Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Christina Michelle Colla

# Functional characterization of cell wall integrity mutants in *Arabidopsis thaliana*

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Supervisor: Thorsten Hamann
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# Abstract

Plants, as stationary organisms, must adapt to their ever-changing environment. The cell wall provides structural support during development and growth, while also acting as a protective barrier against abiotic and biotic stress. To maintain the cell wall integrity (CWI) the plant cell uses a mechanism that monitors and respond to cell wall impairment, known as the CWI maintenance mechanism. Although our understanding of the components and the functioning of this mechanism has advanced, it is still not complete. This project characterized and investigated three pre-selected potential CWI-related genes in *Arabidopsis thaliana*, *BZIP25, CIL1* and *BLUEJAY,* to determine their involvement in CWI maintenance.

First, homozygous plants for T-DNA insertions were isolated and insertion effects verified through gene expression analysis, revealing that all insertion lines, and particularly *bzip25*, exhibited reduced gene expression levels compared to the wildtype. A phenotypic analysis was conducted involving root length in response to cell wall damage (CWD)- and hyperosmotic stress induced using isoxaben and sorbitol. The insertion lines showed significantly reduced root length in non-stressed condition, but no pronounced difference in response to stress treatments, suggesting these genes are involved in root development but not in response to stresses tested. Additionally, a salicylic acid (SA) biosensor analysis was performed to investigate the effect of the insertion lines on SA production induced by isoxaben (a cellulose biosynthesis inhibitor) treatment. Interestingly, *bluejay* seedlings exhibited significantly reduced SA levels compared to wildtype, while *bzip25* and *cil1* showed no significant differences. This suggests that BLUEJAY might play an important role in the SA signaling pathway during stress exposure.

# Sammendrag

Planter, som stasjonære organismer, må tilpasse seg sitt stadig skiftende miljø. Celleveggen gir strukturell støtte under utvikling og vekst, samtidig som den fungerer som en beskyttende barriere mot abiotisk og biotisk stress. For å opprettholde celleveggintegriteten (CWI) bruker plantecellen en mekanisme som overvåker og reagerer på svekkelse av celleveggen, kjent som CWI-vedlikeholds mekanismen. Selv om vår forståelse av komponentene og funksjonen til denne mekanismen har forbedret seg, er den fortsatt ikke fullstendig. I denne studien ble tre forhåndsvalgte og potensielle CWI-relaterte gener i *Arabidopsis thaliana*, *BZIP25*, *CIL1* og *BLUEJAY,* karakterisert og undersøkt for å se om de er involvert i CWI-vedlikehold.

Først ble homozygote planter for T-DNA-innsettinger isolert og effekten av innsettingene ble verifisert gjennom genekspresjonsanalyse, der alle innsettingslinjer, spesielt *bzip25*, viste reduserte genekspresjonsnivåer sammenlignet med villtypen. En fenotypisk analyse ble utført som involverte rotlengde som respons på celleveggskade (CWD)- og hyperosmotisk stress indusert ved bruk av isoxaben (ISX) og sorbitol. Innsettingslinjene viste betydelig redusert rotlengde under ikke-stressende forhold, men ingen tydelig forskjell under stressbehandling, noe som tyder på at disse genene er involverte i rotutvikling, men ikke som respons på testet stress. I tillegg ble det utført en bioanalyse av salisylsyre (SA) for å undersøke effekten av innsettingene på SA-produksjon indusert av behandling med ISX (en cellulosebiosyntese-hemmer). Interessant nok viste *bluejay* frøplanter betydelig reduserte SA-nivåer sammenlignet med villtypen, mens *bzip25* og *cil1* ikke viste noen signifikant forskjell. Dette antyder at BLUEJAY kan spille en viktig rolle i SA-signalveien under stresseksponering.

# Acknowledgment

First and foremost, I would like to thank my supervisor, Thorsten Hamann, for the opportunity to be part of his research group, for great guidance, and for his optimism and encouragement throughout the project. I would also like to thank my co-supervisor, Tereza Tichá, for everything she has taught me in the lab, the valuable learning experience, and for her patience. A big thanks to the members of the Hamann lab for creating an inclusive, fun, and safe environment. Special thanks to Michaela Ticha for additional help in the lab, Luis Alonso Baez for helping me with statistical analyses, Steven Zwartkruis for answering all my questions, and off course, Viktoria Geyer and Bjørnar Alterås for their work assistance.

Additionally, I would like to thank my wonderful friends and study group, my boyfriend, and my lovely family for always being supportive, positive, and loving through both good and challenging times over the past five years. I am very grateful for finishing my education with this project, and I believe it will be helpful for my future profession. Working on this project has provided me with vulnerable knowledge that I hope to use to inspire my future students.

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# List of abbreviations

| | |
|---|---|
| **ACT2** | Actin 2 |
| ***A. thaliana*** | *Arabidopsis thaliana* |
| **BZIP25** | Basic leucine zipper 25 |
| **CESA** | Cellulose synthase |
| **CIL1** | CIB like protein 1 |
| **Col-0** | Columbia-0 |
| ***Cr*RLK** | *Catharanthus roseus* receptor-like kinase |
| **CT** | Cycle threshold |
| **CWD** | Cell wall damage |
| **CWI** | Cell wall integrity |
| **DAG** | Days after germination |
| **GOI** | Gene of interest |
| **HSD** | Honestly Significant Difference |
| **ISX** | Isoxaben |
| **JA** | Jasmonic acid |
| **MS** | Murashige and Skoog |
| **PCR** | Polymerase chain reaction |
| **RT-qPCR** | Reverse transcription quantitative real-time PCR |
| **SA** | Salicylic acid |
| **THE1** | THESEUS1 |
| **T-DNA** | Transfer DNA |
| **TF** | Transcription factor |
| **UBQ10** | Polyubiquitin 10 |

# 1. Introduction

Climate change has resulted in a rise in extreme weather events, such as drought, flooding and extreme temperature, all of which can potentially impact agriculture and crop yields in the future (1). Enhancing our knowledge of plant responses to stress could be used in developing strategies to increase crop production despite more extreme weather (2). Studying the regulatory mechanisms plants use for adaptation to various stressors may contribute to uncover methods to improve food yield (3).

## 1.1 The cell wall

Unlike animals, plants remain stationary and are continually exposed to a wide range of abiotic and biotic stress (4). Nevertheless, they are able to respond to these stressors using mechanisms that allow them to adapt to the continually changing environment (4,5). In both animal and plant cells the extracellular matrix (ECM) plays an important role in defending and supporting the cells, whereas the ECM in plant cells is the cell wall, which acts as a physical barrier (6). This cell wall is highly dynamic, with a complex structure that provides mechanical support during growth and development and acts as a protective barrier against environmental stress (3,7,8). The plant cell wall consist of different polymers, including hemicellulose, pectin and lignin, with cellulose as the main load-bearing element (8,9). Cellulose is synthesized by a cellulose synthase (CESA) complex localized in the plasma membrane, which adds glucose residues to the growing cellulose chain (10). The plant cell wall needs to be flexible during growth, development and in response to abiotic and biotic stress. This flexibility is determined by the composition and organization of its components (7,11). The ability of the plant cell wall to adapt to changes during development and in response to environment interaction is referred to as plasticity (12). The diverse composition of the cell wall reflects its plasticity, with variations in composition and structure observed between different cells, tissues, and also response to various stress conditions (12).

## 1.2 Cell wall integrity maintenance

For the cell wall to adapt to a changing environment (plasticity) the integrity of the cell wall appears to be monitored and regulated through a mechanism called cell wall integrity (CWI) maintenance mechanism (13). During development or when exposed to abiotic and biotic stress, this mechanism monitors and initiates compensatory changes in structure, composition, and cellular metabolism to maintain the CWI. In other words, physical or chemical stimuli is perceived in the cell wall as disturbance/impairment, which is further translated into signals that leads to modifications in the cell wall and cellular metabolism, thereby altering the composition and structure of the cell wall to preserve its integrity (14,15) (Figure 1.1). This modification can be induced by impairment of the cell wall caused by stresses such as wounding, drought or pathogenic infections (14).

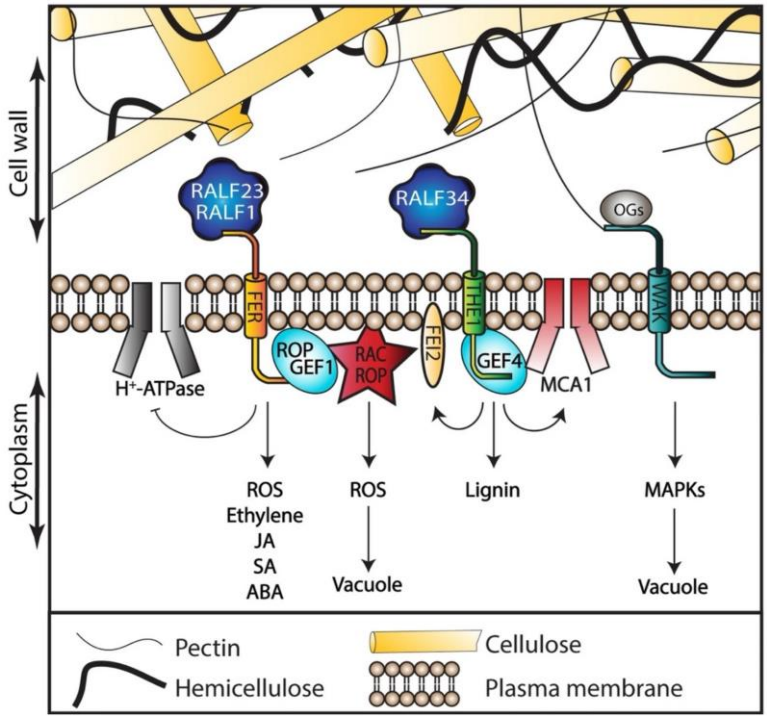


**Figure 1.1:** An overview of different components involved in cell wall integrity (CWI) maintenance in *Arabidopsis thaliana*. Different stimuli, physical or chemical, affect the CWI and are recognized by receptor-like kinases localized in the plasma membrane. This triggers specific defense responses such as downstream signaling events including phytohormone production, lignin accumulation and other modifications to maintain CWI. Figure from Baez et al. 2022 (3).

CWI impairment is detected by receptor-like kinases and ion channels localized in the plasma membrane, which modulate downstream signaling cascade (13). These signals can result in changes in phytohormone production, gene expression and cell wall composition (7,14,16). The activation of signaling processes due to CWI impairment further controls and activates the transcriptional machinery (3) (Figure 1.2). It is likely that CWI maintenance controls the transcriptional regulation of genes related to cell wall metabolism, however, our understanding of how signals precipitated from cell wall damage (CWD) leads to specific transcriptional changes in gene activity seems to be limited (3).

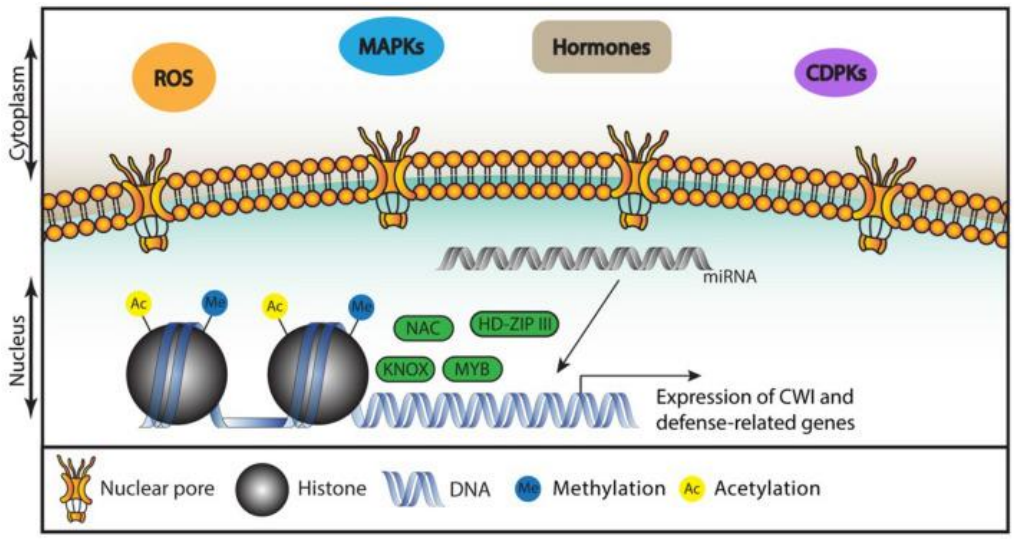


**Figure 1.2:** An overview of transcriptional regulation of genes involved cell wall integrity (CWI) maintenance in *Arabidopsis thaliana*. Generated signals induced by CWI impairment are relayed to the nucleus where the transcriptional machinery is activated and modified, resulting in regulation of genes that mediate responses to maintain CWI. Figure from Baez et al., 2022 (3).

## 1.2.1 Phytohormones

As previously mentioned, phytohormones are involved in CWI maintenance, where phytohormones participate in the downstream signaling cascade by receiving and interpreting signals, ultimately regulating adaptive responses (3). Phytohormones involved are abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (13,16). ABA is usually involved in plant defense against abiotic stress like drought, salinity and heat, while SA, JA and ET are implicated in responses to biotic stress like pathogen

infection (4). However, there are studies showing involvement of other hormones and cross-talk with these in responses to stresses (17,18). The SA pathway is activated by pathogen infection and triggers defense responses throughout the plant to protect undamaged tissue, also including systemic acquired resistance (SAR) (17). SAR is a mechanism that triggers defense that provides prolonged safeguarding against a wide range of microorganisms through accumulation of pathogenesis-related (PR) proteins (19). When plants are exposed to pathogens, SA levels increase, which induces PR proteins, leading to enhanced resistance (17). Mutants that do not respond to SA or are unable to accumulate it effectively are more susceptible to pathogens (4).

### 1.2.2 Growth inhibition

CWI maintenance is essential for both growth and development, as well as for responding to abiotic and biotic stress. However, when the plant is under an attack, the immune response becomes activated, often repressing growth and development (20). The regulation of these responses to stress and during growth appears to be highly intertwined and dynamic (20). Previous studies have shown that induced cell wall stress affects growth, exemplified by inhibition of hypocotyl and root growth (21,22).

### 1.2.3 THESEUS1

An important protein implicated in CWI maintenance is THESEUS1 (THE1), which is a *Catharanthus roseus* receptor-like kinase (*Cr*RLK) that is localized in the plasma membrane (13,20,22). Several THE1 alleles have been studied in detail, including a loss-of-function allele (*the1-1*) and a gain-of-function allele (*the1-4*), which show opposite responses to cellulose deficiency (22). While *the1-1* plants show reduced responses to CWD-induced stress, *the1-4* is producing a truncated THE1 protein that is lacking the kinase domain and exhibits enhanced responses (22) (Figure 1.3). This suggests that while the kinase domain is not required for the activation of THE1, it is necessary for modulating the activity of THE1 (22). Studies involving these mutants have shown that THE1 is activated by CWD-inducing stress, and regulates phytohormones (JA/SA) production, lignin accumulation and growth inhibition (7,16,20,21).

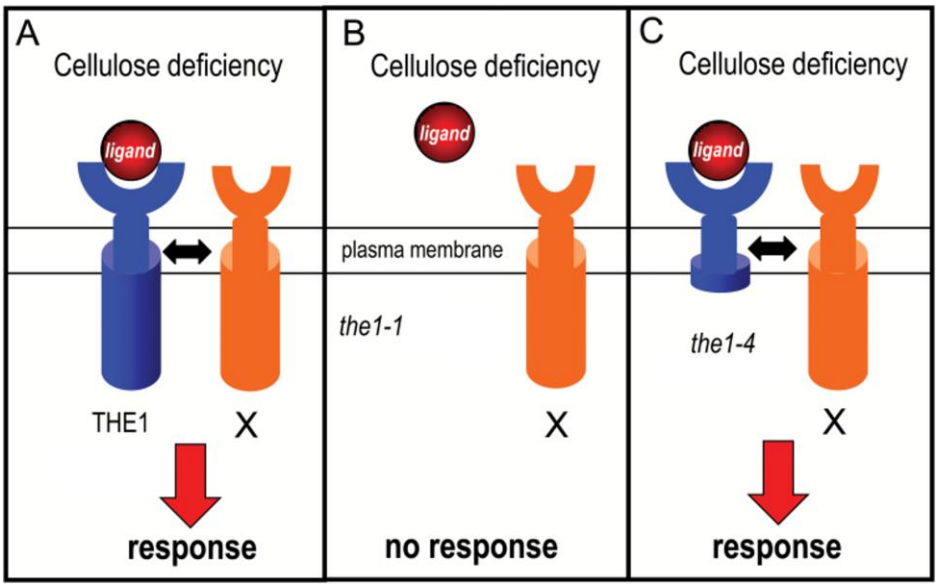


**Figure 1.3:** Responses to cellulose deficiency for THESEUS1 (THE1) loss- and gain-of-function alleles. **A)** In case of cellulose deficiency, a ligand (red) binds to and activates THE1 (blue), leading to a response directly or together with other proteins (orange). Responses includes growth inhibition and hormone production. **B)** Loss-of-function allele (*the1-1*) results in minimal or no expression of THE1, which prevents an efficient response to cellulose deficiency. **C)** Gain-of-function allele (*the1-4*) results in a truncated THE1 protein lacking the cytoplasmic kinase domain, which induces strong responses in cellulose deficiency. Modified figure from Merz et al. 2017 (23).

