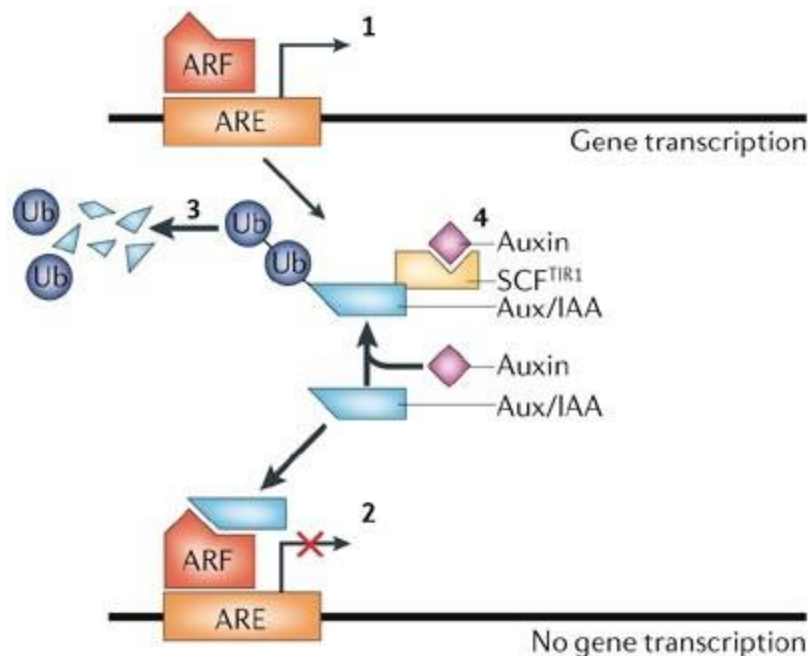

Figure 5: **Auxin signalling pathway** Transcription factor ARF regulates gene expression by binding to ARE element localized in target gene promotor. If AUX/IAA binds to ARF, the ARF is inactivated. AUX/IAA can be degraded by E3 ligase marked by auxin/SCF^{TIR1} complex and so activate the earlier inhibited gene expression. Adapted from (Teale et al., 2006)

Auxin's impact on pluripotency of the stem cells is the opposite of cytokinin, which promotes cell proliferation. It initiates organ primordia in the peripheral zone of the SAM. To retain the balance of SAM and organ primordia, one of ARF, so-called MONOPTEROS (MP) directly represses type A ARR7 and ARR15. As mentioned above A-type ARRs repress the activity of *WUS,* which means that by inactivating these ARRs through ARF, the *WUS* gene expression can be activated.

Another impact of MP is the promotion of *AHP6*, which product transduces cytokinin signal (Fig. 4) into the centre of SAM. The result is a stronger response to cytokinin signal in incipient leaf primordia (Gaillochet et al., 2015).

Ethylene

Ethylene is hydrophobic hydrocarbon gas that plays a role during fruit ripening, fall of leaves, and protection against stress factors. Ethylene is always present in the environment as it is produced by plants, fungi, or bacteria.

Its biosynthesis starts by merging amino acid methionine and ATP to form S-adenosylmethionine. Cleavage reaction catalyzed by ACC-synthase (ACS) gives rise to the straight precursor of ethylene – 1-aminocyclopropane-1-carboxy acid (ACC). The level of this particular

substance is the main regulation point of ethylene synthesis. For ethylene formation catalyzed by ACC-oxidase oxygen is necessary, as cofactors of the enzyme Fe^{3+} and ascorbic acid. In some physiologic processes, the production can be affected by auxin, cytokinin, or abscise acid.

Ethylene is synthesised in all kinds of tissues and spreads through diffusion. The highest level of production is in meristems, where its amount rises during senescence, leaves fall, fruit ripening, and as a reaction to stress. If the signal to synthesis is remoted from the place of its action, it is transported as ACC.

Ethylene itself is degraded by oxidation to ethylene-oxide. This process is probably a chemical character, not physiologically regulated by the plant. Detailed information about ethylene receptors, signal transduction, and its activity will be described further in the individual chapter because elucidation of this topic is one of the aims of this work.

3.3 Ethylene responsive factor

As the plants are immobile, they have to develop mechanisms to defend themselves on the body, cellular and molecular levels. They have adapted their genome and among the genes responsible for defence, transcription factors and their products are very frequent as regulators for the next stress-responsive genes. Ethylene responsive factor (ERF) is one of the largest subfamilies of the APETALA2/ERF (AP2/ERF) transcription factor family, which plays an important role in developmental processes, responses to stress and many others. (Thirugnanasambantham et al., 2014)

3.3.1 Classification and structure of AP2/ERF family

In *Arabidopsis thaliana*, four subgroups of AP2/ERF proteins containing at least one DNA binding domain (the AP2 domain) are defined according to their structure (Licausi et al., 2013).

- ERF – these factors contain only one AP2 domain and have a small number of introns (Nakano et al., 2006).
- AP2 – members of this group are characterized by the presence of two tandem repeats of the AP2 domain. To this group are classified some of the proteins containing one AP2 domain, but report higher similarity to domains of this group that of the ERF.
- RAV – related to ABI3/VP1 group contain the ERF domain and B3 DNA-binding domain.
- Soloist – has one AP2 domain, but differs so much from the ERF, it was classified into an individual group (Licausi et al., 2013).

Sakuma et al. (2002) have introduced another classification, where two main groups were identified according to the similarity of the AP2 domain: Dehydration-Responsive Element Binding-

proteins (DREB) and previously mentioned ERF. Nakano et al. (2006) have considered characteristic intron-exon structures and so subdivided Sakuma's groups into additional twelve subgroups. Both classifications are used equally (Licausi et al., 2013), but in this chapter, we will work with Nakano's classification for the structure of ERF is important to consider.

DNA binding domain of the AP2/ERF family consists of 60 amino acids folded into a layer of three antiparallel beta-sheets followed by alfa-helix (Allen et al., 1998). DNA binding domains of DREB and ERF differ in the identity of residues at specific positions (Sakuma et al., 2002). The differences define the affinity to the sequence of the DNA. DREB proteins often bind to an A/GCCGAC element, which is associated with abscisic acid and genes involved in drought and cold response (Stockinger et al., 1997). ERF subfamily members bind an AGCCGCC element, known as GCC-box usually localized at the N-terminus upstream of the genes induced by ethylene, wound, or pathogen hacking (Ohme-Takagi and Shinshi, 1995).

The transcription factors containing the AP2 domain can act as activators or repressors. The repressors can be further classified as active or passive. The difference is in the repression domain, which in active repressors determines the repressive activity to the transcription factor or the DNA binding domain. On the other hand, passive repressors lack the repression domain, and instead, they compete for the DNA sequence with transcription activators (Licausi et al., 2013).

Activators do not differ in the sequence motifs but are more likely rich in acidic amino acids. For example, tobacco ERF2 and ERF4 have acidic N- or C-terminus, and both act as activator domains (Ohta et al., 2000). In contrast, active repressors contain previously mentioned repressive domains. There are three types of these motifs: ERF-associated amphiphilic repression (EAR) (Ohta et al., 2001), the TLLLFR motif (Matsui et al., 2008), and the B3 repression domain (BRD) (Ikeda, Ohme-Takagi, 2009). ERF containing EAR or BRD motif act as repressors and cooperate with corepressors TOPLESS and TOPLESS-RELATED (Causier et al., 2012).

3.3.2 ERF activity

ERFs often react with other transcription factors. They form complexes with various levels of stability, DNA specificity, amount, or activity. Activity is affected by a special complex of three proteins. In this complex, the EAR domain of ERF is bounded by another protein to histone deacetylase, which results in deacetylation and thereby repression of the ethylene-responsive gene (Kagale, Rozwadowski, 2011). TOPLESS and TOPLESS-RELATED corepressors work in a similar mechanism. The specificity is influenced by interaction proteins. For example, ENHANCER OF SHOOT REGENERATION (ESR) interacts with homeodomain-leucine-zipper transcription factors, which bind to the AP2 domain (Chandler et al., 2007).

ERF play role in responses to environmental or hormone stimuli. The group of ERF regulates ethylene inducible genes downstream of transcription factor ethylene insensitive 3 (Solano et al., 1998). Some of the ERF even influence responses to cytokinin, which can affect the meristems (Licausi et al., 2013). This might result in modulation of the morphology. For example, abnormal expression of *ERF1* or *DREB1* genes ends in dwarf phenotype formation (Solano et al., 1998).

Ectopic expression of ESR follows in shoot regeneration without cytokinin signal (Banno et al., 2001). Another important ethylene-responsive factor is *WOUND INDUCED DEDIFFERENTIATION 1* (*WIND1*), which controls cell dedifferentiation after injury of the plant body and formation of the pluripotent callus (Iwase et al., 2011).