In previous studies, seedlings from *Arabidopsis thaliana* (*A. thaliana*) have been treated with isoxaben (ISX) and sorbitol to study THE1 activity (7). Isoxaben is a chemical that inhibits cellulose synthesis during primary cell wall formation (24). Thus, ISX treatment seems to cause cell wall damage by weakening the main load-bearing element of the cell wall (14). Mutations in *IXR1* cause resistance to ISX (*ixr1-1*), facilitating the use of such resistance mutants to validate phenotypic effects observed when studying CWI maintenance (14,15,25). Several studies have used ISX to induce cell wall damage to study CWI maintenance (7,16,21,22). Sorbitol, a sugar alcohol, is commonly used to induce hyperosmotic stress (26). Sorbitol induces responses similar to those observed when plants are exposed to drought, and are therefore used to mimic drought (27). Studies have shown that co-treatment with osmotic agent, like sorbitol, suppress response to CWI impairment, due to reduction in turgor pressure (7). Therefore, the use of sorbitol with ISX highlights the importance of turgor pressure during CWI maintenance (22).

## 1.3 Candidate genes

Previous transcriptomics experiments performed by the Hamann research group has identified new potential CWI-maintenance-related genes acting downstream of THE1. RNA sequencing (RNA-Seq) was used to study gene expression in *A. thaliana*. The experiments included three genotypes: Col-0, *the1-1* and *the1-4*. The plants were treated with isoxaben and sorbitol, and the expression levels of genes were analyzed, where those that exhibited opposite expression patterns between *the1-1* and *the1-4* genotypes were selected for further investigations. Three of these genes are *BZIP25* (*AT3G54620, BASIC LEUCINE ZIPPER 25*), *CIL1* (*AT1G68920, CIB LIKE PROTEIN 1*) and *BLUEJAY* (*AT1G14580*) (Table 1.1).

**Table 1.1:** Gene expression of candidate genes, *BZIP25, CIL1* and *BLUEJAY*, in isoxaben (ISX) and sorbitol treated seedlings relative to mock treated for the genotypes: Col-0, *the1-1* and *the1-4*. Data provided by T. Tichá.

| **Treatment** | **ISX** | | | **SORBITOL** | | |
|---|---|---|---|---|---|---|
| **Genotype** | Col-0 | *the1-1* | *the1-4* | Col-0 | *the1-1* | *the1-4* |
| ***BZIP25*** | -3.42191 | -4.51988 | 4.51220 | -3.40361 | -4.52715 | 0.381610 |
| ***CIL1*** | 4.20729 | 2.35697 | -2.40846 | -0.007139 | 3.74293 | 0.073541 |
| ***BLUEJAY*** | -1.93444 | 1.61218 | -2.01875 | -0.666739 | 2.90123 | -1.58279 |

*BZIP25* encodes a transcription factor (TF) that is a member of the basic leucine zipper (bZIP) protein family, which is characterized by a basic region for binding DNA and a leucine zipper region to dimerize proteins (28). This bZIP protein family is involved in regulation of stress- and development related processes. This includes members that has been implicated in metabolic changes for adaptations to energy and nutrient deficiency, in addition to growth and development responses such as activation of seed maturation genes (29). Similarly, *CIL1* encodes a basic helix-loop-helix (bHLH) protein, a member of a TF family that contain a basic-helix-loop-helix domain with a basic DNA binding domain at the N-terminus and a domain region for protein interaction at the C-terminus (28). Member of this TF family are implicated in response to drought and cold, and development such as

cell elongation and lateral root initiation (30). Both the bZIP and bHLH families are among the six major TF families, known for their conserved DNA binding domain and roles in development and defense responses (28). *BLUEJAY* encodes a $C_2H_2$-like zinc finger protein, a member of a subclass of the zing finger protein (ZFP) family, characterized by containing a DNA-binding motif with one zinc ion, two cysteine (cys) and two histidine (his) residue arranged in a tetrahedral structure (31). The ZFP family is also involved in growth and development, and respond to both abiotic and biotic stress (31). Several $C_2H_2$ zing finger proteins seem to be implicated in multiple responses to stress, including temperature, drought, osmoregulation and salinity levels (32). Current knowledge about $C_2H_2$ zinc finger proteins role in defense responses seems to be limited (32). Common to these three genes is that they all encode for TFs that belongs to families involved in regulating responses to development and stress.

Transfer DNA (T-DNA) insertion is a great tool to use for studying the gene function by observing the phenotype of the induced-mutants (33). Previous studies have performed genome-wide analysis to generate a large collection of sequence-indexed insertion mutants in *A. thaliana*, indicating a great correlation between the number of genes that were disrupted and the number of ethylene-regulated genes that were induced or repressed (34). The candidate genes, *BZIP25, CIL1* and *BLUEJY* containing T-DNA insertions SALK_119931C, SALK_087424C and SALK_208894C, respectively, is illustrated in Figure 1.4.

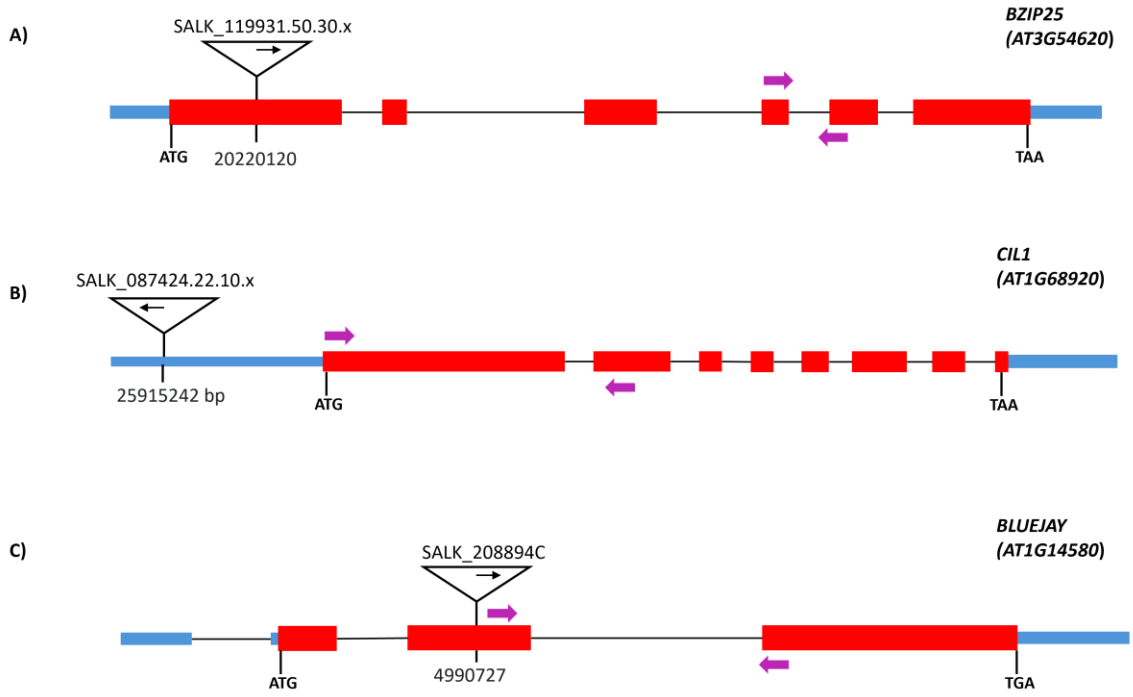


**Figure 1.4:** An overview of the T-DNA insertion mutation in candidate genes. The candidate genes BZIP25 (**A**), CIL1 (**B**) and BLUEJAY (**C**) of *Arabidopsis thaliana*, including the T-DNA insertion. Colors represent UTRs (blue), exons (red) and introns (black lines), while triangles indicate insertion site (including coordinate in base pair) with arrows indicating the direction of the insertion. The purple arrows represent binding sites for primers used in qPCR.

## 1.4 Gene expression- and SA biosensor analyses

A useful technique to quantify gene expression is RT-qPCR which stands for reverse transcription quantitative real-time polymerase chain reaction (35). This technique is a combination of reverse transcription of RNA to cDNA and quantitative PCR, which enables the measurement of RNA levels (35). This allows investigation of changes in gene expression across different genotypes and under different conditions/treatments. RT-qPCR involves synthesis of cDNA through revers transcription PCR where pre-isolated RNA is amplified and converted to cDNA (35). This can either be done together with quantification or it can be in a separate step before. During qPCR the cDNA of interest is amplified using specific primers and quantified with an intercalating dye that emits a fluorescent signal when it binds to newly synthesizes DNA (35). This fluorescent signal is monitored in real time, allowing continuous measurement of DNA amplification, and results in an

amplification curve. As the amount of amplified product increases, so does the fluorescent signal, and eventually the signal will reach a level significantly higher than the baseline (threshold level) (35). When the product of the sample reach this threshold, it is recorded and designated as cycle threshold (CT) value, which can be used to calculate the relative gene expression level by using the $2^{-\Delta\Delta CT}$-method (35).

The $2^{-\Delta\Delta CT}$-method relays on the use of a reference gene, also called housekeeping gene, for normalization (35,36). First the ΔCT is calculated by removing the CT of reference gene from CT of gene of interest (GOI). Then, ΔΔCT is calculated by removing the ΔCT of control sample from the ΔCT of target sample (36). Control could be mock (treatment) or wildtype (genotype), depending on weather the expression levels are compared within treatments or between genotypes. The final value represents a fold change in the expression of GOI in target sample relative to control sample. The $2^{-\Delta\Delta CT}$-method presumes the efficiency of the PCR amplification to be 100%, where the value 2 indicated doubling of product each amplification cycle (36).

Salicylic acid (SA) accumulation, as mentioned previously, is part of the defense response observed in plants. Mutants that are unable to accumulate SA or do not response to SA have shown enhanced susceptibility to pathogens (4). Studying SA accumulation in mutants and investigating how they affect SA production enables the identification of genes that might be important in SA production during defense responses. One way to measure the amount of SA is by using the biosensor *Acinetobacter sp*. ADPWH_*lux*, developed by Huang et al. (37). This biosensor takes advantage of the chromosomal integration of foreign DNA fragments in *Acinetobacter sp*. ADP1. The bacterium has been engineered to detect SA and function as a biosensor by integrating a salicylate-inducible CDABE*lux* operon (37). In the presence of SA, the *LUX* reporter gene will be expressed, leading to production of bioluminescence that can be detected by a luminescence reader, providing an indirect measurement of SA concentration in the medium in which the bacterial cells are growing.

## 1.5 Objectives

In previous transcriptomics experiment, potential CWI-related genes acting downstream of THE1 were identified. Three of these genes, *BZIP25, CIL1* and *BLUEJAY*, have been chosen for this project with the hypothesis that these genes are involved in CWI maintenance. Therefore, the aim of this thesis is to characterize them and investigate their potential role in CWI maintenance. This will involve isolating plants homozygous for T-DNA insertions in the genes of interest, studying whether the insertions in the candidate genes impact gene expression, and determining if they cause mutant phenotypes with respect to root growth and salicylic acid (SA) production under stress conditions induced by isoxaben and sorbitol treatments. The responses observed for the insertion lines will be compared to those in wildtype and THE1 mutant lines (*the1-1* and *the1-4*). Ultimately, this research will contribute to a better understanding of the function of these genes in CWI maintenance.

# 2. Materials and methods

All chemical reagents used are from Sigma Aldrich unless anything else is stated.

## 2.1 Plant material

Seeds from *Arabidopsis thaliana* ecotype Columbia 0 (Col-0) and the following genotypes in the Col-0 background were used: *bzip25* (SALK_119931C), *cil1* (SALK_087424C) *bluejay* (SALK_208894C), *the1-1* ($G^{37}D$), *the1-4* (SAIL_683_H03) and *ixr1-1* (DH47).

## 2.2 Genotyping

Genotyping was preformed to identify homozygous mutant plants, which were then used for propagation of seeds for use in further experiments.

### 2.2.1 Sterilization of seeds

Seeds were sterilized with 1 mL of 70 % EtOH (VWR) in 1.5 mL Eppendorf tubes and mixed for 2 minutes before the supernatant was removed by pipetting. Then 1 mL of 50 % bleach (Klorin) was added before tubes were mixed by flipping a few times and incubated for approximately 8 minutes before the bleach was removed. The seeds were washed three times with 1 mL Milli-Q water each time and the tubes were flipped to disperse the seeds to make sure all the bleach were removed. After the last washing step, the Milli-Q water was replaced with clean 300 µL Milli-Q water for easier planting of seeds.

### 2.2.2 Growing seeds on plates

Approximately 20 sterilized seeds were placed in two rows on plates with 50 mL ½ Murashige and Skoog (MS) growth media (1.1 g MS, 0.25 g MES salt, 5 % sucrose, pH 5.75 adjusted with HCl) and 0,8 % Gellan Gum (Thermo Fisher). The lids of the plates were sealed with parafilm and tape, and the plates were covered in aluminum foil before they were stratified in dark at 4 °C for two days. The plates were then placed in a growth

chamber (16 hour daylight at 150 µmol $m^{-2}s^{-1}$ light intensity at 22 °C / 8 hour dark at 18 °C) for up to 12 days.

### 2.2.3 DNA extraction and Polymerase Chain Reaction (PCR)

For DNA extraction and the polymerase chain reaction (PCR) Thermo Scientific Phire Plant Direct PCR Master Mix were used. 9 days after germination (DAG) the DNA was extracted by cutting a leaf of each seedling and crushing it with a pipet tip in 50 µL dilution buffer (Thermo Scientific) in a 1.5 mL Eppendorf tubes according to the protocol of the kit. Master mixes were prepared on ice with 10 µL of 2x Plant master mix, 1 µL of each primer and 7 µL of $H_2O$ for each sample. Three master mixes were prepared containing: A) forward and reverse primers for GOI, B) forward primer for GOI and LBb1.3 primer for the insertion, and C) reverse primer for GOI and LBb1.3 primer for the insertion (see Table 2.1 for primer list). The primers used for PCR was designed using SIGnAL T-DNA Primer Design tool (38). 19 µL of the master mixes were added in different wells on a 96-well PCR plate, before 1 µL of the template DNA was added to each well, in addition to negative controls. The plate was sealed, mixed by vortex, and centrifuged before placed in a thermocycler (Bio-Rad T100™ Thermal Cycler). The initial denaturation was at 98 °C 30 seconds, while the denaturation, annealing and elongation was respectively 98 °C for 5 seconds, 58 °C for 5 seconds, 72 °C for 30 seconds, and these were repeated x 40. The final elongation was at 72 °C for 2 minutes, with the samples being held afterwards at 12 °C.

**Table 2.1:** Sequences for primers used in PCR for the genes of interest and T-DNA insertion.

| Name | Type of primer | Sequence (5'-3') |
|---|---|---|
| ***BZIP25*** | Forward | GTCAGGCTATGTTTGCAGCTC |
| | Revers | ACAAAGCAACGATACGTGACC |
| ***CIL1*** | Forward | TAACACAGGGCAATGGAAAAG |
| | Revers | TGCTGAAAACTCATCTTTCGC |
| ***BLUEJAY*** | Forward | AAAAATAACCCCTGACATGCC |
| | Revers | AGCGGGGAATTAGAAGAACAG |
| **LBb1.3** | Left border | ATTTTGCCGATTTCGGAAC |

### 2.2.4 Gel electrophoresis

1x TAE buffer (VWR) was mixed with agarose for a final concentration of 1 % and heated up in a microwave. After 5 minutes of cooling down SYBER$^{TM}$ Safe DNA Gel Stain (10x, Thermo Fisher Scientific) was added and the agarose gel was poured into the mold (25 cm x 25 cm) followed by insertion of the combs. After 1 hour the gel had settled and combs were removed before the tray with the gel was transferred to a gel electrophoresis chamber containing 1x TAE buffer. 1 µL of each sample was loaded to each well in addition to the GeneRuler 1kb Plus ladder (Thermo Fisher). Gels were run at 130 V for approximately 1.5 hour. Next, a G:Box Chemi XRQ (Syngene) was used to image the gels.