In this work, I have focused on the group VIII ERF based on Nakano et al. (2006) classification. This group was divided into two subgroups according to their function.

Subgroup VIIIa consists of ERF containing the EAR motif, which is responsible for the repression of the target gene (Ohta et al., 2001) and so this subgroup functions as transcriptional repressors. Subgroup VIIIb differs from VIIIa by the presence of the CMVIII-3 motif at the C-terminus (Nakano et al., 2006). We have studied ESR1 and ESR2 function *in vitro*. These transcription factors are known to enhance the shoot regeneration through positive regulation of the *CUP SHAPED COTYLEDON 1*, the gene which positively regulates other SAM-related genes (Hibara et al., 2003).

3.3.3 Protein degradation of ERF

Short-lived proteins are degraded by the Ubiquitin 26S Proteasome system (Jackson and Hewitt, 2016). Transcription factors that affect genes important for development are degraded by this system too. The system itself is strictly regulated to secure the correct timing and localization of the processes.

In general, the ubiquitin molecule is activated by E1 ubiquitin-activating enzyme, while in this process, ubiquitin is bounded to E2 ubiquitin-conjugating enzyme, which is in the next step alongside the target protein recognized by E3 ubiquitin ligase. This results in the formation of the E2-substrate-E3 complex and ubiquitin transfer to the substrate. The reaction is called polyubiquitination and the protein marked by the chain of ubiquitin is recognized by the 26S proteasome followed by its degradation into amino acids and individual ubiquitin molecules (Vierstra, 2009).

There are several types of E3 ligases in plants. One of four groups, into which these ligases are classified, can bind to AP2/ERF and so becomes another way of transcriptional control (Weber et al., 2009).

CULLIN-RING (REALLY INTERESTING NEW GENE) ligases are proteins with three subunits with various roles. The RING-box1 (RBX1) protein binds E2, a subunit that can recognize

specific target proteins and CULLIN protein, which connects them (Viestra, 2009). This group contains four families, for this work we focused on family CUL3 which has shown to play a role in ERF degradation (Weber et al., 2009).

Some proteins contain the BRIC-A-BRAC – TRAMTRACK – BROADCOMPLEX/POX VIRUS and ZINC FINGER (BTB/POZ) domain, which is bounded by the N-terminus of the CUL3 (Figueroa et al., 2005). BTP/POZ is localized at the C-terminus of the protein. At the N-terminus, there might occur MEPRIN and TRAF (TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED FACTOR) HOMOLOGY (MATH) domain. Weber et al. (2009) studied BTP/POZ protein interactions with MATH. They have found that BTP/POZ interact *via* MATH, which is present in AP2/ERF transcription factors too. This led them to the hypothesis that AP2/ERF might be a possible substrate for degradation by CUL3 related E3 ligase degradation (Fig. 6)

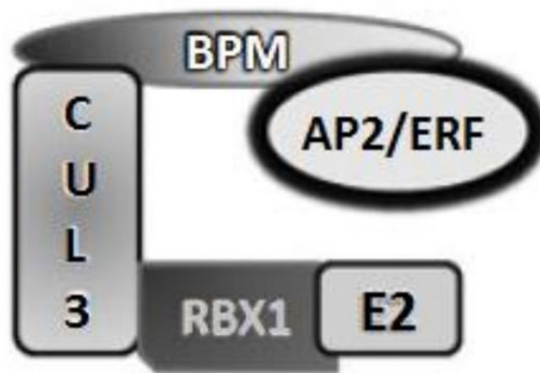

Figure 6: **Model for AP2/ERF transcription factor degradation *via* CUL3 based E3 ligase** Adapted from: Weber et al., 2009), edited

4 MATERIALS AND METHODS

4.1 Biologic materials

- For the phenotypic analyses, the following *Arabidopsis thaliana* mutant lines seeds were used. All seeds were harvested from the plants cultivated in the phytotron.

  Col-0
  erf 4-1,8 dm
  erf 9-1,12 dm
  erf 4-1,8,9,11-1
  erf 4-1,8,9,12
  erf 4-1,8,9,11-2,12
  erf 4-1,8,9,10-2,11-2,12
  pKSVM38 ERF4 #2
  pKSVM38 ERF4 #5
  pKSVM38 ERF4 #9
  pKSVM38 ERF9 #1
  pKSVM38 ERF9 #8
  pKSVM38 ERF9 #10
  pKSVM38 ERF11 #1
  pKSVM38 ERF11 #2
  pKSVM38 ERF12 #11
  pKSVM38 ERF12 #12
  pKSVM38 ERF12 #13
  pKSVEM40 ERF9
  pKSVEM40 ERF11
  pkl-1
  cre-2 *ahk* 3-8
  pkl-1 *cre*-2 *ahk* 3-8
  erf 4,8,9,10,11,12 *cre* 1-2 *ahk* 3-8
  esr 1-1 *esr* 2-2

- For the histological analysis of *ERF12* expression by GUS staining. From each genotype, ten independent lines were chosen for the experiment. All the seeds were harvested from the plants grown in the phytotron.

  pHLGUS ERF12

4.2 Growth conditions

- The intact plants were grown in a phytotron with a 16 h light and 8 h dark photoperiod at 21 °C and 70 % humidity.

4.3 Chemicals and solutions

4.3.1 Chemicals

- Surface sterilization of *Arabidopsis* seeds
  Ethanol p.a. (Penta chemicals unlimited, Cat. No.: 71250-11001)
- DNA extraction
  Ethanol p.a. (Penta chemicals unlimited, Cat. No.: 71250-11001)
  Chloroform isoamyl alcohol p.a. (VWR chemicals, Cat. No.: 301696S)
- PCR reaction
  5x GoTaq® Flexi Green Buffer (Promega, Cat. No.: M891A)
  GoTaq Flexi DNA polymerase (Promega, Cat. No.: M7802)
  25mmol·l^{-1} , $MgCl_2$ (Promega, Cat. No.: A351H)
  10mmol·l^{-1} dNTP mixture
- Electrophoresis separation
  Agarose (Sigma, A9539)
  0,05% ethidium bromide (SERVA, Cat. No.: 39803.01)
- Shoot regeneration assay medium preparation
  B5 Gamborg salt (Duchefa Biochemie, Cat. No.: G0415.0100)
  Glucose (Sigma, Cat. No.: G8270)
  MES (Duchefa Biochemie, Cat. No.: M1503.0250)
  Gellan gum (Alfa Aesar, J63423)
  0,5mg·l^{-1} dichlorophenoxyacetic acid (2, 4- D) (Duchefa Biochemie, Cat. No.: D0911.0100)
  0,5mg·l^{-1} kinetin (SIGMA, Cat. No.: 48130-1G-F)
  MS salt (Duchefa Biochemie, Cat. No.: M0222.0100)
  Saccharose (Lach-Ner, Cat. No.: 40135-AP0-G1000-1)
  1mg·l^{-1} isopentenyl adenine (2 ip) (Duchefa Biochemie, Cat. No.: 001 0233)
  0,15mg·l^{-1} indole-3-acetic acid (IAA) (SIGMA, Cat. No.: D2629-1G)
  5mol·l^{-1} KOH (Lach-ner, Cat. No.: 10003-AP3-G1000-1)
- GUS staining
  0,2mol·l^{-1} Na_2HPO_4 . 12 H_2O (Lach-Ner, Cat. No.: 30061-AP0-G0500-1)

0,5mol·l^{-1} EDTA (Penta, Cat. No.: 253 416 333 803)

200mmol·l^{-1} Potassium ferricyanide (Neolab, Cat. No.: 9565-0250)

200mmol·l^{-1} Potassium ferrocyanide (Neolab, Cat. No.: 4580-0250)

0,25mg·µl^{-1} 5-Bromo-4-chloro-3-indoyl-β-D-glucopyranoside (X-Gluc) in DMF (x 500) (Apollo Scientific, Cat. No.: 15548-60-4)

10% Triton X-100 (SIGMA, Cat. No.: X100-1L)

- Medium for phenotypic analyses of transgenic plants

  Chemicals for preparation of MS medium

  Estradiol (SIGMA, Cat. No.: E8875-25G)

4.3.2 Solutions and their preparation

- Surface sterilization of Arabidopsis seeds

  1x sterilizing solution: for 1 ml of the solution add 500 µl of sodium hypochlorite, 100 µl of 96% ethanol and 400 µl of deionized H_2O

- DNA extraction

  DNA extraction buffer: for preparation of 500 ml add 100 ml of 1M Tris HCl, 20 ml of 0,5mol·l^{-1} EDTA, 31,25 ml of 4M NaCl, 75 ml 10% SDS and add deionized H_2O to fill up to 500 ml in total

  TE buffer: for 50 ml add 500 µl 1mol·l^{-1} Tris HCl (pH8), 100 µl 0,5mol·l^{-1} EDTA (pH8), 49,4 ml sterile deionized H_2O