### 2.2.5 Propagation of seeds

After genotyping, homozygous seedlings were selected and planted in pots containing autoclaved soil (Emmaljunga Så-JORD, Torvmull AB). In each pot 1-3 seedlings were planted, pots were covered with lids and placed in a growth room (16 hour daylight at 150 µmol $m^{-2}s^{-1}$ light intensity at 22 °C / 8 hour dark at 18 °C) for two days before the lids were removed. The plants were watered every 4 days and grown for 9 weeks before seeds were collected.

## 2.3 Gene expression analysis

RT-qPCR was used to investigate the expression of genes of interest to determine if the insertions affected gene expression.

### 2.3.1 Growing plant tissue in liquid media

Seed were sterilized (section 2.2.1), and approximately 30 mg of sterilized seeds were transferred to Erlenmeyer flasks (250 mL) with autoclaved 125 mL ½ MS growth media. The flasks were stratified for two days at 4 °C in dark before they were transferred to a growth chamber (section 2.2.2) and grown on a shaker (IKA® KS 501 digital) at 120 rpm. Tissue from 7 days old seedlings was collected in Eppendorf tubes (2 mL) containing a sterile metal bead and placed in liquid nitrogen before long-term storage at -80 °.

### 2.3.2 RNA extraction and purification

A Plant RNA Kit (Omega Bio-Tek) was used for RNA isolation and preformed according to the protocol for RNA Extraction and Purification from Fresh or Frozen Plant Tissue. Samples were homogenized using a TissueLyser II (QIAGEN), before they were stored in liquid nitrogen. 500 µL RNA binding (RB) buffer containing 10 µL 2-mercaptoetanol was added, sample was vortex and pipetted onto a Homogenizer Mini Column in a collection tube (2 mL). The tubes with the columns were centrifuged at 13000 x g for 5 minutes at room temperature before the columns were removed. 70 % ethanol were added to the flowthrough (1:1), the mix was vortexed and centrifuged for 20 seconds. 700 µL of sample were transferred to a HiBind RNA Mini Column inserted in a collection tube (2 mL), before centrifuged at 12000 x g for 1 minute. The flowthrough was discarded, and the step repeated with the rest of the sample. 250 µL RNA Wash Buffer I was added and centrifuged at 10000 x g for 1 minute before flowthrough was discarded and 75 µL DNase I stock solution was added to membrane of the column and incubated for 15 minutes at room temperature. 250 µL RNA Wash Buffer I was added and incubated for 2 minutes before samples were centrifuged and flowthrough discarded.

Next, 700 µL RNA Wash Buffer II diluted with 100 % ethanol was added to the column, centrifuged at 10000 x g for 1 minute and flowthrough discarded. This was repeated with 500 µL RNA Wash Buffer II. Then the columns in the collection tubes were centrifuged at maximum speed for 3 minutes to dry the columns. The HiBind RNA Mini Column was transferred to a new Eppendorf tube (1.5 mL) and 50 µL Nuclease-Free Water was added to the membrane of the column before being centrifuged at maximum speed for 1 minute. The eluted RNA was placed on ice and the concentration was measured using a NanoDrop One (Thermo Fisher Scientific) before storage at -80 °C.

### 2.3.3 RT-qPCR

The cDNA synthesis preparation was performed on ice. The RNA concentrations for the samples were standardized (1000 ng/µL) and a final volume of 5 µL purified RNA was added to each well of an autoclaved and sterilized 96-well PCR plate. 15 µL cDNA synthesis master mix (4 µL buffer, 2 µL $MgCl_2$, 1.25 µL dNTPs (10 µM), 0.5 µL random

primer, 5.75 µL RNase-free water, 0.5 µL oligo(dT)15 primer and 1 µL ImProm-II™ Reverse Transcriptase (Promega)) was added to each well. The plate was then placed in a thermocycler (Bio-Rad T100™ Thermal Cycler) and run using the following program: 25 °C for 15 minutes, 42 °C for 60 minutes, 75 °C for 15 minutes followed by hold at 4 °C. The cDNA was then diluted by adding 180 µL Milli-Q water. The plate was sealed with a lid and vortexed before being centrifuged, followed by measurement of cDNA concentration using NanoDrop One (Thermo Fisher Scientific) to ensure that each well had approximately the same concentration before running the qPCR.

The qPCR-primers for genes of interest were designed using the PrimerQuest™ Tool from Integrated DNA technologies (39) (Table 2.2). The primers were blasted using TAIR BLAST 2.9.0+ to confirm specificity (40). Master mixes for qPCR reactions for the genes of interest and two housekeeping genes *ACTIN2* (*ACT2)* and *POLYUBIQUITIN 10* (*UBQ10*) were prepared for each sample (3 µL Milli-Q water, 1 µL of each primer (5 µM), and 10 µL SYBER® Green I Master). 15 µL of the master mixes were transferred to particular wells on a 96-well qPCR plate sitting on ice, along with 5 µL of the samples (*bzip25, cil1, bluejay* and Col-0). The plate was sealed with qPCR film, vortexed and centrifuged at 15000 x g for 2 minutes, before being placed in a LightCycler 96 (Roche). The program used was preincubation at 95 °C for 10 minutes, while denaturation, annealing and extinction respectively were at 95 °C for 30 seconds, 60 °C for 25 seconds, 72 °C for 25 seconds with x 45 repeats. Lastly the melting curve was performed at 95 °C for 5 seconds, 60 °C for 60 seconds and 95 °C for 1 seconds.

**Table 2.2:** Sequences for primers used for qPCR for the genes of interest and housekeeping genes.

| Name | Type of primer | Sequence (5'-3') |
|---|---|---|
| ***BLUEJAY*** | Forward | AGCACGGTGAGAAAAAGTGG |
| | Reverse | GGGTTTCTTGCTGACTCCTG |
| ***CIL1*** | Forward | GGTGGTAATGGTCAGAAAGG |
| | Reverse | TTTGTTTGCCCTGTTGTTTC |
| ***BZIP25*** | Forward | CACGCTTCACATCAGTAGG |
| | Reverse | AGTGCTTCAGCTAGCAATC |

| | | |
|---|---|---|
| ***ACT2*** | Forward | CTTGCACCAAGCAGCATGAA |
| | Reverse | CCGATCCAGACACTGTACTTCCTT |
| ***UBQ10*** | Forward | AACTTTCTCTCAATTCTCTCTACC |
| | Reverse | CCACGGAGCCTGAGGACCAAGTGG |

## 2.4 Phenotyping

Measurements of root lengths for the different genotypes (controls and insertion lines) were performed under different conditions to determine if the genes of interest are required for root growth/development responses to different stress. The same procedure was performed 4 times (different biological replicates).

### 2.4.1 Stress treatments

Three treatments were prepared for each genotype: control, ISX and sorbitol. The genotypes tested included controls (Col-0, *the1-1, the1-4* and *ixr1-1*), *bzip25, cil1* and *bluejay.* ½ MS growth media was prepared as described before (Section 2.2.2). For ISX treatment, 50 mL of ½ MS growth media was mixed with 2.5 µL ISX (20 µM) in a Falcon tube and then poured onto each plate for a final concentration of 1 nM ISX. To determine seedling response to hyper-osmotic stress 500 mL ½ MS growth media was prepared as described before (Section 2.2.2), including 18.21 g sorbitol for a final concentration of 200 mM.

### 2.4.2 Planting and measurement of root length

The seeds from each genotype were sterilized with ethanol and bleach (Section 2.2.1), but here close to 50 seeds were plated on each plate in two rows. The plates were imaged on 5, 6, 7, 8 and 9 DAG together with a ruler using a scanner (Ricoh IM C4500). The seedling root lengths of each genotype and treatment were measured using ImageJ. The scale bar was set at 1 cm by tracing the ruler used for normalization. Each root length was manually measured by tracing the roots of the seedlings, and the average length was calculated using excel.

## 2.5 Salicylic acid biosensor

The outmost rows of an ethanol-sterilized 96-well PCR plate were filled with 300 µL Milli-Q water, while the rest of the wells were filled with 150 µL ¼ MS5 (0.275 g MS, 0.125 g MES and 0.5 % glucose) solution. Seeds were sterilized with ethanol and bleach (Section 2.2.1) and transferred on to glass plates with sterile filter paper to dry. One seed was transferred to each well with media, before the PCR plate was sealed with film and wrapped in aluminum foil for stratification for two days (dark at 4 °C). Next the plate was placed in a growth room (125 µml $m^{-2}s^{-1}$ light at 22°C) for seven days. 5 DAG, 20 µL ¼ MS0 (0.275 g MS, 0.125 g MES and 0 % glucose) mixed with 6.7 µL isxoaben (600 µL) for a final concentration of 200 nM was added to the wells with seeds before the plate was sealed and placed back to grow for another 24 hours.

A single colony of *Acinetobacter sp.* ADPWH_*lux* was inoculated in 10 mL Luria-Bertani (LB) media for 1 day (37 °C), before diluted (1:20) and grown for 2 hours (37 °C). Then, 50 µL of the media used to grow seedlings treated for 24 hours were combined with 50 µL of bacterial culture into a white 96-well flat-bottom plate and incubated for 60 minutes. The luminescence was measured using a Cytation$^{TM}$ 5 imaging reader (BioTek) with the following settings: Set temperature with setpoint of 37°C, gradient 0°C and preheat before moving to next step, integration time on 0:01.00 (MM:SS.ss), Filter set 1, emission: full light, Top optics and gain: 135, normal read speed with delay: 100 msec, extended dynamic range and read height of 1 mm.

## 2.6 Data analysis

### 2.6.1 Calculations of relative expression of genes of interest

To visualize the average relative fold change in gene expression for the insertion lines compared to Col-0, the $2^{-\Delta\Delta CT}$-method was used. All calculations were performed in excel.

For each biological replicate, the average CT values for the technical replicates were calculated for both the gene of interest (*BZIP25, CIL1* and *BLUEJAY)* and the reference gene (*ACT2*) across all genotypes.

The ΔCT was calculated by subtracting the average CT value for the reference gene from the average CT value for the gene of interest. This was carried out for the insertion lines and their respective genes, as well as for Col-0 and all the genes of interest.

Subsequently, the ΔΔCT was calculated by subtracting the ΔCT for Col-0 from ΔCT for genes of interest. Finally, the relative gene expression of *BZIP25, CIL1* and *BLUEJAY* were calculated by using the ΔΔCT for each gene of interest in the equation: $2^{-\Delta\Delta CT}$, where 2 was set as the primer efficiency.

### 2.6.2 Statistical analysis

For the relative expression of genes of interest, a student's t-test was performed among *bzip25, cil1, bluejay,* and Col-0. Similarly, a student's t-test was performed for the amount of luminescence normalized to Col-0 between *the1-1, the1-4, bzip25, cil1, bluejay,* and Col-0. Additionally, a one-way ANOVA and post hoc Tukey HSD (Honestly Significant Difference) test was carried out for the root lengths across each genotype and treatment. All p-values are provided in Appendix 2.

## 2.7 Use of AI

ChatGPT has been used as an aid in parts of the text for inspiration purposes. The tool has been used to improve self-written text to make the flow of the text better. It has also been used as inspiration for synonyms.

# 3. Results

In this section, the key findings obtained over the course of the project are going to be presented to give a deeper understanding of the characterization and the potential role in CWI maintenance of genes of interest: *BZIP25, CIL1 and BLUEJAY*.

## 3.1 Genotyping

To investigate the role of the candidate genes in CWI maintenance, it was necessary to establish if the T-DNA insertions were at the intended position in the candidate genes before proceeding with further experiments. A genotyping approach involving PCR was used for this purpose. Images of the gels from the gel electrophoresis (1 % agarose) of the PCR product for *bzip25, cil1* and *bluejay* are presented in Figure 3.1, 3.2 and 3.3, respectively. For *bzip25* all plants exanimated were homozygous since PCR reactions only produced fragments indicating the presence of the insertion and no wildtype fragment (bands shown in Figure 3.1A, B). For *cil1* plants 2-4, 8, 10, 12, 14-17 and 19-24 were homozygous for the insertion due to only producing fragments for the insertion and not wildtype fragments in the PCR reactions (bands shown in Figure 3.2A, B). Same applied for *bluejay*, where plants 1, 5-9, 11-14, 16-21, 23 and 24 were homozygous with the insertion (bands shown in Figure 3.3A, B). For the insertion lines *bzip25, cil1* and *bluejay*, there were plants exhibit homozygous plants containing the intended T-DNA insertions at the expected position.

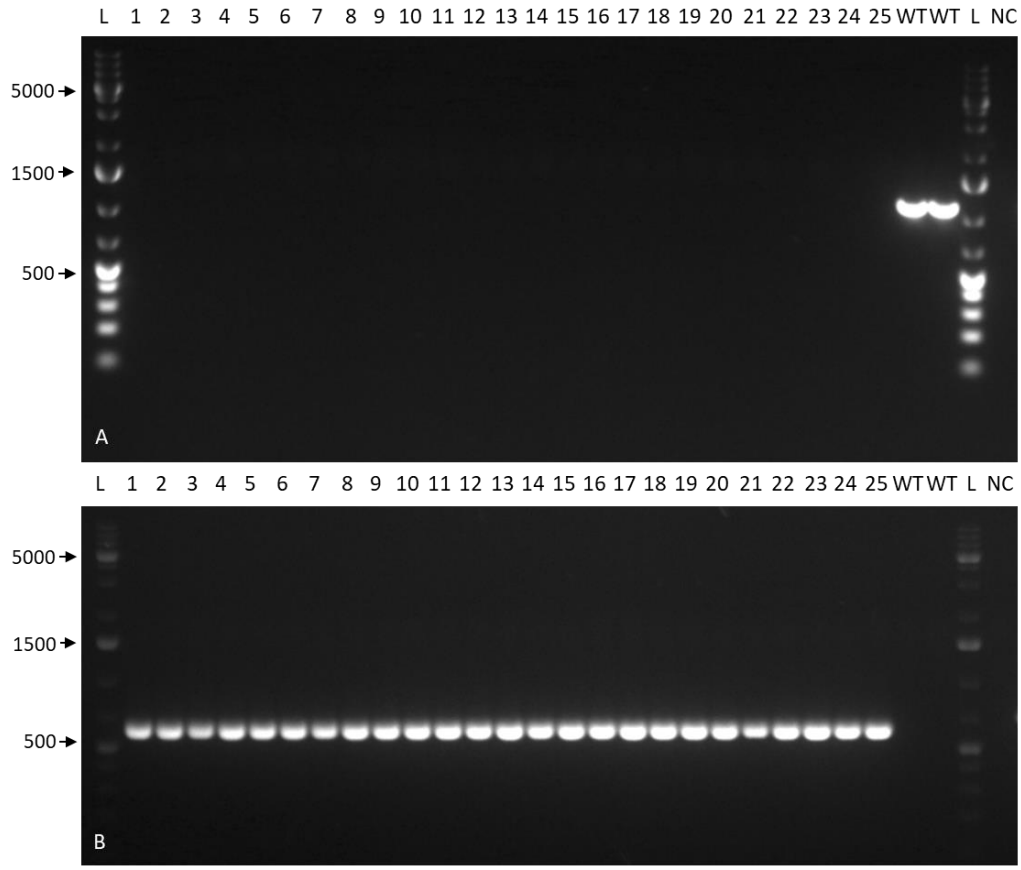


**Figure 3.1:** Gel electrophoresis (1 % agarose) of PCR products derived from plants for the *A. thaliana* insertion line *bzip25*, wildtype Col-0 (positive control for wt), 1 kb plus DNA ladder (L), negative control (NG) and plants 1-25. **A)** Samples with forward and reverse primers for *BZIP25*, and **B)** samples with reverse (*BZIP25*) and LBb1.3 (insertion) primers.