- Electrophoresis separation

  50x TAE buffer: for preparation of 500 ml add 50 ml of 0,5mol·l^{-1} EDTA (pH 8), 400 ml of Tris-HCl and 27 ml of acetic acid p.a. and 23 ml of deionized H_2O

- GUS staining buffer pH = 7,2

  Stock solution of 0,2mol·l^{-1} phosphate buffer A: 3,58 g of $Na_2HPO_4 \cdot 12\ H_2O$ dissolved in 50 ml of deionized H_2O

  Stock solution of 0,2mol·l^{-1} phosphate buffer B: 1,56 g of $Na_2HPO_4 \cdot 12\ H_2O$ dissolved in 50 ml of deionized H_2O

  GUS staining buffer: For preparation of 20 ml, add 10 ml of A + B phosphate buffer mixture, 40 µl of 0,5mol·l^{-1} EDTA, 100 µl of 200mmol·l^{-1} $K_3Fe(CN)_6$, 100 µl of 200mmol·l^{-1} $K_4Fe(CN)_6 . 3\ H_2O$, 40 µl of 0,25mg·µl^{-1} X-Gluc, 200 µl of 10% Triton X-100 and 17,02 ml of deionized H_2O

4.4 Primers

Chart 1: Primers for erf higher-order mutants genotyping

Amplified gene	Primer name	Oligo sequence
erf 4	F3178	TGCATGTTGCGACGAAGATGG
	R3585	ATGGGGTGAAACCAAGTCGC
	LBa1m	GGTTCACGTAGTGGGCCATCGccctg
erf 8	F1846	gtgatttgtgcagtctttgttacaaagg
	RFSeI	ctcGGCCGGCCTTCCGCCGGAGGAGCTAAG
	FLAG LB4	CCTTGATAAAGTGATGATGCTGTTCC
erf 9	F2ASCI	TTTggcgcGCcATGGCTCCAAGACAGGCGAACGG
	R3244	CCAGGTTTAGTTCTCTTCTCGGTTC
	LBλ-d	GTGAAGGGCAATCAGCTGTTGC
erf 10-2	F2890	TGTAAGCTGCGAATGGAACCG
	R3119	acgtcaacacgaaatccattgg
	GKL8760	GGGCTACACTGAATTGGTAGCTC
erf 11-1	F2591	AACGGAGTCCGTTACAGAGG
	R2697	GTGTCGAAAGTACCGAGCCAGACACG
erf 11-2	F2591	AACGGAGTCCGTTACAGAGG
	R2697	GTGTCGAAAGTACCGAGCCAGACACG
erf 12	F2804	TTTTTCTCCGAACCGGTGTGC
	R3066	atagaaaagtaggcaaaacggcg
	LB3m	CTGAATTTCATAACCAATCTCGATACAC

Chart 2: Primers for pKSVM38 ERF 4, 9, 11, 12 transgenic plants genotyping

Orientation	Genotype	Primer	Oligo sequence
Forward	pKSVM38 ERF 4	ERF 4 FASCI	ATCTggcgcgccATGGCCAAGATGGGCTTGA
	pKSVM38 ERF 9	ERF 9 FASC2	TTTggcgcGCcATGGCTCCAAGACAGGCGAACGG
	pKSVM38 ERF 11	ERF 11 FASCZ	TTGggcgcGcCATGGCACCGACAGTTAAAAC
	pKSVM38 ERF 12	ERF 12 FASCZ	AGAGgCgcgCcATGGCGTCAACGACGTGTGCA
Reverse	all above	1NOSR	CGAAACGAACTAGTTCCGGTGCAG

4.5 Instruments

- Surface sterilization of Arabidopsis seeds

  Flow box Merci

- DNA extraction

  Eppendorf centrifuge 5417R

  Biosan Combi-spin 2400N centrifuge

  Retsch MM 400 mixer mill

Labobase SBC840.40 vacuum system

- PCR reaction

  Scotsman AF80 ice maker

  Eppendorf thermomixer comfort 1,5 ml

  Veriti 96 Well fast thermal cycler

- Electrophoresis separation

  Major science MP-300V power supply

  Bio-Rad Gel-Doc EE imager UV transilluminator

- Shoot regeneration assay medium preparation

  Sterivap HP autoclave

  IKA RCT basic magnetic mixer

  Ohaus Pioneer PX 5202 laboratory scales

  XS instruments pH meter

- Phenotype analyses

  Zeiss Axio microscope + Axiocam 305 Color

  Nikon SMZ800 microscope

- Software

  Nikon NIS-Elements

  Gel Doc EZ imaging system (Bio-Rad)

  Image Lab

4.6 Methods

4.6.1 Surface sterilization of *Arabidopsis* seeds

A fresh sterilizing solution is always prepared before the sterilization. Firstly, the *Arabidopsis thaliana* seeds transferred into a 1,5 ml Eppendorf tube were washed in 70% ethanol for 2–3 minutes. Ethanol was removed by pipetting and then 1 ml of 1x sterilizing solution was added to the tube and incubated for 10–12 minutes while occasionally inverting the tube. The solution was removed by pipetting and the seeds were rinsed twice with 1 ml of sterile deionized water. 200 µl of sterile deionized water was added and the seeds were kept in the fridge for 1–4 days before planting on a medium.

4.6.2 DNA extraction

From the tested plant of *Arabidopsis thaliana,* a small piece (3x3 mm) of a true leaf was transferred into a 1,5 ml safe-lock Eppendorf tube with a tungsten bead. 300 µl of genomic DNA extraction buffer and 70 µl of chloroform isoamyl alcohol were added. The tubes were placed into the

TissueLyser Adapters and stabilized in the oscillator. The leaves were homogenized for 2 minutes with the frequency set to 25 Hz. Then, the tubes were transferred into the centrifuge and centrifuged for 3 minutes at 14 000 rpm.

After centrifugation, the supernatant containing genomic DNA was transferred to a new Eppendorf tube of 1,5 ml. 700 µl of 96% ethanol was added and the tubes were centrifuged for 10 ml at 14 000 rpm. All ethanol was then removed with the vacuum suction device and the precipitated DNA was washed from the tube wall with 75 µl of 70% ethanol. The samples were centrifuged for 3 minutes at 14 000 rpm, all liquid was removed and the DNA was dissolved in 50 µl of TE buffer using vortex and stored at -20 °C.

4.6.3 PCR genotyping

Preparation of PCR samples was performed on ice to prevent enzyme or DNA degradation. Master mix for PRC reaction was prepared into a new 1,5 ml Eppendorf tube. One reaction (9 µl) was prepared by adding:

3,4 µl	deionized H_2O
2 µl	5 x GoTaq® Flexi Green Buffer
1 µl	$MgCl_2$ 25 mmol·l^{-1}
1 µl	dNTP mixture 10 mmol·l^{-1}
0,8 µl	Forward primer
0,8 µl	Reverse primer
0,05 µl	GoTaq Flexi DNA polymerase 5000 U·ml^{-1}

Genotyping was often performed while using three primers – in that case, the volume of deionized water was reduced by the amount of the third primer to keep the same reaction volume.

9 µl of the master mix was transferred into the PCR strip compartment per sample. 1 µl of DNA was added and carefully mixed. All samples were transferred into a thermo cycler with the conditions set to:

95 °C 40 s initial denaturation

36 cycles:
- 95 °C 15 s denaturation
- 58 °C 15 s annealing
- 72 °C 1 min amplification

72 °C 5 s final amplification

In the meantime, electrophoresis was prepared. Into 50 ml of warm 1% agarose gel, approximately 2 µl of ethidium bromide was added. The gel was mixed and poured into the tray with attached combs. After 20 min, the solidified gel was transferred into the electrophoresis chamber and the tank was filled with TAE buffer.

After the finish of the PCR reaction, the samples were pipetted into the prepared gel, the electrophoresis was set to run for approximately 20 min on 120 V.

The gel was then visualized under ultraviolet light and the photo was kept.

4.6.4 Shoot regeneration assay

To test the ability of regeneration of the plants, the shoot regeneration assay was performed. First, the sterilized seeds were grown in a vertical position on MS medium for 5–6 days. Then, root explants were made by cutting the middle parts of the root with a special tool to keep the same size of the explants (8 mm). The root explants were transferred onto the callus-inducing medium (CIM). After 3–4 days of incubation, the root explants were transferred onto a shoot-inducing medium (SIM). The number of regenerated shoots was counted on days 11, 14, and 18 after the last transfer. All work was performed in sterile conditions in a flow box to prevent sample contamination.