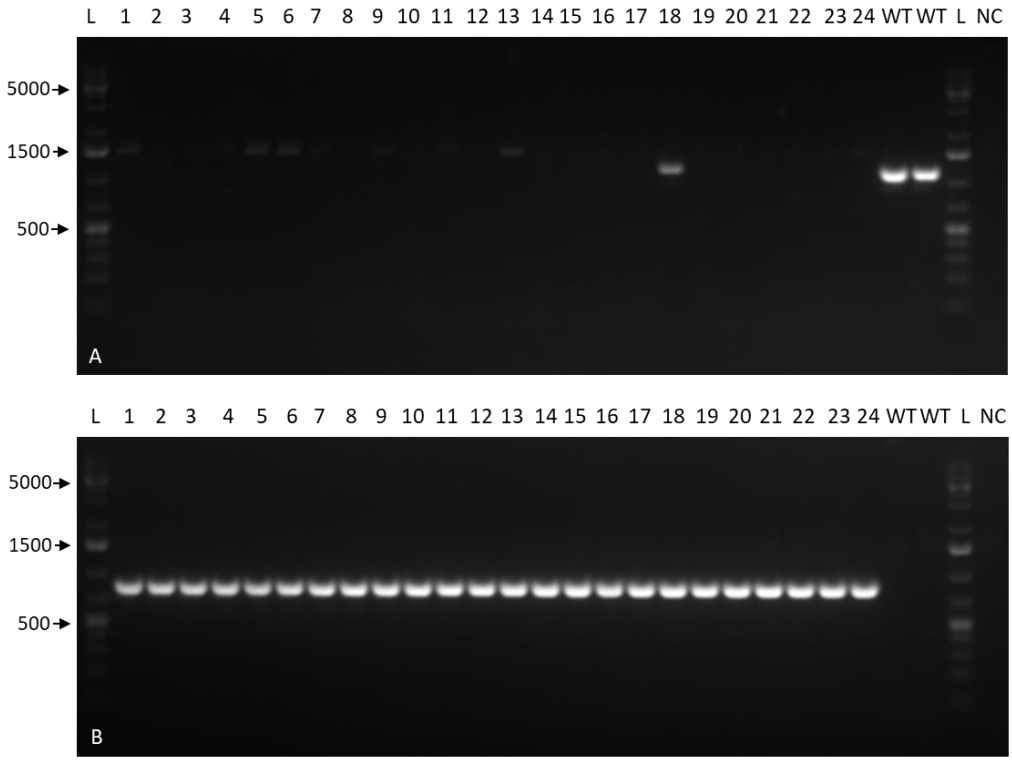


**Figure 3.2:** Gel electrophoresis (1 % agarose) of PCR products derived from plants for the *A. thaliana* insertion line *cil1*, wildtype Col-0 (positive control for wt), 1 kb plus DNA ladder (L), negative control (NG) and plants 1-24. **A)** Samples with forward and reverse primers for *CIL1*, and **B)** samples with reverse (*CIL1*) and LBb1.3 (insertion) primers.

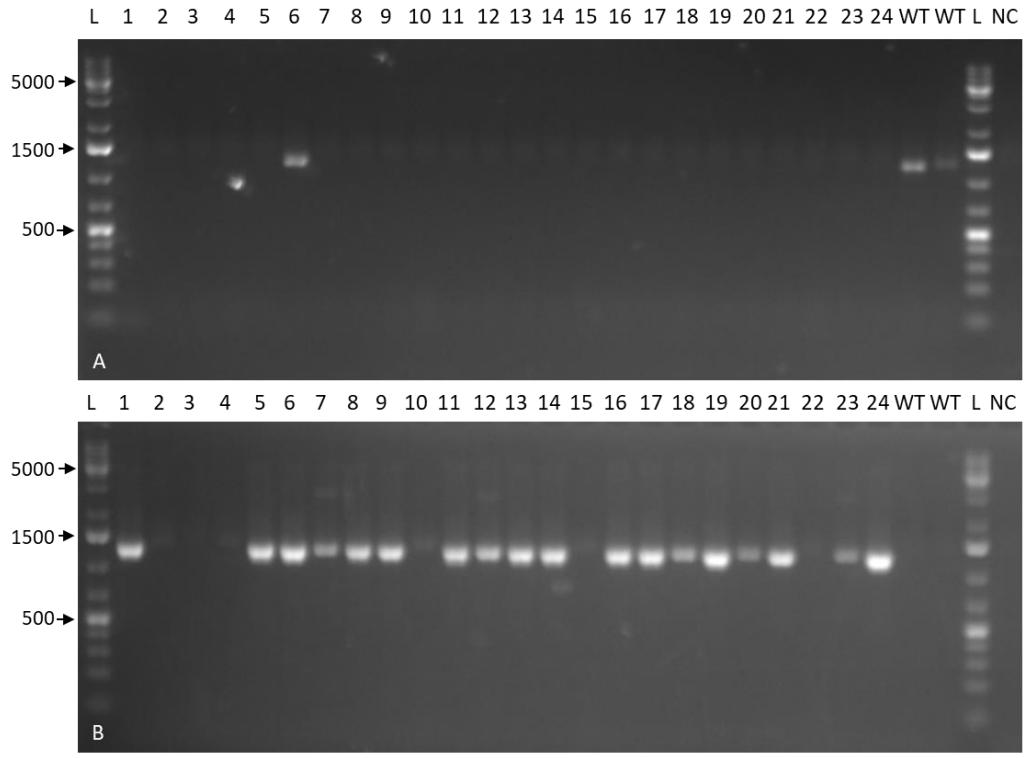


**Figure 3.3:** Gel electrophoresis (1 % agarose) of PCR products derived from plants for the *A. thaliana* insertion line *bluejay*, wildtype Col-0 (positive control for wt), 1 kb plus DNA ladder (L), negative control (NG) and plants 1-24. **A)** Samples with forward and reverse primers for *BLUEJAY*, and **B)** samples with reverse (*BLUEJAY*) and LBb1.3 (insertion) primers.

## 3.2 Relative gene expression

To determine how the T-DNA insertions affects expression of the GOI, RT-qPCR was conducted. To accomplish this the $2^{-\Delta\Delta CT}$-method was used to visualize the average relative fold change in gene expression in insertion lines compared to Col-0. In the gene expression analysis, two housekeeping genes, *ACT2* and *UBQ10*, were initially considered. However, *ACT2* was ultimately used for normalization due to substantial differences in the cycle threshold (CT) values observed for the expression of *UBQ10* among *cil1*, *bluejay*, and Col-0 (Appendix 1, Table A1.2). The expression of the genes of interest in their respective insertion lines relative to Col-0 is illustrated in Figure 3.4. For the insertion lines examined the expression of their respective genes is reduced relative to Col-0, where *cil1* and *bluejay* plants exhibit approximately half of the expression levels observed in Col-0, while *bzip25* is particularly reduced in gene expression.

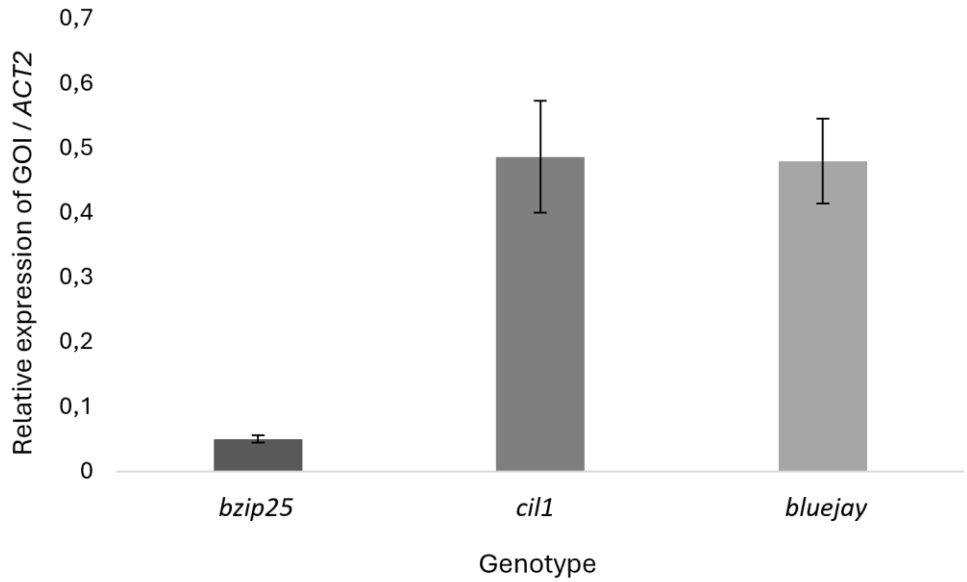


**Figure 3.4:** Gene expression of the genes of interest (GOI) in their respective T-DNA insertion lines (*bzip25, cil1* and *bluejay*) relative to expression level in wildtype Col-0. The graphs represent average relative fold change ($2^{-\Delta\Delta CT}$) with *ACT2* used for normalization. Error bars are based on average standard deviation, and all are significant different to Col-0 with $p < 0.001$ when using a student's t-test (see Appendix 2. Includes 4 biological and 1-3 technical replicates).

## 3.3 Phenotypic analysis

The root length of the insertions lines and controls were measured to check the phenotype of the insertion lines when they were exposed to stress isoxaben (ISX) and sorbitol (SORB). This enabled investigation on how the mutation in the GOI affected the root growth under different condition. The average root length (cm) for each genotype exposed to three treatments (A) mock, B) isoxaben and C) sorbitol) are presented in Figure 3.5. Three biological replicates for each genotype and treatment are included in the analyses, while the fourth replicate is excluded due to problems with growth conditions (see Appendix 1). A negative control including Col-0 in mock treatment is included in Appendix 1.

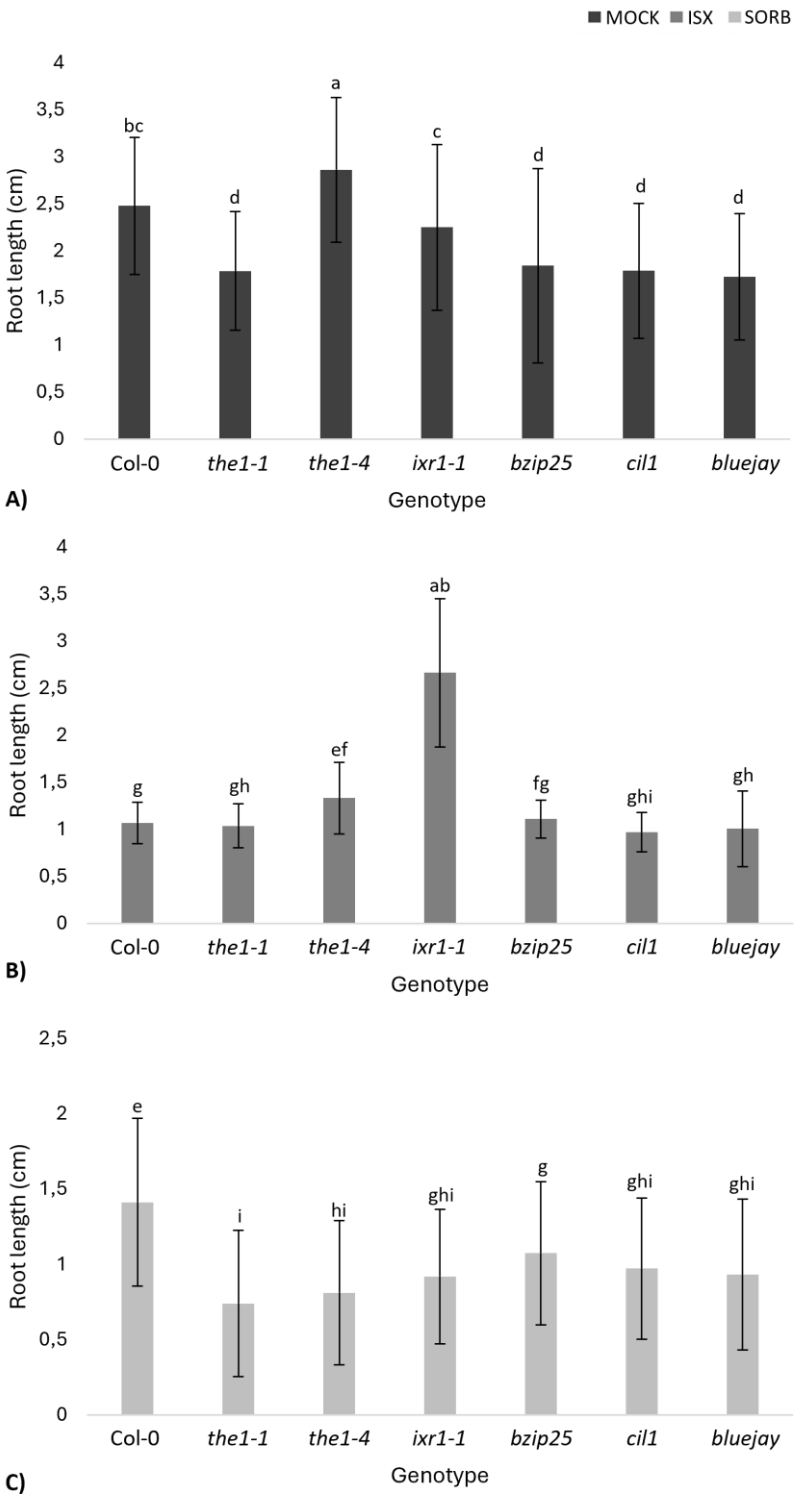


**Figure 3.5:** The average root length (cm) for each genotype, including controls; Col-0, *the1-1, the1-4* and *ixr1-1*, and insertion lines; *bzip25, cil1* and *bluejay*. For each genotype the root length is measured for three treatments: **A)** control (MOCK, 1/2 MS media), **B)** isoxaben (ISX, 1 nM) and **C)** sorbitol (SORB, 200 mM). Error bars represent standard deviation, while different lowercase letters represent significant differences between groups ($p < 0.05$) according to a one-way ANOVA and post hoc Tukey HSD test (3 biological replicates and 17-58 technical replicates).

In mock treatment *ixr1-1* seedlings showed no significant difference to the wildtype Col-0 (Figure 3.5A). The other genotypes exhibited significant differences to the wildtype, with *the1-4* seedlings exhibiting a slightly enhanced root length compared to the wildtype. The rest, *the1-1, bzip25, cil1* and *bluejay,* showed significantly reduced root length compared to the wildtype. In treatment with ISX *ixr1-1* seedlings exhibited significantly enhanced root length compared to wildtype, while *the1-4* showed an enhancement in root length, but not as pronounced (Figure 3.5B). The other genotypes, *the1-1, bzip25, cil1* and *bluejay,* were not significantly different to wildtype. In hyperosmotic stress induced by sorbitol, all genotypes showed significantly reduced root length compared to the wildtype, where *bzip25* seedlings showed slightly less reduction compared to *the1-1* and *the1-4* (Figure 3.5C). For each genotype there were a reduction in root length in the stress treatments (ISX and SORB) compared to control (MOCK), expert for ISX-treated *ixr1-1* seedlings, which showed enhanced root length compared to control treatment.

## 3.4 SA Biosensor analysis

To further characterize the role of candidate genes in CWI maintenance, a SA biosensor analysis was performed to assess if loss of the candidate genes affected the production of SA. SA levels were indirectly visualized using luminescence as readout. Controls used were Col-0, *the1-1* (reduced SA levels after ISX treatment) and *the1-4* (enhanced SA levels after ISX treatment). The amount of luminescence for each genotype was normalized to the average levels observed in wildtype Col-0. When comparing the results for the different genotypes investigated to control, only *bluejay*, in addition to *the1-1* and *the1-4*, exhibited a significant difference to Col-0 according to a student's t-test (Figure 3.6). A one-way ANOVA and post hoc Tukey HSD test exhibited only significant difference between THE1 mutant lines and Col-0.

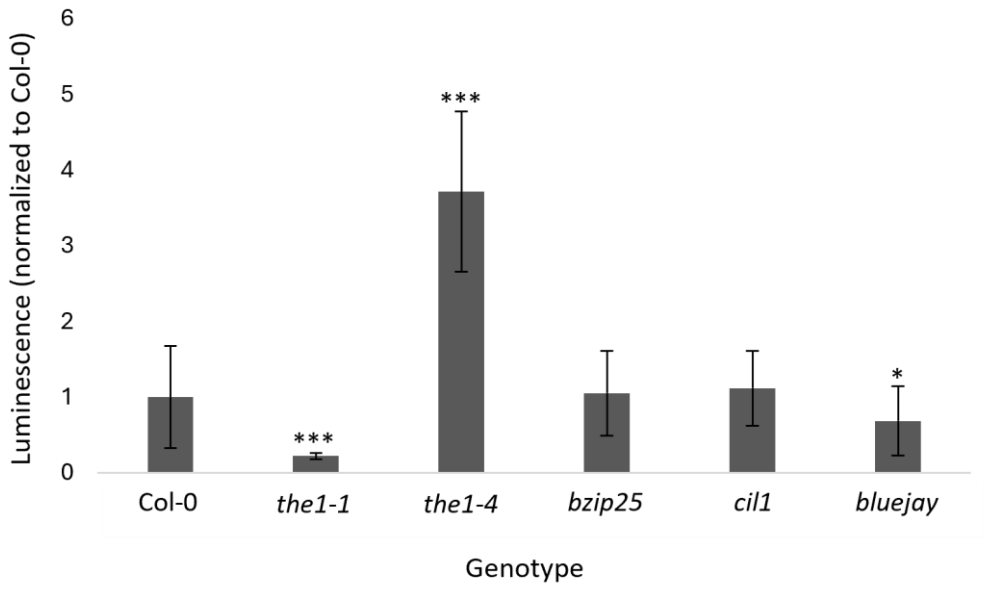


**Figure 3.6:** Amounts of luminescence for each genotype examined normalized to average luminescence for wildtype Col-0. Genotypes include the controls (Col-0, *the1-1* and *the1-4)* and insertion lines (*bzip25*, *cil1* and *bluejay*). Error bares show standard deviation, while * and *** indicate significant differences to wildtype with $p < 0.05$ and $p<0.001$ according to a student's t-test (4 biological replicates and 6-12 technical replicates).

# 4. Discussion

The plant cell wall must adapt to various physical and chemical challenges from both the environment and the plant itself, necessitating the monitoring and maintenance of the cell wall integrity (CWI) though the (CWI) maintenance mechanism (13). Based on previous work, THESEUS1 has been particularly interesting and been implicated in CWI maintenance during CWD-induced stress, including regulation of phytohormones and other stress responses (21,22). As the understanding of THE1 and its involvement in CWI maintenance mechanism has grown, there also been an interest in identifying correlated components. The Hamann research group identified genes potentially acting downstream of THE1. Three of these genes, *BZIP25, CIL1* and *BLUEJAY,* were selected for this project, with the hypothesis that these genes are involved in CWI maintenance. To assess this, hypersensitivity assays, along with gene expression- and SA biosensor analyses were conducted.

## 4.1 Verification of insertion lines

Before proceeding with further experiments, it was necessary to establish if the T-DNA insertions were at the intended position in the candidate genes, thus possibly generating loss of or knock-down alleles. Plants for insertion lines where the PCR reactions only produced fragments for the insertion and no fragments for wildtype were considered to be homozygous plants.

To determinate how the T-DNA insertions affect expression of genes of interest, qPCR was utilized to assess expression levels of genes of interest for the insertion lines relative to the observed expression levels in wildtype Col-0. Based on the results from the RT-qPCR based gene expression analysis, all the insertion lines were significantly different compared to the wildtype (details provided in Appendix 2) and showed reduced gene expression levels (Figure 3.4). However, primers that were used for the qPCR were binding after the insertion site, which might have influenced the results, and the analysis in this case provides insight into the downstream effects of the insertions. The insertion line *bzip25* displayed particularly pronounced reduction in expression level relative to wildtype. The T-DNA insertion was located within the first exon of the gene (Figure 1.4A), and therefore, minimal or no gene transcription downstream of the insertion site were expected (41). This suggests

that the insertion disrupt the coding sequence, potentially leading to premature termination of transcription or translation, and having a gene knockout effect.

*bluejay* and *cil1* plants show reduced expression of their respective genes, but not as pronounced as observed in *bzip25*. For *bluejay* and *cil1,* gene expression levels were approximately 0.5 times that of Col-0, suggesting that the insertions only partially affect the expression of the genes. Given that the T-DNA insertion in the *BLUEJAY* gene occurred within the second exon (Figure 1.4C), it could disrupt the regulation of the gene by interrupting the coding sequence. A review of several studies on T-DNA insertions in genes of *A. thaliana* shows that an insertion in an exon are likely to affect the transcription level and usually causes knockout or knockdown of the gene of interest (41). However, since the primers bind downstream of the insertions, it is difficult to clearly assess the impact of the insertion on gene expression. To assess the extent of the disruption caused by the insertion, it would have been better to use primers spanning the insertion. On the other hand, it seems that if the insertions is closer to the middle of the gene in an exon or intron, detection of transcript can be monitored both up- and downstream of the insertion, potentially leading to truncated transcript, which might not produce a functional protein (41). This could support the reduced transcript level observed downstream of the insertion in *bluejay* plants, suggesting that the insertion in *BLUEJAY* could have caused a truncated transcript. However, lack of quality control makes it impossible to exclude the possibility of genomic DNA (gDNA) contamination. It would be reasonable to proceed with gel electrophoresis for *bluejay* samples to assess if there is multiple bands or bands of different sizes, which indicates contamination.

Similarly, in the case of *cil1*, where the T-DNA insertion was within the 5' untranslated region (UTR) (Figure 1.4B), the insertion could significantly impact gene expression due to the involvement of this region in transcriptional regulation. The 5'UTR includes cis-regulatory elements which regulates different aspect of mRNA, including stability, transport and translation (42). However, there seems to be a trend where insertions in regions upstream of the start codon are slightly less effective compared to insertions in exons or introns (41). Additionally, it seems to be a trend that the effectiveness of the insertion on transcription decrease as the distance between the start codon and the insertion

upstream increase (41). In other words, the closer the insertion is to the start codon the more the knockdown rate increase, while the knockout rate decrease. (41). This appears to be the trend for insertions up to 500 base pairs upstream of the start codon. This could be the case for *CIL1* where the insertion is 377 base pairs upstream, suggesting that the reduced expression level is due to knockdown of the gene. However, these observed trends are based on published T-DNA mutants, which could have bias for insertions leading to a knockout or knockdown of the gene (41). Additionally, the same applies for *cil1* as *bluejay* plants, where no quality control were performed, and there could be enhanced transcription levels due to gDNA contamination. Since the primes were binding downstream of the insertion site it may not fully reflect the impact that the insertion had in the 5'UTR. For further experiments, using primers spanning the insertion site could provide a more comprehensive understanding of the effects of T-DNA insertions on gene expression. It would also be reasonable to proceed with further analysis to assess exactly what part of the UTR and regulatory elements the insertion affects.

## 4.2 Phenotypic analysis

A phenotypic analysis was conducted assessing the root length in response to two different stress treatments (ISX and sorbitol), where seedlings for the different insertion lines were compared to Col-0, *the1-1* and *the1-4*. In response to mock treatment, *ixr1-1* seedlings showed no significant difference compared to wildtype, while *the1-4* exhibit slightly enhanced root length compared to Col-0 (Figure 3.5A). *the1-1* and insertion lines seedlings exhibit reduced root length compared to wildtype. Previous studies on stiffness in THE1 seedling roots showed that mock-treated *the1-1* seedling roots exhibited significantly lower stiffness compared to wildtype, suggesting that THE1 is required for regulation of stiffness during growth (7). This could support the phenotypic observation of *the1-1* in root length. However, it is presumed that THE1 only becomes active when CWI has been impaired, due to absence of phenotypic defects in hypocotyl for THE1 knockout mutants under non-stressed condition (3,21). Also, in the study of Engelsdorf et al. there were no significant difference between root length in *the1-1, the1-4,* and Col-0 (22). This suggests that the differences observed in root length of these genotypes may have other causes such as suboptimal growth conditions. This is supported by considerable variation observed in root length for each genotype between different experiments. The mock-treated Col-0 seedling

roots exhibited considerable variation between the biological replicates, indicating that the results are not reproducible, which needs to be taken into account.

However, previous studies have implicated *BLUEJAY* in root growth with respect to organizing and maintaining ground tissue, including endodermis differentiation (43,44). This could support the reduced root length for mock-treated *bluejay* plants observed. It seems to be little evidence currently available, which implicates *CIL1* and *BZIP25* in root growth regulation, necessitating further experiment for conclusive findings. Nevertheless, the pronounced reduction observed in mock treated *bzip25, cil1* and *bluejay* seedling roots compared to wildtype, must be considered to prevent bias when analyzing the responses to the stress treatments.

When comparing root length in ISX-treated seedlings, *ixr1-1* exhibits significantly enhanced root growth compared to wildtype (Figure 3.5B). This aligns with the expectations, as *ixr1-1* is known to be resistant to ISX treatment (45), functioning effectively as a positive control for resistance to ISX. In addition, ISX-treated *the1-4* seedlings also exhibit enhanced root growth compared to wildtype, though to a lesser extent than *ixr1-1*. Previous studies have shown no observed difference in root growth inhibition between Col-0 and *the1-4* under ISX treatment (22), suggesting that the minor differences observed are likely inconsequential and may be due to dataset variation. Similarly, the study found no differences in root growth inhibition between *the1-1* and Col-0 (22), supporting the observation of ISX-treated *the1-1* seedlings showing no significant difference to wildtype. None of the seedlings for the different insertion lines exhibited significant differences compared to the wildtype, implying that these genes do not affect the root growth response to CWD-induced stress fundamentally.

Regarding the root lengths in response to sorbitol treatment, all genotypes exhibited reduced root length compared to wildtype (Figure 3.5C). *bzip25* seedlings seem to be slightly less sensitive compared to the other genotypes but still showed a reduction compared to wildtype. Interestingly, *ixr1-1* seedlings displayed a significant reduction in root length compared to wildtype, which has not been reported before. Furthermore, the

reduced root growth observed in the insertion lines compared to the wildtype may not be biologically relevant, considering the reduced phenotype in non-stressed condition. Experimental difficulties, especially in the sorbitol treatment experiments, led to pronounced variation among the experiments, making it challenging to draw conclusions. For the analysis presented in the results section, only replicates 1-3 were included, as the fourth replicate showed very strong reduction in root length compared to the others (see Appendix 1 Figure A1-A7).

Additionally, as mentioned earlier, there are notable variation among the biological replicates of the control (mock-treated Col-0 seedlings), indicating a lack of reproducible results. This highlights the need for improvement in experimental conditions to establish a strong control baseline before proceeding with insertion lines and stress treatments. It would be reasonable to check the factors influencing the experiment, including seed sterilization, media preparation and other growth conditions such as light and humidity. The substantial variation in the treatments for all genotypes emphases the need for further investigations to generate a dataset allowing to clarify the involvement of *BZIP25, CIL1* and *BLUEJAY* in root growth and the responses to CWD- and sorbitol- (drought) induced stress.

## 4.3 SA Biosensor Analysis

SA production is important for the regulation of plant defense responses and studies have investigated changes in SA levels induced by CWD-induced stress (4,22). To investigate the role of the candidate genes in CWI maintenance, a SA biosensor analysis was conducted to evaluate the impact of the insertions on SA production. *the1-1* and *the-1-4* have been previously characterized, with *the1-1* exhibiting loss-of-function and *the1-4* showing gain-of-function phenotypic effects (23). Previous studies revealed opposite responses of these mutants to ISX treatment, with *the1-1* displaying reduced and *the1-4* showing enhanced SA production (22). Thus, these mutant lines were included as controls.

In the analysis *the1-4* seedlings exhibited significantly increased luminescence compared to wildtype, whereas *the1-1* exhibited a significant reduction in luminescence (Figure 3.6).

This indicates that *the1-4* has heightened SA production, while *the1-1* has reduced SA production compared to Col-0, aligning with findings from previous studies (22), and affirming their role as controls. Interestingly, among the insertion lines, *bluejay* seedlings showed a significant difference compared to Col-0, exhibiting a slight reduction in luminescence, though not as pronounced as *the1-1*. This suggest that the insertion in *BLUEJAY* results in a decreased response in SA production to CWD-induced stress, potentially implicating the gene in the signal pathway associated with SA response. There is little evidence of *BLUEJAY* implicated SA responses to CWD -induced stress, but there are studies that shows interaction between *BLUEJAY* (*AtIDD6*) and DELLA (aspartic acid–glutamic acid–leucine–leucine–alanine) proteins (46). DELLA proteins mediate gibberellin (GA) signaling during growth and development (47), and other studies have suggested that there is hormone cross-talk between GA and SA in seedling growth during abiotic stress (18). Based on the results it is not possible to say whether the reduced SA response observed in *bluejay* seedlings could be due to an indirect regulation, but further investigation into this interaction could be interesting.

In contrast, *bzip25* and *cil1* seedlings did not exhibit significant differences compared to wildtype, suggesting that they likely do not play a role in SA production response to CWD-induced stress. However, the one-way ANOVA and post hoc Tukey HSD test only identified *the1-1* and *the1-4* as significantly different from the wildtype, possibly due to variations in biological replicates between insertion lines and the wildtype. This emphasizes the need for further experiments into the potential role of *BLUEJAY* in SA response before reaching any definitive conclusions.

# 5. Conclusion

Potential CWI-related genes, *BZIP25, CIL1* and *BLUEJAY,* were characterized to determine their potential role in CWI maintenance. Homozygous T-DNA insertion lines were isolated to generate knockout alleles for the genes of interest. This involved genotyping and RT-qPCR based gene expression analysis. The responses of seedling roots, homozygous mutant for the different genes, to isoxaben-induced CWD and sorbitol-induced hyperosmotic stress was investigated to determine if the genes of interest are required for responses to the stresses examined. Additionally, biosensor for SA was used to compare SA concentrations after ISX treatment in controls (Col-0 and THE1 mutant lines) and insertion lines, to evaluate if insertions in the candidate genes affected ISX-induced SA accumulation. The relative gene expression levels for the insertion lines were all significantly reduced compared to wildtype, where particularly *bzip25* seedlings exhibited a substantial decrease, suggesting that the T-DNA insertion in the first exon leads to a gene knockout. The other two insertion lines exhibited approximately half of the expression levels of Col-0, suggesting that the insertions lead to a knockdown. However, primers used for the RT-qPCR were binding downstream of the insertion site, so it is conceivable that truncated proteins are generates. For future studies, it would be beneficial to include primers that span the insertion site.

For the phenotypic analysis, the root length for the insertion lines in non-stress condition was significantly reduced compared to wildtype, suggesting that that these genes may be involved in seedling root development and growth. When being exposed to isoxaben there were no significant differences in root length compared to wildtype. In response to sorbitol treatment, the seedlings of the insertion lines seemed to exhibit reduced root lengths compared to Col-0. Since there was already a reduction in root growth in control media and there were challenges with experimental conditions it remains to be determined if the insertions really affected responses to sorbitol. Repeating the experiments in the future after ensuring experimental conditions are reproducible will clarify these results. Assuming the findings are reproducible, *BZIP25, CIL1* and *BLUEJAY* are apparently required for proper root growth, which may in turn affect root growth responses to sorbitol stress.

Regarding SA accumulation in response to ISX treatment, *bluejay* was the only insertion line that exhibited a slight significant reduction to Col-0. The controls, *the1-1* and *the1-4*, both exhibited the expected effects on SA production with *the1-1* showing reduced and *the1-4* enhanced responses. Thus, the insertion in *bluejay*, which seems to downregulate the gene expression, seems to result in a reduced SA response to ISX treatment. This suggest that *BLUEJAY* might be important for the SA signaling pathway that is activated in response to CWI impairment. However, a one-way ANOVA and post hoc Tukey HSD test showed no significant different between *bluejay* and Col-0, emphasizing the need for more experiments to draw definitive conclusions.

In the future, it could be interesting to explore the connection between THE1 and these candidate genes, as previous experiments suggested that they are acting downstream of THE1. For instance, investigating the gene expression of these candidate genes in THE1 mutant lines exposed to stress, or examining the gene expression of THE1 in the different insertions, could reveal whether these genes act upstream or downstream of THE1. Additionally, it would be interesting to further investigate the potential involvement of BLUEJAY in SA response to stress induced by CWD.

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# Appendix 1 – Raw data

Following are raw data for the different experiments presented. RT-qPCR based gene expression analysis was used to investigate the effect of the insertions on the genes of interest in *Arabidopsis thaliana*. The CT values for gene expression of reference genes *ACT2* and *UBQ10*, and genes of interest (*BZIP25, CIL1* and *BLUEJAY*) are in Table A1.1-A1.3, respectively. A biosensor for salicylic acid (SA) was utilized to compare SA concentrations in isoxaben (ISX)-treated seedling for Col-0, *the1-1, the1-4, bzip25, cil1* and *bluejay*, and the detected luminescence for each genotype is in Table A1.4. A control including luminescence for mock- and ISX-treated Col-0 seedlings are presented in Table A1.5.

Additionally, a phenotypic analysis was conducted involving root length of Col-0, *the1-1, the1-4, ixr1-1, bzip25, cil1* and *bluejay* seedlings treated with mock, ISX and sorbitol. Images of seedlings with treatments for each genotype are represented in Figure A.1-A.7 respectively. The root length measured using ImageJ for three biological replicates are included in Table A1.6 (mock), Table A1.7 (ISX) and Table A1.8.