Preparation of the media

The media were prepared by weighing individual components (the list of components and their quantity are in chart 3). They were dissolved in deionized water in a beaker and mixed with a magnetic stirrer. pH was adjusted with KOH to 5,7 and all liquid was transferred into a 1l glass bottle. The media were autoclaved and after cooling down, the phytohormones were added and the media was poured into Petri dishes and left to solidify (this work was performed in sterile conditions)

Chart 3: Components for preparation of 1l of the media used for the shoot regeneration assay

	MES [g]	MS salts [g]	B5 Gamborg salt [g]	Sucrose [g]	Glucose [g]	Gellan gum [g]	2 ip [µl]	IAA [µl]	2,4-D [µl]	Kinetin [µl]
MS	0,5	4,3	x	10	x	10	x	x	x	x
SIM	0,5	4,3	x	10	x	5,7	2000	200	x	x
CIM	0,5	x	3,1	x	20	5	x	x	100	100

4.6.5 Histological analysis of *ERF12* expression by GUS staining

The seeds for the experiment were sterilized and left for 4 days in the cold for stratification. The seeds were planted onto MS medium and grown in a vertical position for 1–14 days. The plants were

transferred into 6 well plates, each well containing 2,8 ml of GUS-staining buffer. The samples were incubated overnight at 36 °C. The chlorophyll was removed by going through ethanol series (from 70% to 96%). After rinsing, the samples were then observed under the binocular microscope.

5 RESULTS

5.1 Analysis of loss-of-function *erf* higher order mutant phenotypes in intact plants

To find out if there are any changes in the phenotype of *Arabidopsis* loss-of-function *erf* higher order mutants and transgenic plants pKSVM38: ERF–/AP2, there were some characteristic structures counted. Observed organs were phytomers, sepals, petals and stamens. In analyses of intact plants, there were not many changes from wild type in the number of these organs and the results are not statistically significant, so this experiment is determined for further studies with more samples.

The individual data are shown in the 'supplements' section in chart 5. The graph shows the comparison of Col-0 to *erf* higher order mutants in the mean of flower organs numbers (Fig 7) The numbers were counted as an arithmetic means of abnormal data. Because of the small number of measurements, the increased numbers of these organs in the graph are shown more likely to explain the hypothesis and support another work.

In Col-0, there are usually 4 sepals and petals and 6 stamens. We observed increased numbers of stamen and in two of the mutants, some flowers had 5 petals or petals of abnormal shape.

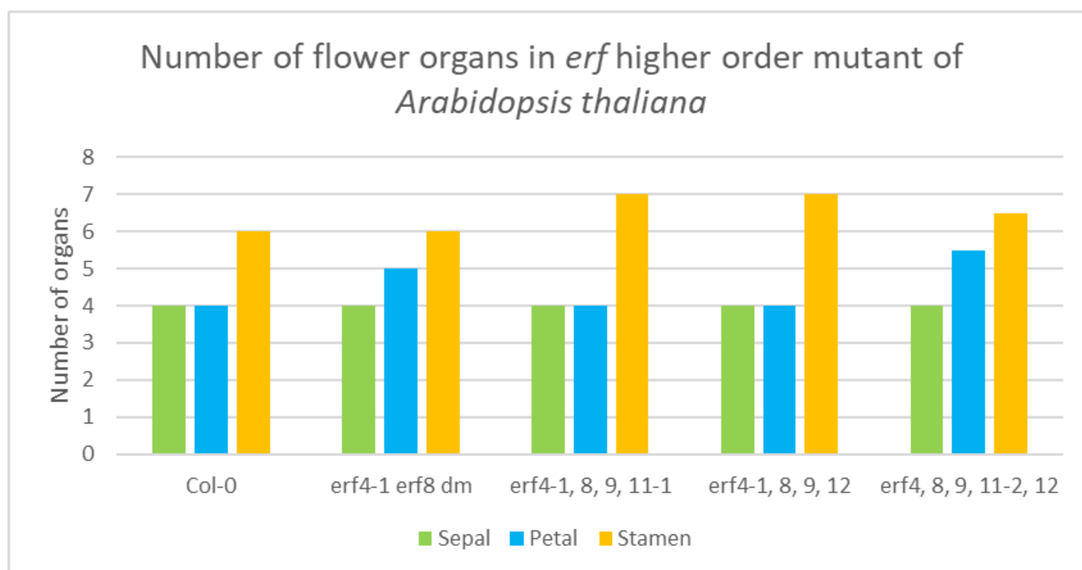

Figure 7 **Number of flower organs in erf higher order mutants** The graph shows statistically unsignificant data where only measurements confirming the hypothesis were considered

According to chart 4, increased numbers of stamen were observed. The photos of the flowers showing phenotype were taken and compared with each other (Fig. 8).

Figure 8 **Arabidopsis Col-0 (right) flower (adapted from Huertas et al., 2019; edited), *erf* 4, 8 (middle) flower showing 5 petals phenotype and *erf* 4, 8, 9, 11 (right) flower showing 7 stamen phenotype**

The other parameter we have remarked was the phyllotaxis. According to Palauqui and Laufs (2011), there is a golden angle of 137,5 degrees. We have found out that in loss-of-function *erf* higher order mutants, there occurs a disturbance in this rule (Fig 9).

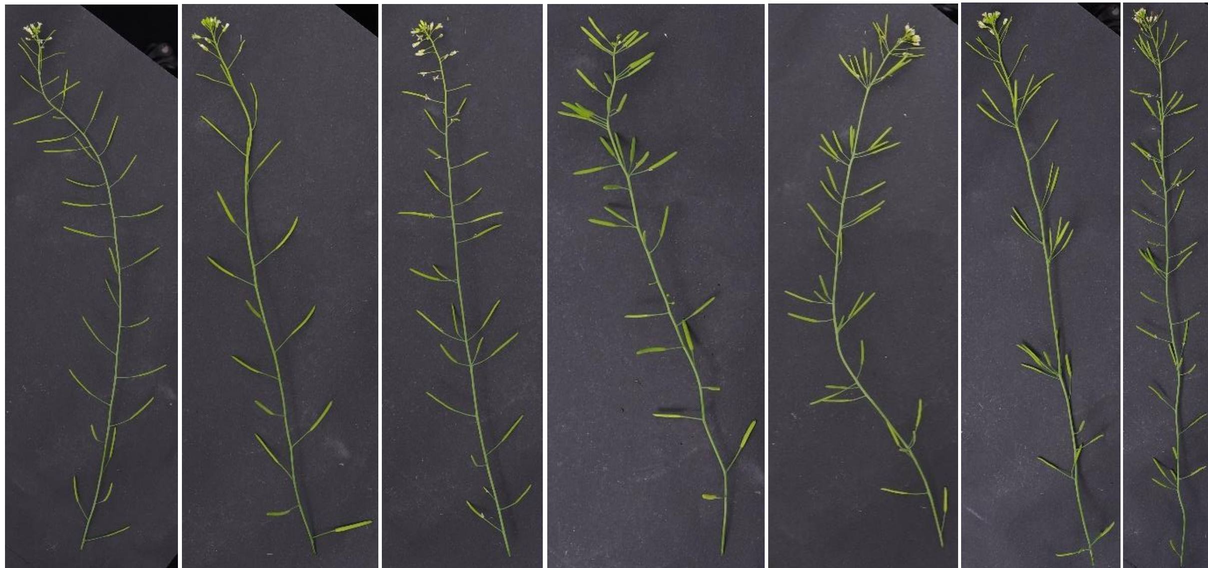

Figure 9 **Disturbance of golden angle in Arabidopsis loss of function erf higher-order mutants** From left: Col-0; *erf* 4, 8; *erf* 9, 12; *erf* 4, 8, 9, 11; *erf* 4, 8, 9, 12; *erf* 4, 8, 9, 11, 12; *erf* 4, 8, 9, 10, 11, 12

It appears that while in *erf* 4, 8 there is no difference from wild type Col-0, in another double mutant, *erf* 9, 12, the disturbance of the golden angle is visible. This confirms that *erf* 9 and *erf* 12 are important transcription factors which play a role in shoot meristem and primordia organization. As visible in fig 9, with an increased number of mutations, the phenotype gets stronger. Another confirmation of the importance of *erf* 9 and 12 is the stronger phenotype in *erf* 4, 8, 9, 12 than in *erf* 4, 8, 9, 11 higher order mutants.

We also noticed that in *erf* higher order mutants, the overall body structure changes a little. The more mutations the plat carries, the smaller it is (dwarf phenotype) (Fig 10). The phenotype is not strong, but visible well in comparison to wild type. The phenotype is more frequent and stronger in transgenic plants.

Figure 10 **Overall body structure of erf higher order mutants (dwarf phenotype)** Plants from left: Col-0; *erf* 4, 8, 9, 12; *erf* 4, 8, 9, 11-1; *erf* 4, 8, 9, 11, 12; *erf* 4, 8, 9, 10, 11, 12

I have performed genotyping of all observed plants to find out if they have the mutations that we were studying. The data found were written into the chart (chart 4) and the pictures of result gels from electrophoresis are in supplements. We tested the presence of genomic DNA and the transgene.