**Table A1.1:** CT values from gene expression of *ACT2* in *bzip25*, *cil1*, *bluejay* and Col-0 seedlings. Includes CT values obtained from qPCR for 4 biological replicates with three technical replicates.

| ***ACT2*** | | | | |
|---|---|---|---|---|
| **Genotype** | ***bluejay*** | ***cil1*** | ***bzip25*** | **Col-0** |
| Rep 1.1 | 17.83 | 17.83 | 16.77 | 16.39 |
| Rep1.2 | 17.79 | 17.69 | 16.8 | 16.41 |
| Rep1.3 | 17.73 | 17.8 | 16.79 | 16.41 |
| Rep 2.1 | 16.85 | 16.69 | 17.52 | 16.9 |
| Rep 2.2 | 16.91 | 16.7 | 17.5 | 16.96 |
| Rep 2.3 | 16.9 | 16.67 | 17.4 | 16.98 |
| Rep 3.1 | 17.21 | 17.15 | 17.2 | 16.07 |
| Rep 3.2 | 17.18 | 17.19 | 17.23 | 16.14 |
| Rep 3.3 | 17.22 | 17.42 | 17.21 | 16.2 |

| | | | | |
|---|---|---|---|---|
| Rep 4.1 | 17.14 | 17.08 | 17.21 | 16.71 |
| Rep 4.2 | 17.18 | 17.05 | 17.21 | 16.8 |
| Rep 4.3 | 17.18 | 17.15 | 17.2 | 16.74 |

**Table A1.2:** CT values from gene expression of *UBQ10* in *bzip25*, *cil1*, *bluejay* and Col-0 seedlings. Includes CT values obtained from qPCR for 4 biological replicates with three technical replicates.

| ***UBQ10*** | | | | |
|---|---|---|---|---|
| **Genotype** | ***bluejay*** | ***cil1*** | ***bzip25*** | **Col-0** |
| Rep 1.1 | 17.09 | 17.25 | 16.26 | 15.03 |
| Rep1.2 | 17.1 | 17.34 | 16.26 | 15.11 |
| Rep1.3 | 17.12 | 17.39 | 16.26 | 15.16 |
| Rep 2.1 | 16.61 | 16.16 | 16.62 | 16.24 |
| Rep 2.2 | 16.61 | 16.55 | 16.62 | 16.39 |
| Rep 2.3 | 16.61 | 16.07 | 16.62 | 16.46 |
| Rep 3.1 | 16.87 | 17.11 | 16.69 | 15.11 |
| Rep 3.2 | 16.73 | 17.1 | 16.69 | 15.1 |
| Rep 3.3 | 16.88 | 17.01 | 16.69 | 15.19 |
| Rep 4.1 | 16.91 | 16.62 | 16.48 | 15.77 |
| Rep 4.2 | 16.86 | 16.54 | 16.48 | 15.73 |
| Rep 4.3 | 16.93 | 16.47 | 16.48 | 15.82 |

**Table A1.3:** CT values from qPCR for gene expression of *BZIP25*, *CIL1* and *BLUEJAY* in the insertion lines (*bzip25, cil1* and *bluejay*) and the wildtype Col-0. Includes CT values from 4 biological replicates with three technical replicates, where "–" indicates no value.

| **Gene** | ***BZIP25*** | | ***CIL1*** | | ***BLUEJAY*** | |
|---|---|---|---|---|---|---|
| **Genotype** | ***bzip25*** | **Col-0** | ***cil1*** | **Col-0** | ***bluejay*** | **Col-0** |
| Rep 1.1 | 24.88 | 20.27 | 23.79 | 21.62 | 23.02 | 20.22 |
| Rep1.2 | 24.88 | 20.27 | 23.79 | 21.62 | 23.02 | - |
| Rep1.3 | 24.88 | - | 23.79 | 21.62 | 23.02 | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Rep 2.1 | 25.32 | 20.54 | 22.8 | 22.25 | 22.31 | 21.33 |
| Rep 2.2 | 25.32 | 20.54 | 22.8 | 22.25 | 22.31 | 21.33 |
| Rep 2.3 | 25.32 | 20.54 | 22.8 | 22.25 | 22.31 | 21.33 |
| Rep 3.1 | 25.61 | 19.89 | 23.69 | 21.22 | 22.42 | 20.38 |
| Rep 3.2 | 25.61 | 19.89 | 23.69 | 21.22 | 22.42 | 20.38 |
| Rep 3.3 | 25.61 | 19.89 | 23.69 | 21.22 | 22.42 | 20.38 |
| Rep 4.1 | 25.33 | 20.66 | 23.32 | 21.69 | 22.34 | 21.06 |
| Rep 4.2 | 25.33 | 20.66 | 23.32 | 21.69 | 22.34 | 21.06 |
| Rep 4.3 | 25.33 | 20.66 | 23.32 | 21.69 | 22.34 | 21.06 |

**Table A1.4:** Values of detected luminescence for Col-0, *the1-1, the1-4, bzip25, cil1* and *bluejay* seedlings treated with isoxaben (ISX).

| **Col-0** | ***the1-1*** | ***the1-4*** | ***bzip25*** | ***cil1*** | ***bluejay*** |
|---|---|---|---|---|---|
| 3633 | 2872 | 69490 | 5097 | 4469 | 14865 |
| 3810 | 2983 | 31656 | 6592 | 13477 | 12601 |
| 14210 | 2935 | 27054 | 5174 | 4755 | 5025 |
| 7292 | 2991 | 56190 | 6332 | 11955 | 3475 |
| 40396 | 3034 | 39381 | 4958 | 11760 | 7715 |
| 5916 | 2969 | 65953 | 7730 | 9948 | 4157 |
| 14047 | 2692 | 43859 | 15380 | 19797 | 6154 |
| 4886 | 2773 | 38830 | 8668 | 17953 | 3004 |
| 10124 | 2856 | 43137 | 8227 | 8195 | 3821 |
| 5159 | 2923 | 42672 | 8855 | 21991 | 3710 |
| 13502 | 2936 | 49126 | 9047 | 19902 | 7021 |
| 16213 | 3034 | 36985 | 15403 | 21131 | 3153 |
| 10506 | 2611 | 39784 | 29238 | 10651 | 6507 |
| 4647 | 2760 | 31278 | 20741 | 3840 | 17756 |
| 11985 | 2489 | 32372 | 14901 | 22181 | 3152 |
| 7150 | 2812 | 45466 | 19478 | 12017 | 4140 |
| 12565 | 2937 | 48287 | 19526 | 17883 | 6728 |

| | | | | | |
|---|---|---|---|---|---|
| 22873 | 2905 | 50417 | 13905 | 16930 | 21040 |
| 8326 | 1657 | 37013 | 5929 | 20302 | 10713 |
| 16473 | 1738 | 35912 | 8369 | 10894 | 20177 |
| 3848 | 1909 | 42369 | 6805 | 10738 | 14559 |
| 10317 | 1898 | 39231 | 15294 | 19048 | 4683 |
| 17618 | 1905 | 39058 | 15045 | 17891 | 16093 |
| 17288 | 1920 | 52530 | 13828 | 14873 | 8388 |
| 3843 | | | 12562 | 5221 | 21664 |
| 18206 | | | 8226 | 7438 | 6447 |
| 25109 | | | 16405 | 12100 | 7635 |
| 14408 | | | 16969 | 13588 | 2815 |
| 9253 | | | 2821 | 1739 | 7045 |
| 3044 | | | 5961 | 14489 | 3535 |
| 23517 | | | 23617 | | 5388 |
| 8534 | | | 12514 | | 2688 |
| 10973 | | | 16165 | | |
| 4786 | | | 16142 | | |
| 12241 | | | | | |
| 18320 | | | | | |
| 26945 | | | | | |
| 14204 | | | | | |
| 11217 | | | | | |
| 3060 | | | | | |
| 7246 | | | | | |
| 3939 | | | | | |
| 2771 | | | | | |

**Table A1.5:** Values of detected luminescence for wildtype Col-0 seedlings with and without isoxaben (ISX) treatment provided by T. Tichá.

| Col-0 with ISX | Col-0 without ISX |
|---|---|
| 7552 | 450 |
| 8488 | 422 |
| 10996 | 503 |
| 10398 | 495 |
| 10615 | 572 |
| 10639 | 476 |
| 10545 | 401 |
| 10578 | 450 |
| | 562 |
| | 538 |

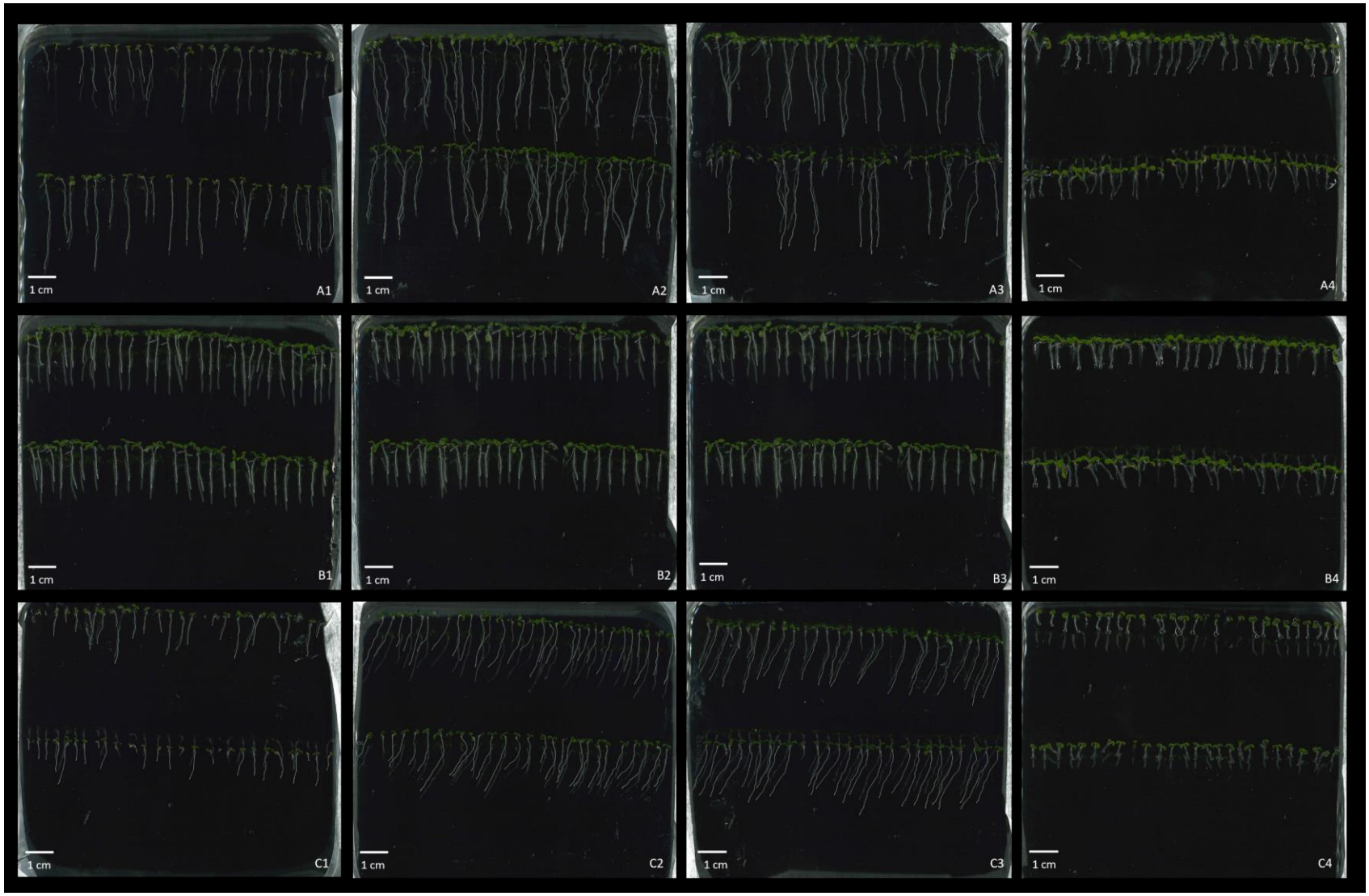



**Figure A.1:** Images of wildtype Col-0 seedlings of *Arabidopsis thaliana.* Numbers represent biological replicates (experiments) 1-4, while letters indicate treatment: A) mock, B) isoxaben and C) sorbitol. Scale bars represent 1 cm.

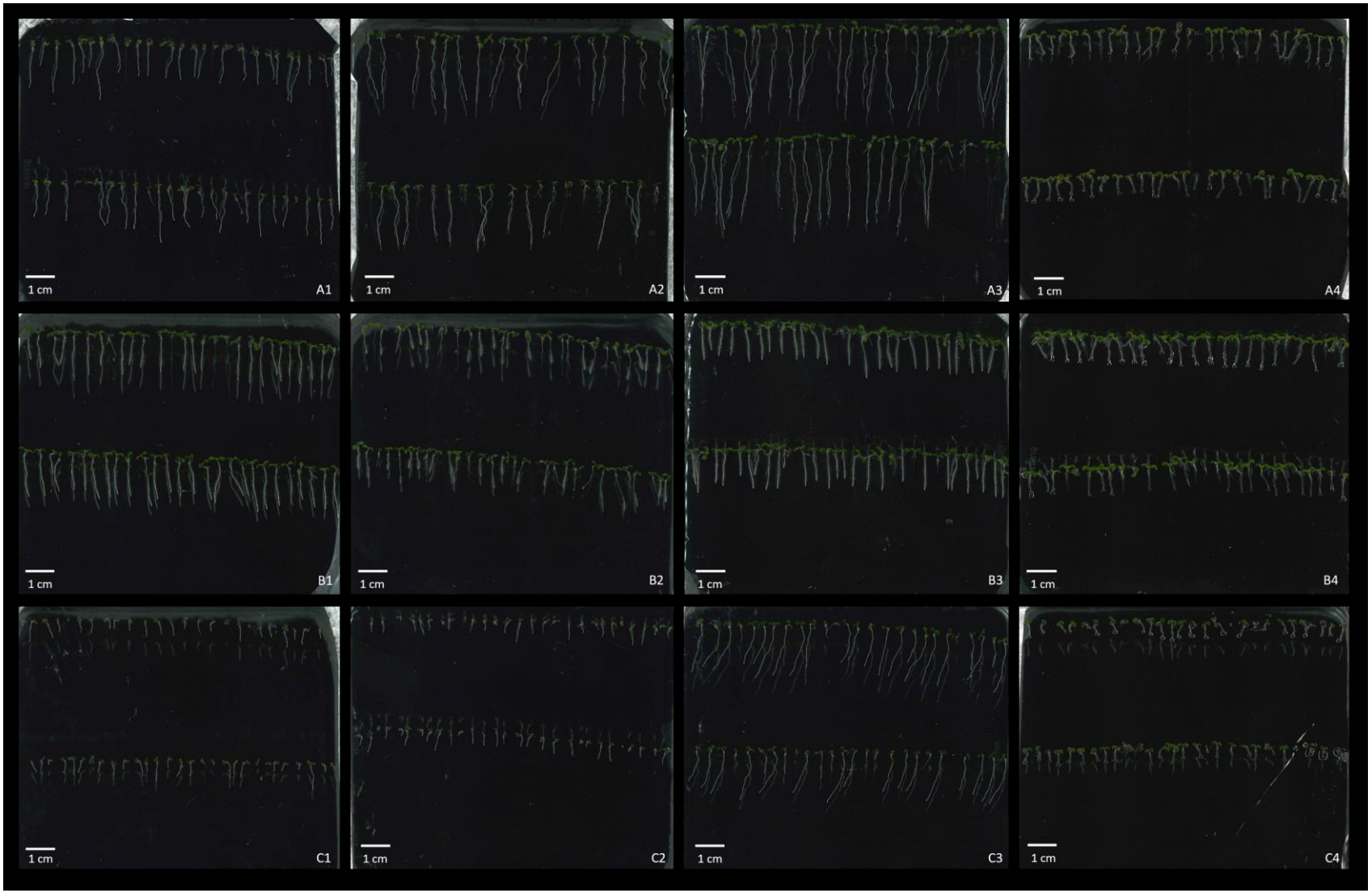



**Figure A.2:** Images of loss-of function allele *the1-1* seedlings of *Arabidopsis thaliana.* Numbers represent biological replicates (experiments) 1-4, while letters indicate treatment: A) mock, B) isoxaben and C) sorbitol. Scale bars represent 1 cm.

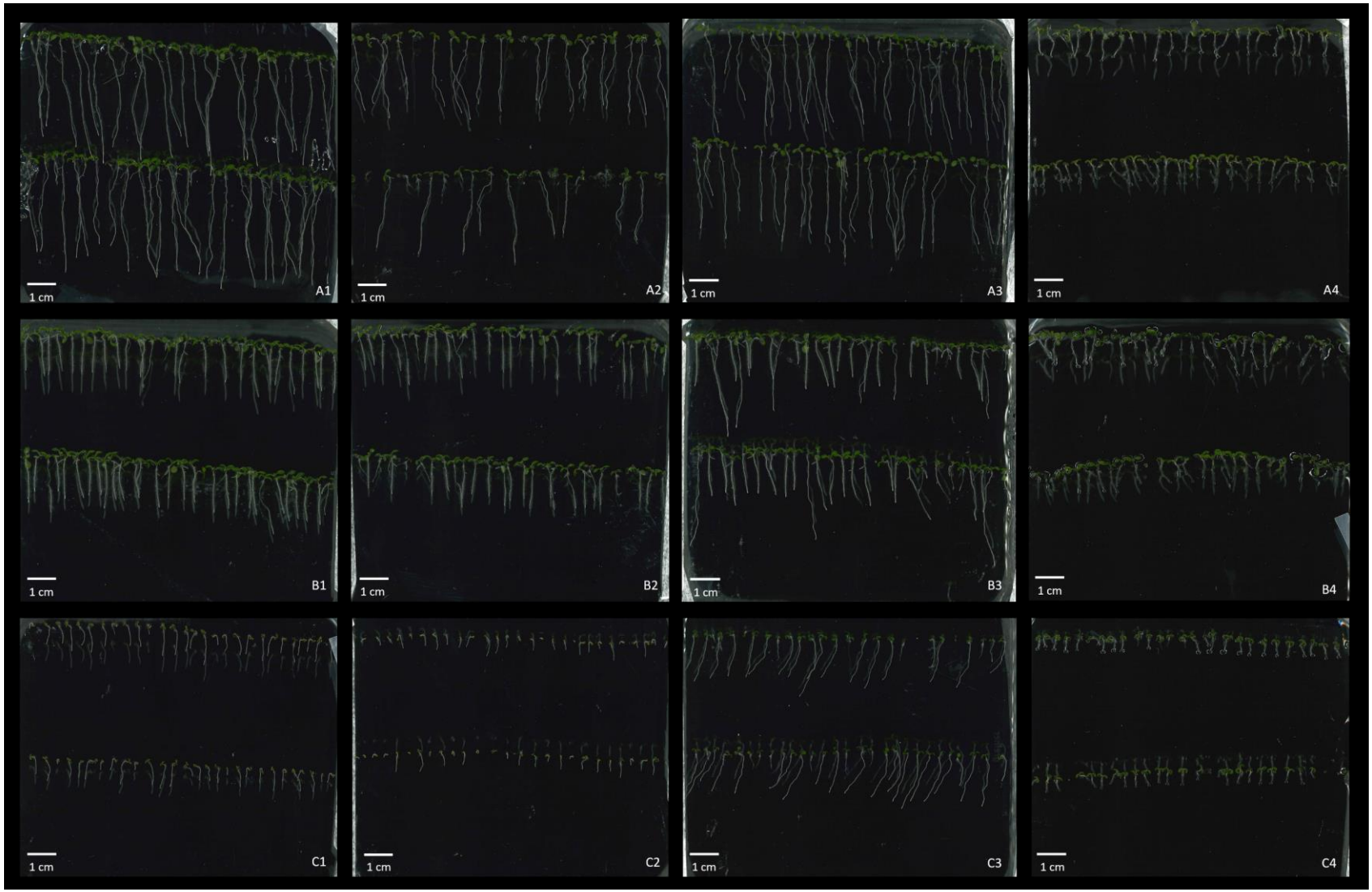



**Figure A.3:** Images of gain of function allele *the1-4* seedlings of *Arabidopsis thaliana.* Numbers represent biological replicates (experiments) 1-4, while letters indicate treatment: A) mock, B) isoxaben and C) sorbitol. Scale bars represent 1 cm.

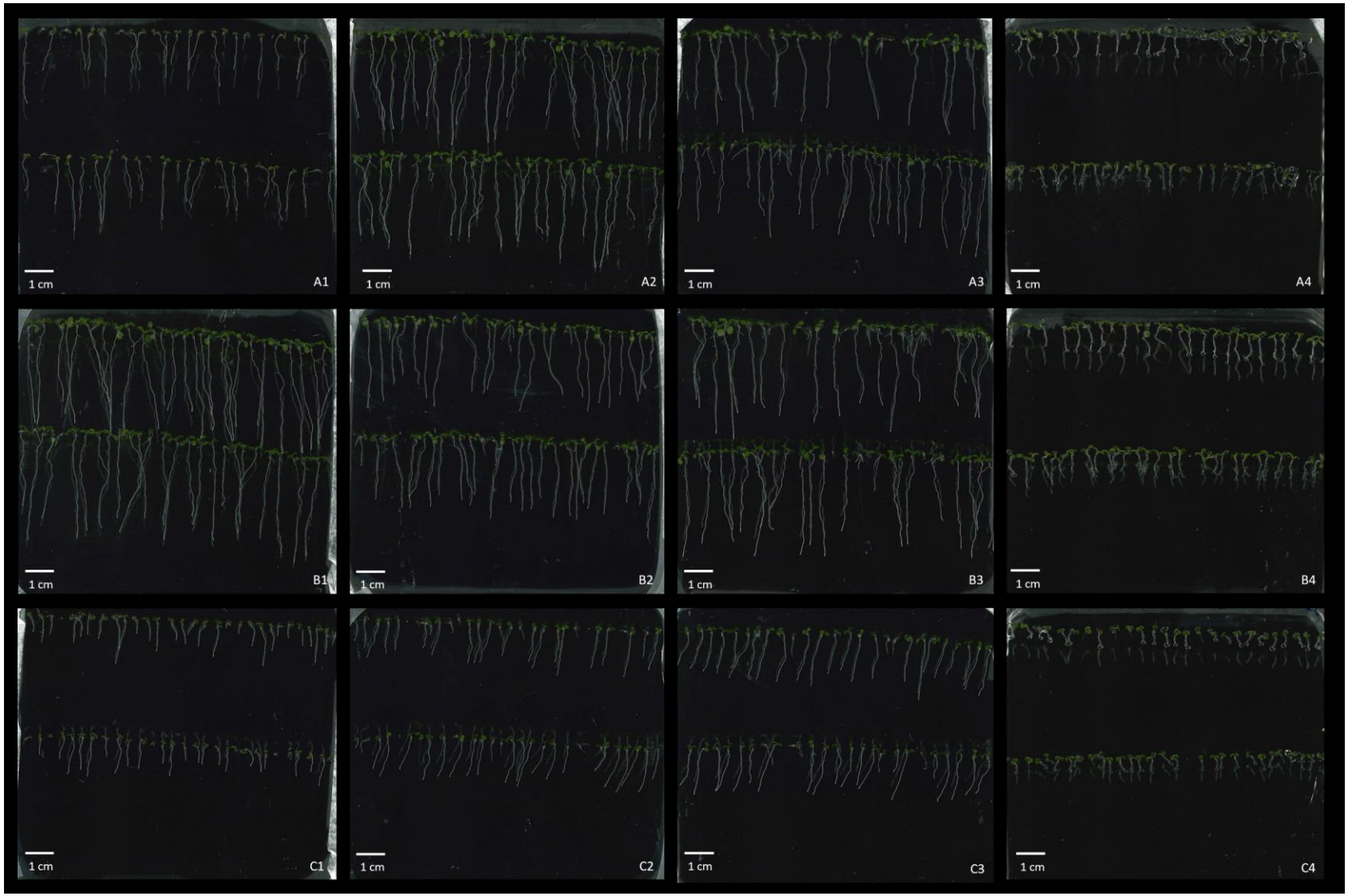



**Figure A.4:** Images of isoxaben resistant *ixr1-1* seedlings of *Arabidopsis thaliana.* Numbers represent biological replicates (experiments) 1-4, while letters indicate treatment: A) mock, B) isoxaben and C) sorbitol. Scale bars represent 1 cm.

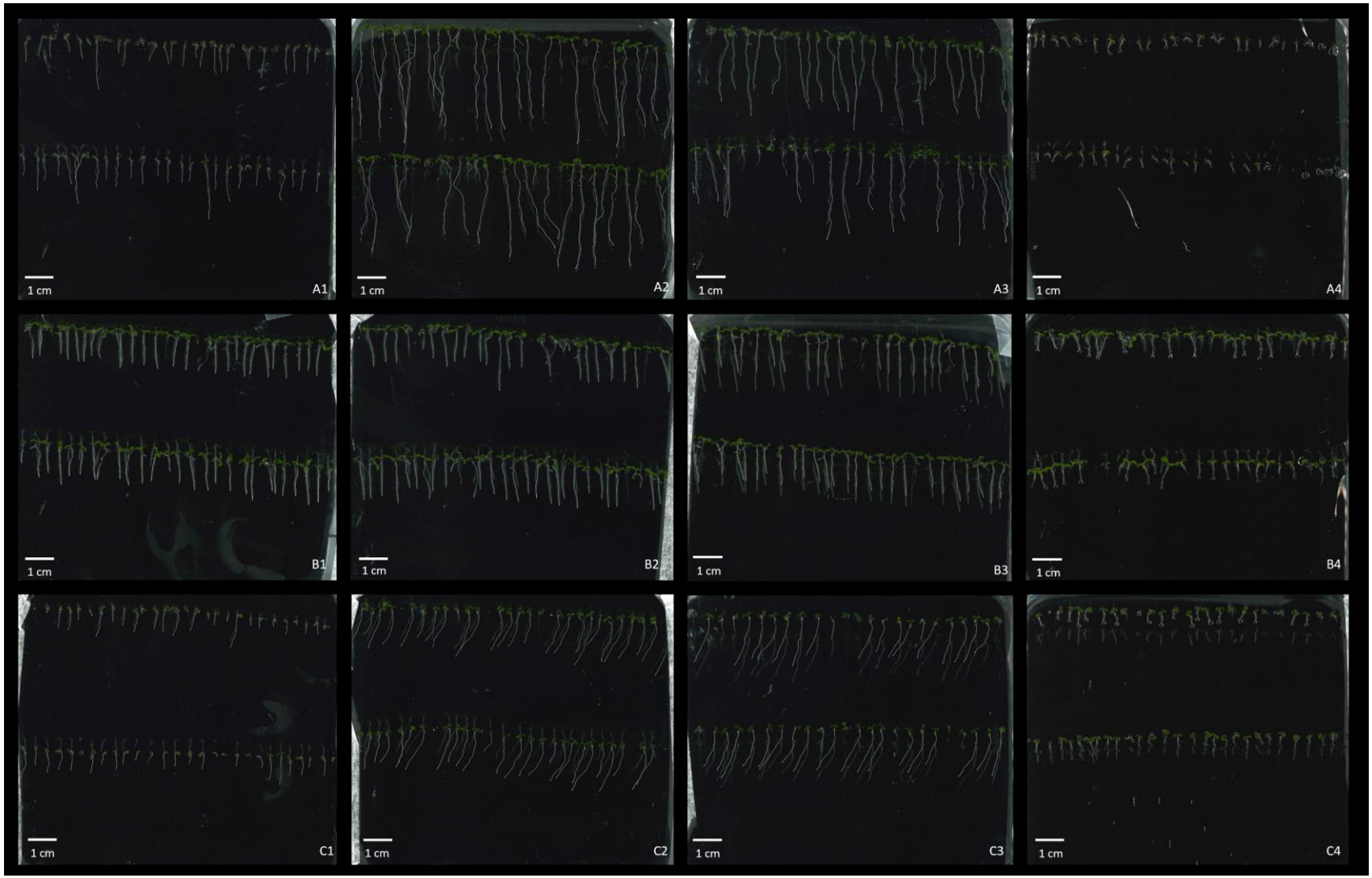



**Figure A. 5:** Images of insertion line *bzip25* seedlings of *Arabidopsis thaliana.* Numbers represent biological replicates (experiments) 1-4, while letters indicate treatment: A) mock, B) isoxaben and C) sorbitol. Scale bars represent 1 cm.

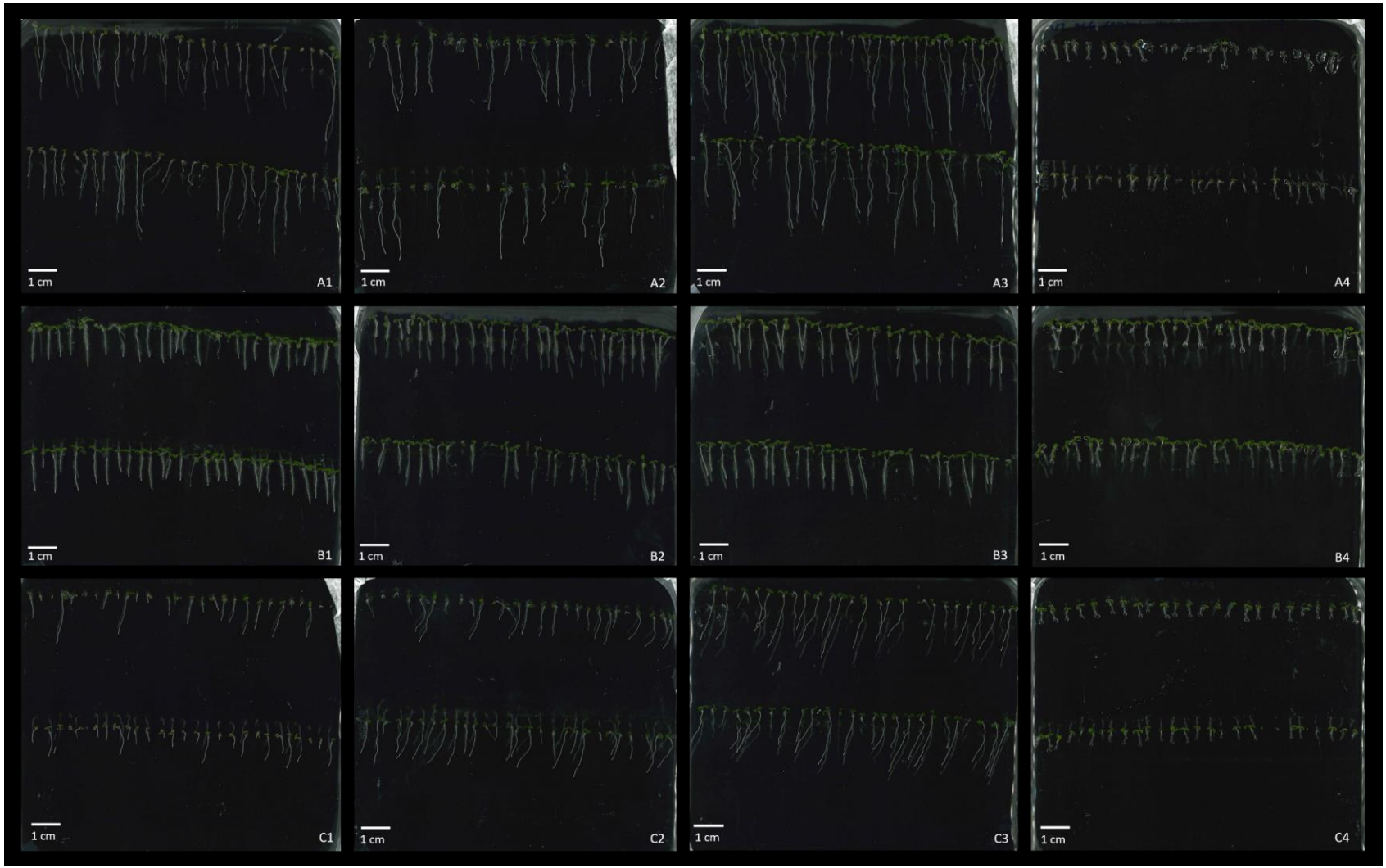



**Figure A.6:** Images of insertion line *cil1* seedlings of *Arabidopsis thaliana.* Numbers represent biological replicates (experiments) 1-4, while letters indicate treatment: A) mock, B) isoxaben and C) sorbitol. Scale bars represent 1 cm.