We found that all of the plants from *erf* higher order mutants were homozygous (for some genes, we did not gen the results – possible causes in chapter Discussion). The results from genotyping transgenic plants were more various (chart 5).

Chart 4 The genotype of tested plants. *erf* 4, 8, 9, 10, 11 and 12 genes were genotyped individually.

gene	**plant no.**	**mutant combination**					
		erf 4, 8	*erf* 9, 12	*erf* 4, 8, 9, 11	*erf* 4, 8, 9, 12	*erf* 4, 8, 9, 11, 12	*erf* 4, 8, 9, 10, 11, 12
***erf* 4**	1	-/-		-/-	-/-	-/-	-/-
	2	-/-		-/-	-/-	-/-	-/-
	3	-/-		-/-	-/-	-/-	-/-
	4	-/-		-/-	-/-	-/-	-/-
	5	-/-		-/-	-/-	-/-	-/-
***erf* 8**	1	-/-		-/-	-/-	-/-	-/-
	2	-/-		-/-	-/-	-/-	-/-
	3	-/-		-/-	-/-	-/-	-/-
	4	-/-		-/-	-/-	-/-	-/-
	5	-/-		-/-	-/-	-/-	-/-

gene	plant no.	mutant combination				
		erf 4, 8	*erf* 9, 12	*erf* 4, 8, 9, 11	*erf* 4, 8, 9, 11, 12	*erf* 4, 8, 9, 10, 11, 12
***erf* 9**	1		-/-	no result	-/-	-/-
	2		-/-	no result	-/-	-/-
	3		-/-	no result	-/-	-/-
	4		-/-	no result	-/-	-/-
	5		-/-	no result	-/-	-/-
***erf* 10**	1					-/-
	2					-/-
	3					-/-
	4					-/-
	5					-/-
***erf* 11**	1				-/-	-/-
	2				-/-	-/-
	3				-/-	-/-
	4				-/-	-/-
	5				-/-	-/-
***erf* 12**	1		-/-	-/-	-/-	-/-
	2		-/-	-/-	-/-	-/-
	3		-/-	-/-	-/-	-/-
	4		-/-	-/-	-/-	-/-
	5		-/-	-/-	-/-	-/-

Chart 5 The genotype of pKSVM38: ERF–/AP2 transgenic plants

transgenic plant line pKSVM38	plant no.	genotype
ERF 4 # 2	1	+/-
	2	+/-
	3	-/-
	4	+/-
	5	+/-
ERF 4 # 5	1	+/-
	2	+/-
	3	+/-
	4	+/-
	5	+/-
ERF 4 # 9	1	+/-
	2	+/-
	3	+/-
	4	+/-
	5	+/-
ERF 9 # 1	2	-/-
	3	-/-
	4	-/-
	5	+/-
ERF 9 # 8	1	+/-
ERF 9 # 10	1	-/-
	2	-/-
	3	-/-

transgenic plant line pKSVM38	**plant no.**	**genotype**
ERF 9 # 10	4	+/-
	5	+/-
ERF 11 # 1	1	+/-
	2	+/-
ERF 11 # 2	1	+/-
ERF 12 # 11	1	+/-
	2	+/-
	3	-/-
	4	-/-
	5	-/-
ERF 12 # 12	2	--
	3	-/-
	4	+/-
	5	-/-
ERF 12 # 13	1	-/-
	2	-/-
	3	+/-
	4	+/-
	5	+/-

5.2 Analysis of loss-of-function *erf* higher order mutant phenotypes in *de novo* organogenesis

To elucidate the role of *erf* higher order mutants, we tested these mutants on shoot regeneration. In three time points, I have counted the numbers of regenerated shoots and pin structures. I have worked these data into graphs to see the increase in the numbers (Fig 11).

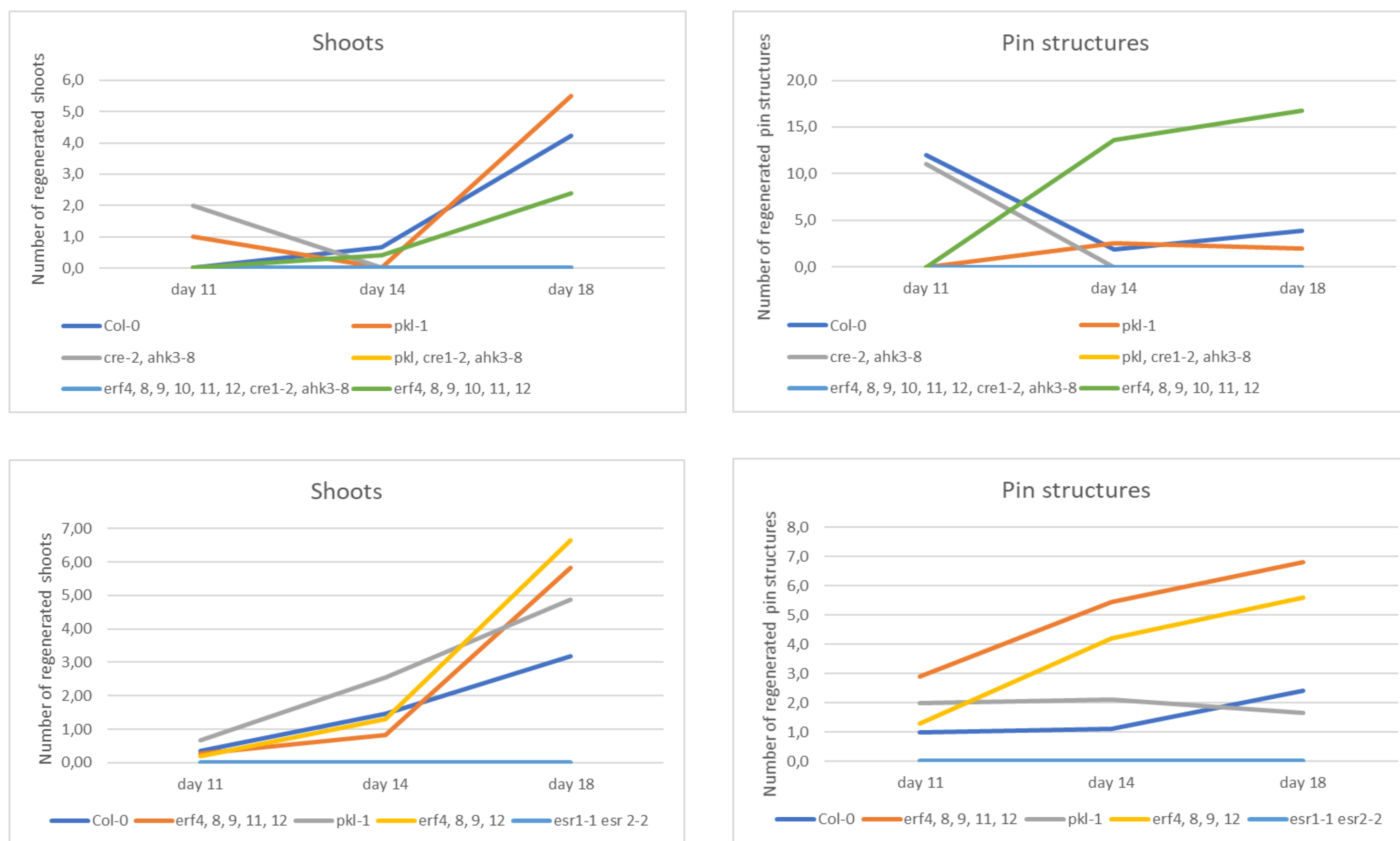

Figure 11 **Regeneration of shoots and pin structures in different loss-of-function erf higher order mutants during 7 days of incubation on SIM**

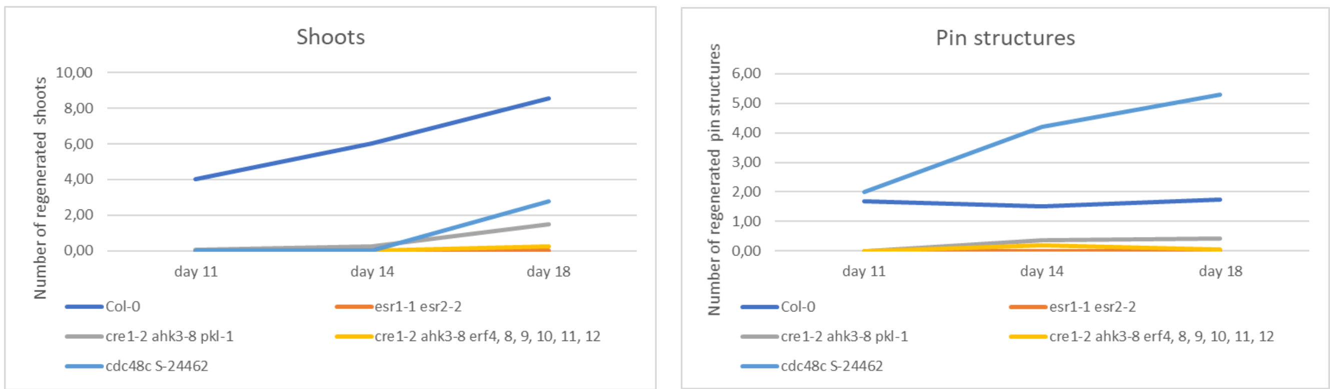

Figure 12 **Continue of fig 10, Regeneration of shoots and pin structures in different loss-of-function erf higher order mutants during 7 days of incubation on SIM**

During the counting of regenerated structures, I have observed two types of structures: shoots and pin structures, which lead us to the information that *erf* genes are responsible for the establishment of shoot apical meristem from which the shoots come. By knocking out the *erf*, there is a problem in the definition of the shoot and so the pin structure is created. The difference between these two structures and their ratio is shown in fig 13.

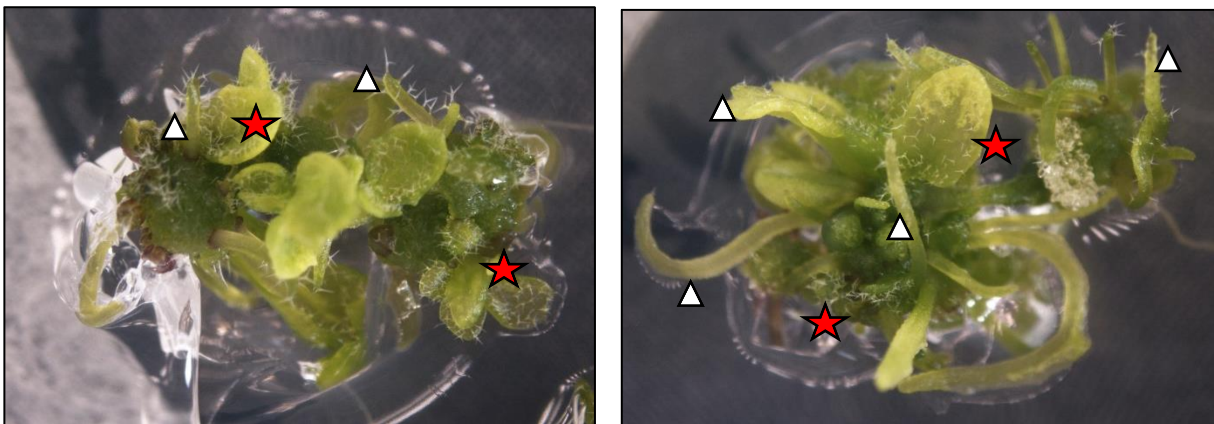

Figure 13 **Regenerated structures observed under microscope** in Col-0 (left) and *erf* 4, 8, 9, 10, 11, 12 (right) during the shoot regeneration assay. The shoots are marked * and pin structures Δ

5.3 Histological analysis of *ERF12* expression by GUS staining

We have looked for the specific tissues where the ERF12 is expressed. After GUS staining we observed the shoot and root tissues individually.

In the shoot part of the plant, the staining was stronger in young tissues, as the second pair of true leaves and part of the shoot apical meristem (Fig 14).

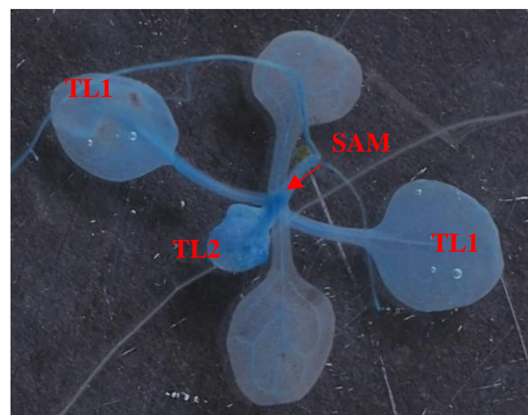

Figure 14 **Histological analysis of pHLGUS ERF12 transgenic plant by GUS staining** C – cotyledon, SAM – shoot apical meristem, TL1–2 – pairs of true leaves

In root tissues, we sow a similar model of stronger staining in younger tissues. In the main root, the expression of ERF12 was observed in the root cap and the QC (Fig 15A). From the lowest part of the root, the staining was localized in pericycle and root primordia (Fig 15B), but the older the root cells were (in the upper parts of the main root), the expression became less specific and appeared in endodermal cells (Fig 15C). In Fig 15C, it is shown that we observed a strange expression. In some of the lateral roots, the signal was strong while in the others, the tip of the roots were completely stainless. The uppermost part of the root was stainless (Fig 15D).

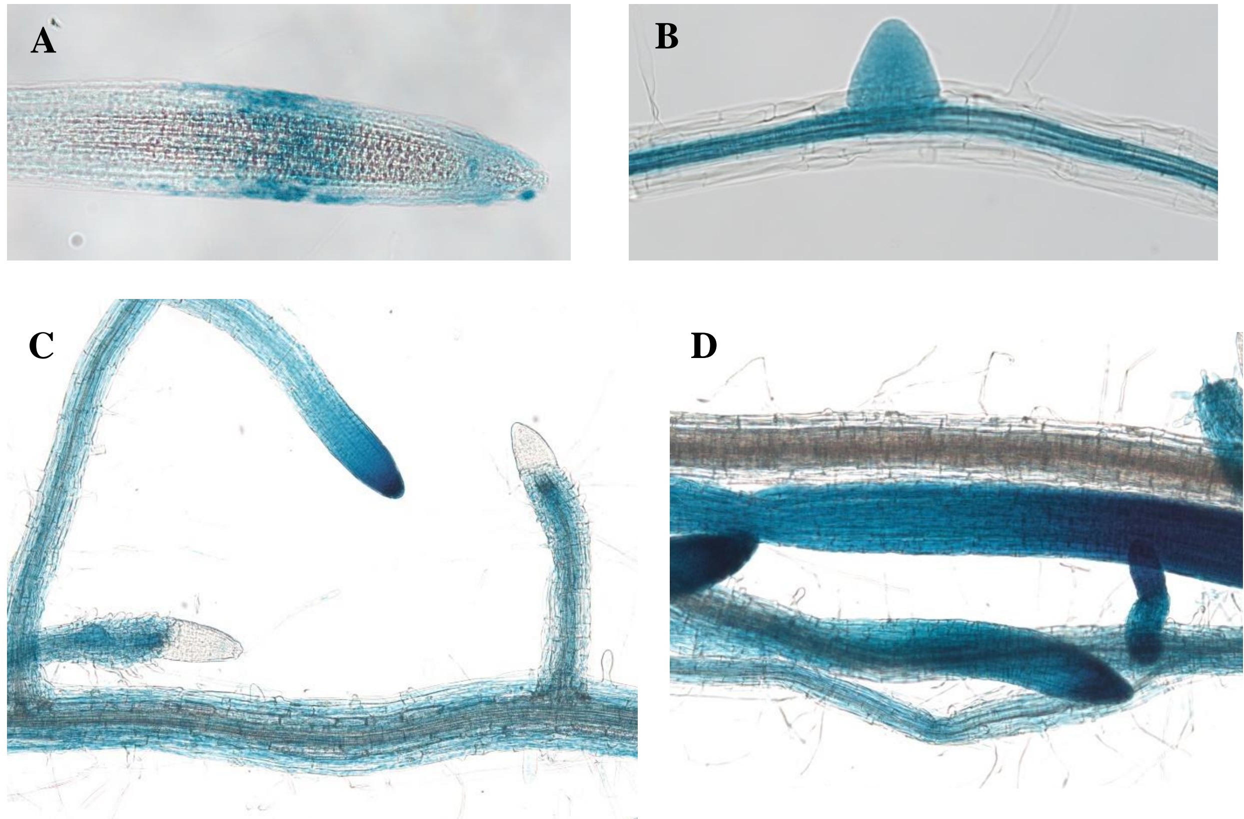

Figure 15 **Histological analysis of ERF12 expression in root tissue by GUS staining** A – Root tip, expression in the root cap and QC; B – The expression of ERF12 in pericycle and lateral root primordia; C – Middle part of the main root with tissue unspecific staining and lateral roots with various expression of ERF12; D – The uppermost part of the main root without staining and lateral roots

6 DISCUSSION

The first of the experiments was phenotypic analyses of intact plants. In previous studies, it was shown that in loss-of-function *erf* higher order mutants, the activity of the meristem was increased. We set a hypothesis that an increased number of flower organs might occur. I have measured 3 – 5 flowers from each plant. In common studies of the phenotype, one mutant is measured a few hundred times, so I have found only a small number of flowers with increased numbers of organs. The reality of the presence of these abnormalities supports the hypothesis.

I have genotyped all the plants for the mutations. Because of lack of time, I was not able to get the results from all the reactions. There are several primers used for genotyping different *erf* transcriptional repressor genes, so some of the reactions did not work. But most of the plants were homozygous or showed a strong phenotype, so we have used all these plants in the experiment. Transgenic plants having pKSVM38: ERF–/AP2 were genotyped too. Their genetic constitutions were more variable because the seeds were from T2 generations, where wild types may occur.

In the shoot regeneration assay, we continued confirming the hypothesis about meristem activity in *erf* mutants. We observed an increased level of regeneration, but also developmental issues. The plants regenerated normal shoots and pin-like structures. This showed that *erf* transcriptional repressors play role in development processes and shoot apical meristem maintenance (and proper organ development).

The histological analyses of ERF12 expression by GUS staining showed some interesting results. We expected the expression in the meristem, which showed to be true. We have also observed that the expression is higher in younger tissues and it is also more specific. In older root tissue the staining faded. This was the first time observing this phenotype so there is not any guessing about why is this happening or what it means. More strange observations were in lateral roots, some of which had very strong staining in the tip but some were completely faint. Again, this is meant for further studies.

7 CONCLUSION

Work aimed to elucidate the role of *erf* and *esr* transcriptional repressors in pluripotency. In the literature review, I have found that these transcription factors can act through cytokinin and auxin signalling pathways and so modulate the main meristems and developmental processes.

In the experimental part, I have used molecular biology methods to find out how *erf* affects the regeneration of the wounded plant, where it is expressed and how it changes the overall body structure of the plant. I observed many phenotype abnormalities such as dwarf phenotype, increased level of regeneration and developmental issues in shoot part.

This leads to the conclusion that *erf* transcription factors play an important role in the life of plants. It affects the crucial processes of survival and its study may move on our knowledge about pluripotency in plants on yet unknown levels.

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9 SUPPLEMENTS

Attachment 1

Chart 6 shows an individual measurement of the flowers from phenotypic analyses summed up on page 26. Interesting data that was used to create the graph (Fig 7) are marked yellow.

Chart 6 Number of flower organs in erf higher order mutants.

Name	Plant	Measurement	Reproductive organ			Stem
		flower no.	Sepal	Petal	Stamen	Phytomers
Col-0	1	1	4	4	6	4
		2	4	4	6	
		3	4	4	5	
	2	1	4	4	6	2
		2	4	4	6	
		3	4	4	6	
	7	1	4	4	6	4
		2	4	4	6	
		3	4	4	5	
		4	4	4	5	
erf4-1 erf8 dm	1	1	4	5	4	3
		2	4	4	4	
		3	4	4	6	
		4	4	4	6	
		5	4	4	6	
	2	1	4	4	6	4
		2	4	4	6	
		3	4	4	6	
	3	1	4	4	6	4
		2	4	4	6	
		3	4	4	6	
	4	1	4	4	6	5
		2	4	4	5	
		3	4	4	5	
		4	4	4	4	
		5	4	4	4	

Name	Plant	Measurement	Reproductive organ			Stamen
		Flower no.	Sepal	Petal	Stamen	Phytomers
erf4-1 erf8 dm	5	1	4	4	6	4
		2	4	4	5	
		3	4	4	6	
		4	4	4	5	
		5	4	4	5	
erf9-1 erf 12 dm	1	1	4	4	4	3
		2	4	4	6	
		3	4	4	6	
		4	4	4	6	
		5	4	4	6	
	2	1	4	4	6	3
		2	4	4	6	
		3	4	4	6	
	3	1	4	4	6	3
		2	4	4	6	
		3	4	4	6	
		4	4	4	6	
		5	4	4	5	
	4	1	4	4	6	3
	5	1	4	4	6	5
		2	4	4	6	
		3	4	4	6	
erf4-1, 8, 9, 11-1	1	1	4	4	6	1
		2	4	4	6	
		3	4	4	7	
		4	4	4	6	
		5	4	4	5	
	2	1	4	4	6	3
		2	4	4	6	
		3	4	4	6	
		4	4	4	6	
	3	1	4	4	6	5
		2	4	4	3	
		3	4	4	5	
		4	4	4	6	
		5	4	4	5	
	5	1	4	4	5	3
		2	4	4	3	
erf4-1, 8, 9, 12	1	1	4	4	6	4
		2	4	4	6	
		3	4	4	5	
		4	4	4	6	
		5	4	4	6	

Name	Plant no.	Measurement	Reproductive organ			Stem
		Flower no.	Sepal	Petal	Stamen	Phytomers
erf4-1, 8, 9, 12	2	1	4	4	6	3
		2	4	4	6	
		3	4	4	6	
	3	1	4	4	6	4
		2	4	4	5	
		3	4	4	6	
		4	4	4	7	
	5	1	4	4	6	3
		2	4	4	6	
		3	4	4	6	
erf4, 8, 9, 11-2, 12	1	1	4	4	6	5
		2	4	7	5	
		3	4	4	6	
		4	4	4	6	
		5	4	4	6	
	2	1	4	4	5	3
		2	4	4	7	
		3	4	4	5	
	5	1	4	4	6	
		2	4	4	5	
		3	4	4	6	
		4	4	4	5	
erf4, 8, 9, 10-2, 11-2, 12	2	1	4	4	6	3
	2	2	4	4	6	3
	3	1	4	4	5	4
	3	2	4	4	5	4
		3	4	4	5	
	4	4	4	4	6	3
		1	4	4	6	
	4	2	4	4	6	3

Attachment 2

Chart 7 Number of flower organs in pKSVM38:ERF–/AP2 transgenic plants.

Name	Line	Plant no.	Measurement flower no.	Reproductive organ			Stem
				Sepal	Petal	Stamen	Phytomers
pKSVM38 ERF4	# 2	3	1	4	4	4	4
			2	4	4	5	
			3	4	4	5	
			4	4	4	6	
			5	4	4	5	
		4	1				4
			2				
		5	1	4	4	4	3
			2	4	4	4	
			3	4	4	5	
	# 5	4	1				4
	# 9	4	1				3
pKSVM38 ERF9	# 1	2	1	4	4	3	3
			2	4	4	0	
			3	4	4	5	
			4	4	4	4	
		3	1				2
			2				
			3				
			4				
		4	1				2
			2				
	# 10	1	1		4	5	3
			2		6	5	
			3		5	6	
			4		5	6	
		2	1		7	2	2
			2		7	4	
		3	1				3
			2				
		4	1				
			2				
pKSVM38 ERF12	# 11	3	1	4	4	4	3
			2	4	4	6	
			3	4	4	5	
			4	4	4	6	
			5	4	4	5	
		4	1	4	4	0	4
			2	4	4	4	
			3	4	4	0	
			4	4	4	5	

Name	Line	Plant no	Measurement flower no.	Reproductive organ			Stem
				Sepal	Petal	Stamen	Phytomers
pKSVM38 ERF12	# 11	5	1	4	4	5	5
			2	4	4	5	
			3	4	4	6	
			4	4	4	5	
			5	4	4	6	
	# 12	2	1	4	4	6	3
			2	4	4	5	
			3	4	4	6	
		5	1	4	4	5	4
			2	4	4	5	
			3	4	4	3	
			4	4	4	6	
pKSVM38 ERF12	# 13	1	1	4	4	4	5
			2	4	4	6	
			3	4	4	5	5
		2	1	4	4	5	3
			2	4	4	5	
			3	4	4	5	
		5	1				3

Attachment 3

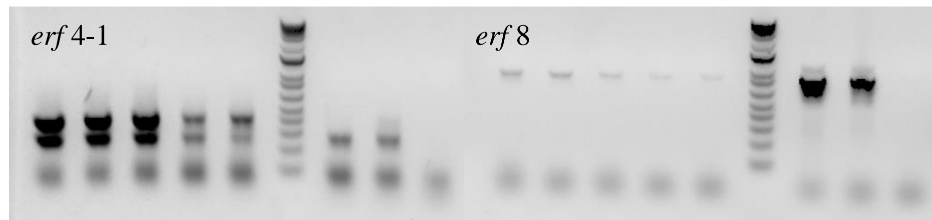

Figure 16 ***erf* 4-1, 8 genotyping** order of pits:1st–5th are samples, molecular weight marker, control (Col-0, +/-, -/-)

Attachment 4

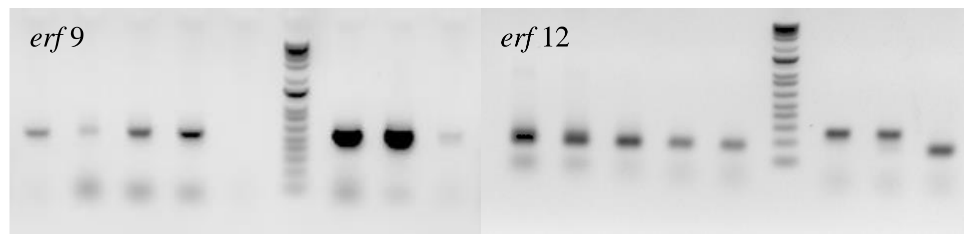

Figure 17 ***erf* 9, 12 genotyping** order of pits:1st–5th are samples, molecular weight marker, control (Col-0, +/-, -/-)

Attachment 5

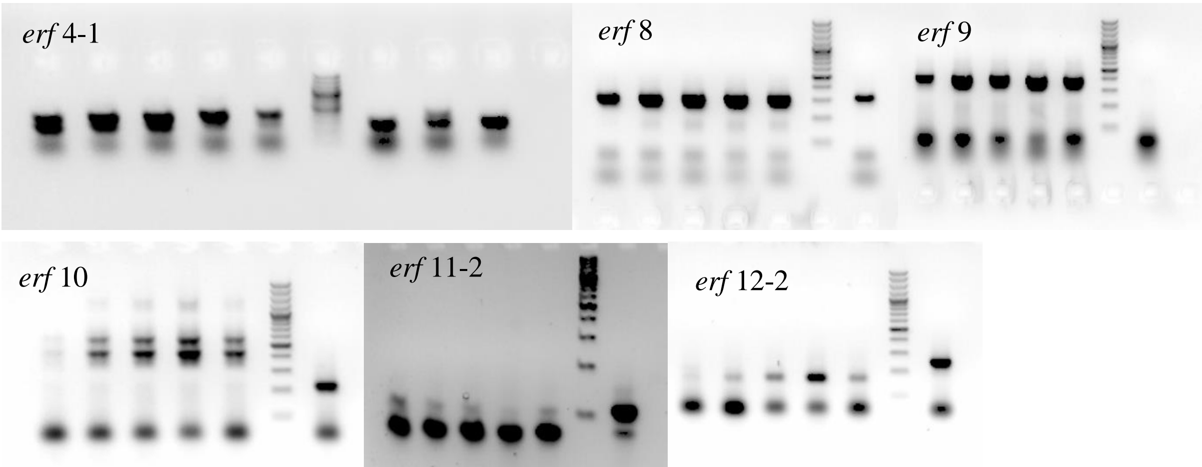

Figure 18 ***erf* 4, 8, 9, 10, 11, 12 genotyping** order of pits:1st–5th are samples, molecular weight marker, control (Col-0, +/-, -/-)

Attachment 6

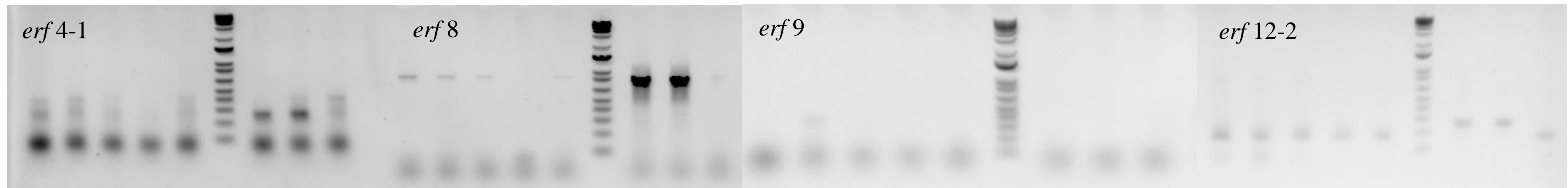

Figure 19 ***erf* 4, 8, 9, 12-2 genotyping** order of pits:1st–5th are samples, molecular weight marker, control (Col-0, +/-, -/-)

Attachment 7

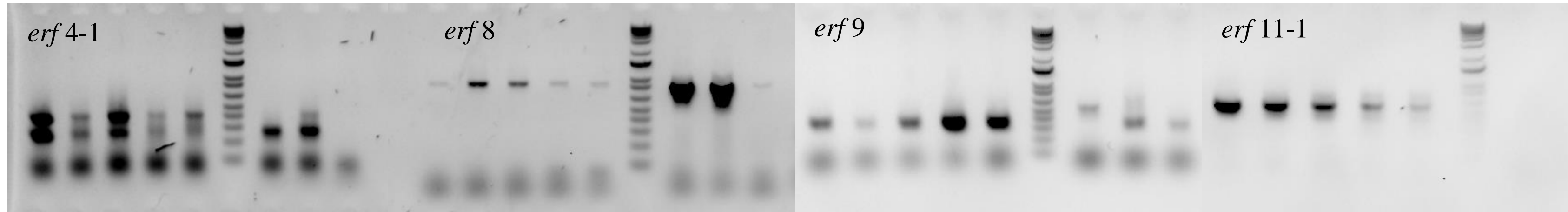

Figure 20 ***erf* 4, 8, 9, 11-1 genotyping** order of pits:1st–5th are samples, molecular weight marker, control (Col-0, +/-, -/-)

Attachment 8

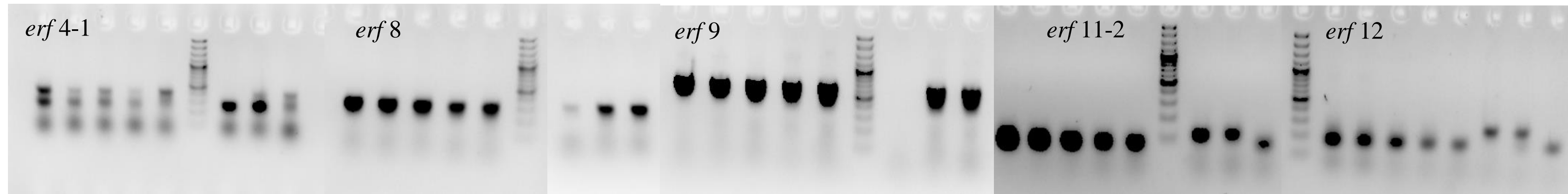

Figure 21 ***erf* 4, 8, 9, 11, 12 genotyping** order of pits:1st–5th are samples, molecular weight marker, control (Col-0, +/-, -/-)

Attachment 9

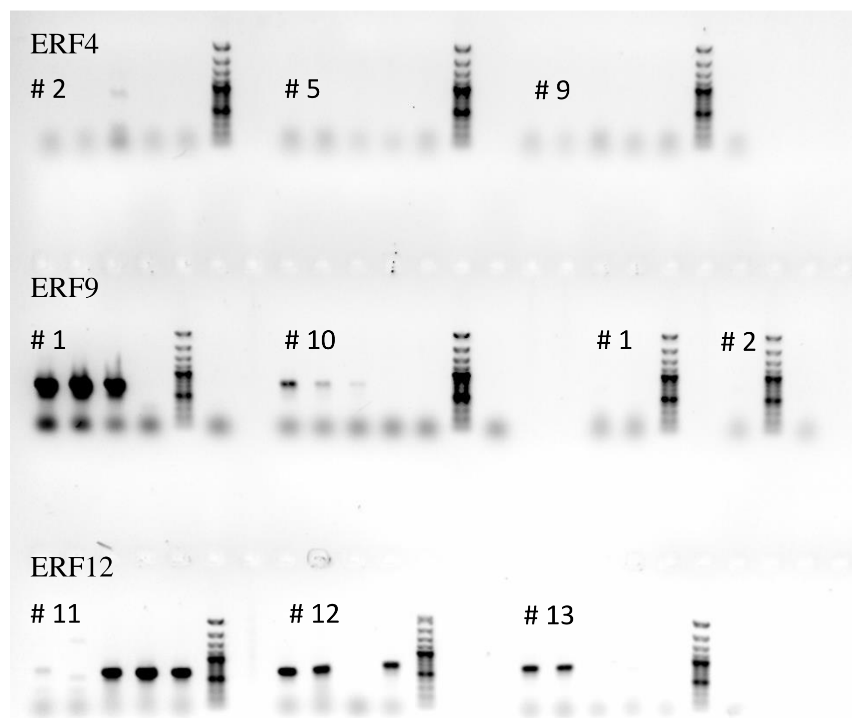

Figure 22 **Transgenic plants pKSVM38: ERF–/AP2 genotyping** order of pits from left, first row:1st–5th are samples, molecular weight marker (M), control (Col-0); 8th–12th are samples, M, control (Col-0, +/-, -/-); second row: 1st–4th are samples, M, Col-0; 8th–12th are samples, M, Col-0; 17th, 18th are sample, M; 21st is sample, M, Col-0; third row: 1st–5th are samples, M; 8th–11th are samples, M; 15th–19th are samples, M, Col-0