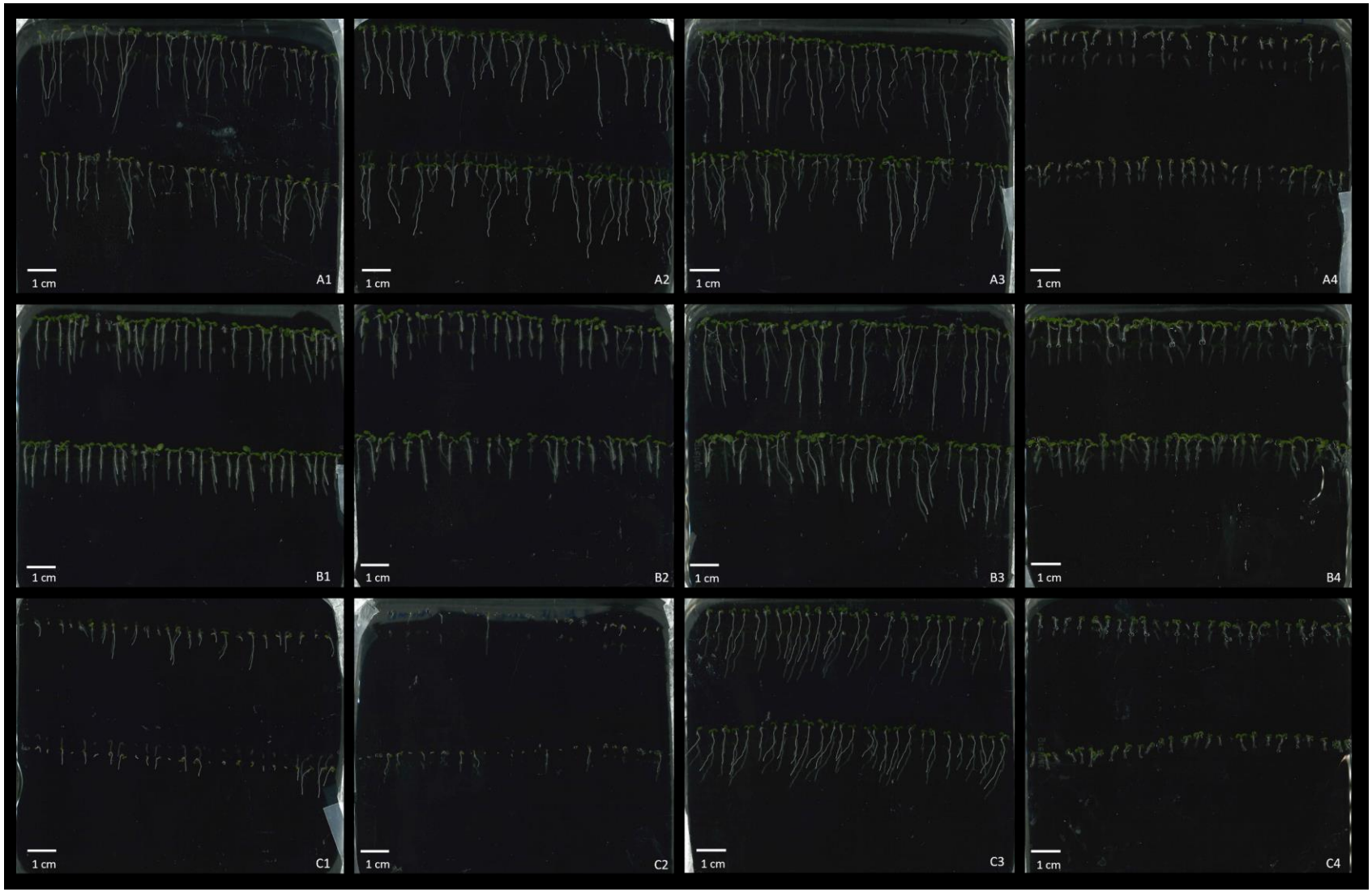



**Figure A.7:** Images of insertion line *bluejay* seedlings of *Arabidopsis thaliana.* Numbers represent biological replicates (experiments) 1-4, while letters indicate treatment: A) mock, B) isoxaben and C) sorbitol. Scale bars represent 1 cm.

**Table A1.6:** Measured root length (cm) in mock treatment for seedlings of *Arabidopsis thaliana*. Includes measurements from 3 biological replicates (independent experiments) for genotypes: Col-0 (wildtype), *the1-1* and *the1-4* (THE1 alleles), *ixr1-1* (resistant to isoxaben) and *bzip25, cil1* and *bluejay* (insertion lines). Measurement of seedling roots was performed using ImageJ where the scale bar was set to 1 cm.

| **MOCK** | | | | | | |
|---|---|---|---|---|---|---|
| Col-0 | *the1-1* | *the1-4* | *ixr1-1* | *bzip25* | *cil1* | *bluejay* |
| 1.086 | 1.528 | 2.669 | 2.617 | 0.789 | 1.043 | 2.271 |
| 2.326 | 0.924 | 3.368 | 1.509 | 0.614 | 1.871 | 2.43 |
| 1.247 | 1.718 | 2.964 | 1.464 | 0.716 | 1.418 | 2.037 |
| 1.647 | 1.496 | 3.692 | 1.332 | 0.57 | 1.793 | 0.698 |
| 1.977 | 1.141 | 4.073 | 1.679 | 0.735 | 1.468 | 1.341 |
| 1.679 | 0.81 | 3.218 | 0.656 | 0.264 | 1.368 | 2.282 |
| 1.256 | 1.986 | 4.16 | 1.568 | 1.694 | 1.284 | 1.04 |
| 1.229 | 1.409 | 3.519 | 1.494 | 0.355 | 1.992 | 2.877 |
| 1.922 | 1.48 | 1.772 | 1.325 | 0.907 | 0.984 | 1.357 |
| 0.63 | 1.18 | 3.596 | 1.484 | 0.576 | 0.659 | 1.508 |
| 1.237 | 1.128 | 3.796 | 1.098 | 0.749 | 1.133 | 1.863 |
| 2.076 | 1.161 | 3.806 | 1.297 | 0.666 | 1.632 | 1.063 |
| 1.521 | 1.213 | 3.014 | 1.413 | 0.795 | 1.43 | 0.817 |
| 1.861 | 1.026 | 3.319 | 1.927 | 0.534 | 0.984 | 2.177 |
| 1.421 | 1.065 | 3.603 | 0.566 | 0.802 | 1.75 | 1.265 |
| 1.939 | 1.001 | 3.432 | 1.233 | 1.002 | 1.158 | 1.402 |
| 1.615 | 1.296 | 4.119 | 0.726 | 0.818 | 0.777 | 2.125 |
| 1.214 | 1.309 | 3.629 | 1.518 | 0.881 | 0.894 | 1.707 |
| 2.402 | 1.169 | 3.562 | 1.401 | 0.864 | 1.746 | 0.901 |
| 1.983 | 0.935 | 3.714 | 1.34 | 0.436 | 0.958 | 1.645 |
| 2.215 | 1.039 | 3.602 | 1.487 | 1.089 | 1.097 | 0.873 |
| 1.983 | 1.606 | 3.174 | 1.268 | 0.85 | 1.502 | 1.533 |
| 0.96 | 1.54 | 3.13 | 1.282 | 0.84 | 0.671 | 1.158 |
| 3.356 | 0.944 | 3.611 | 1.553 | 0.885 | 1.027 | 1.151 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2.102 | 1.106 | 3.406 | 2.463 | 0.566 | 3.023 | 1.206 |
| 2.311 | 0.704 | 3.72 | 1.727 | 0.693 | 1.003 | 1.128 |
| 1.801 | 1.196 | 3.187 | 2.333 | 1.136 | 1.389 | 1.776 |
| 3.257 | 1.365 | 3.196 | 2.271 | 0.84 | 1.255 | 1.873 |
| 1.638 | 1.313 | 0.848 | 1.433 | 0.792 | 1.244 | 1.728 |
| 2.674 | 1.653 | 3.823 | 1.172 | 1.06 | 0.779 | 2.623 |
| 1.556 | 1.42 | 3.313 | 2.092 | 0.897 | 1.292 | 1.583 |
| 1.34 | 1.631 | 3.217 | 0.674 | 1.808 | 1.825 | 1.484 |
| 2.596 | 1.489 | 2.871 | 1.413 | 1.121 | 1.046 | 1.468 |
| 2.142 | 0.635 | 2.841 | 1.713 | 0.725 | 0.923 | 1.424 |
| 2.595 | 1.922 | 3.542 | 1.771 | 1.043 | 2.079 | 2.339 |
| 1.643 | 1.162 | 3.737 | 1.653 | 0.689 | 1.789 | 2.527 |
| 1.403 | 1.21 | 3.793 | 0.738 | 0.964 | 2.719 | 0.649 |
| 1.784 | 1.416 | 3.683 | 1.283 | 0.55 | 2.002 | 1.093 |
| 1.892 | 0.952 | 3.634 | 1.941 | 0.711 | 0.865 | 0.76 |
| 1.223 | 0.992 | 3.716 | 1.503 | 0.732 | 0.524 | 0.611 |
| 3.278 | 1.544 | 3.04 | 1.281 | 0.537 | 1.011 | 1.346 |
| 2.688 | 0.666 | 0.658 | 0.63 | 1.841 | 0.61 | 0.846 |
| 2.552 | 1.135 | 3.561 | 2.28 | 0.735 | 1.522 | 0.615 |
| 2.884 | 1.432 | 3.843 | 2.936 | 1.098 | 2.272 | 1.609 |
| 2.789 | 0.895 | 2.911 | 3.416 | 0.75 | 1.814 | 0.844 |
| 3.357 | 1.039 | 3.768 | 3.105 | 0.543 | 0.978 | 1.623 |
| 2.193 | 1.285 | 3.47 | 3.362 | 0.5 | 1.814 | 1.791 |
| 3.319 | 1.542 | 3.474 | 3.019 | 1.082 | 2.771 | 1.438 |
| 2.473 | 0.984 | 3.605 | 2.709 | 0.667 | 1.787 | 1.153 |
| 3.564 | 2.292 | 3.228 | 2.805 | 0.646 | 1.133 | 1.824 |
| 3.262 | 1.984 | 3.239 | 3.337 | 0.561 | 1.08 | 1.466 |
| 2.253 | 1.947 | 3.229 | 3.88 | 1.017 | 0.758 | 1.822 |
| 2.682 | 2.137 | 3.37 | 3.122 | 3.368 | 0.7 | 1.272 |
| 2.603 | 2.422 | 3.288 | 3.647 | 2.718 | 1.448 | 1.016 |
| 3.517 | 2.19 | 2.668 | 3.599 | 3.366 | 0.95 | 2.097 |
| 2.51 | 2.139 | 2.72 | 2.856 | 3.416 | 2.517 | 2.269 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2.736 | 1.938 | 2.865 | 0.976 | 3.921 | 2.182 | 2.263 |
| 2.414 | 1.876 | 2.14 | 2.77 | 2.329 | 1.798 | 2.39 |
| 2.169 | 1.675 | 2.518 | 3.272 | 2.319 | 2.072 | 2.406 |
| 2.986 | 2.261 | 2.478 | 2.068 | 3.192 | 1.428 | 1.657 |
| 0.666 | 1.136 | 2.681 | 3.476 | 3.477 | 2.316 | 1.082 |
| 2.813 | 2.071 | 2.908 | 3.425 | 0.755 | 1.195 | 2.127 |
| 3.368 | 1.842 | 2.314 | 3.064 | 3.108 | 0.635 | 2.211 |
| 0.855 | 2.177 | 2.547 | 3.168 | 3.383 | 2.096 | 0.599 |
| 2.471 | 1.723 | 2.305 | 3.104 | 3.309 | 2.037 | 1.922 |
| 3.26 | 2.036 | 2.075 | 2.976 | 2.621 | 2.248 | 1.564 |
| 2.559 | 1.921 | 2.502 | 3.019 | 2.883 | 2.432 | 1.521 |
| 3.022 | 2.14 | 2.507 | 2.841 | 2.386 | 1.744 | 1.051 |
| 2.198 | 1.925 | 1.957 | 2.769 | 0.995 | 2.166 | 1.751 |
| 3.13 | 2.041 | 2.172 | 2.726 | 3.588 | 1.972 | 0.863 |
| 2.685 | 1.967 | 2.402 | 2.966 | 2.94 | 1.621 | 2.348 |
| 3.247 | 1.744 | 2.626 | 2.626 | 0.264 | 1.512 | 2.13 |
| 2.929 | 1.686 | 2.57 | 0.824 | 3.562 | 2.437 | 2.021 |
| 2.03 | 1.826 | 2.642 | 3.278 | 3.109 | 2.291 | 1.016 |
| 2.831 | 2.225 | 2.516 | 1.135 | 2.81 | 2.568 | 0.472 |
| 0.647 | 2.082 | 2.655 | 3.041 | 0.77 | 1.706 | 2.612 |
| 3.259 | 1.862 | 0.861 | 2.703 | 2.495 | 1.835 | 0.66 |
| 2.293 | 1.286 | 0.616 | 2.986 | 2.988 | 1.847 | 2.072 |
| 2.724 | 1.928 | 2.76 | 2.906 | 2.672 | 2.792 | 0.505 |
| 3.262 | 0.592 | 0.677 | 2.125 | 3.282 | 1.758 | 1.089 |
| 3.489 | 1.726 | 0.606 | 3.12 | 2.485 | 2.427 | 1.728 |
| 3.34 | 0.657 | 2.748 | 2.385 | 2.538 | 1.989 | 2.255 |
| 3.217 | 0.771 | 2.291 | 2.498 | 2.81 | 1.408 | 2.57 |
| 2.495 | 2.161 | 2.988 | 2.766 | 0.75 | 0.724 | 1.798 |
| 2.685 | 1.783 | 2.38 | 2.601 | 2.594 | 0.694 | 2.065 |
| 3.283 | 1.843 | 1.779 | 2.436 | 2.783 | 1.761 | 2.025 |
| 3.235 | 0.602 | 1.921 | 3.398 | 0.793 | 2.886 | 0.681 |
| 3.004 | 2.507 | 0.474 | 1.053 | 0.86 | 2.47 | 2.357 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2.523 | 2.012 | 1.976 | 2.7 | 2.964 | 1.661 | 0.818 |
| 2.285 | 2.363 | 2.35 | 3.547 | 3.249 | 1.529 | 0.899 |
| 2.586 | 2.892 | 3.595 | 3.364 | 2.453 | 1.799 | 0.718 |
| 3.134 | 2.695 | 1.529 | 3.103 | 3.075 | 2.231 | 1.429 |
| 3.114 | 2.4 | 2.441 | 1.945 | 0.7 | 1.86 | 0.63 |
| 3.093 | 2.367 | 3.28 | 3.005 | 2.708 | 2.553 | 0.786 |
| 2.814 | 2.468 | 2.985 | 2.405 | 3.43 | 2.001 | 0.869 |
| 3.149 | 1.99 | 2.825 | 2.581 | 3.541 | 3.347 | 0.943 |
| 2.736 | 2.166 | 3.19 | 3.089 | 3.301 | 2.598 | 2.475 |
| 2.55 | 2.619 | 2.788 | 2.257 | 2.711 | 2.987 | 0.906 |
| 2.905 | 1.911 | 3.435 | 3.478 | 2.658 | 2.112 | 0.719 |
| 2.87 | 2.633 | 3.1 | 2.955 | 3.237 | 0.805 | 1.945 |
| 3.324 | 2.707 | 2.628 | 0.992 | 2.521 | 2.407 | 0.336 |
| 3.308 | 2.778 | 3.02 | 2.055 | 3.376 | 3.243 | 1.336 |
| 2.816 | 2.646 | 2.076 | 2.615 | 3.33 | 2.527 | 2.424 |
| 3.02 | 2.151 | 0.827 | 2.986 | 2.192 | 0.723 | 2.769 |
| 2.716 | 2.736 | 2.811 | 2.467 | 2.501 | 1.262 | 0.887 |
| 2.577 | 2.362 | 2.948 | 0.614 | 1.947 | 2.394 | 2.443 |
| 3.033 | 2.819 | 3.288 | 2.793 | 2.86 | 1.68 | 2.073 |
| 2.784 | 2.233 | 2.549 | 2.903 | 1.946 | 2.422 | 1.833 |
| 3.282 | 1.869 | 3.445 | 2.691 | 2.121 | 0.737 | 1.95 |
| 2.779 | 2.41 | 2.431 | 0.671 | 2.537 | 3.001 | 1.858 |
| 2.043 | 2.862 | 2.887 | 3.208 | 2.246 | 2.536 | 2.05 |
| 3.222 | 2.479 | 3.12 | 3.295 | 2.242 | 3.151 | 2.733 |
| 3.129 | 2.5 | 2.561 | 0.785 | 2.678 | 1.873 | 2.308 |
| 3.228 | 1.895 | 3.152 | 2.885 | 2.549 | 1.937 | 2.455 |
| 2.974 | 3.119 | 2.982 | 2.737 | 3.23 | 2.37 | 3.049 |
| 3.574 | 2.802 | 1.817 | 2.652 | 1.649 | 3.07 | 2.627 |
| 2.956 | 2.75 | 3.287 | 0.935 | 2.485 | 2.012 | 2.435 |
| 2.581 | 2.48 | 2.179 | 2.282 | 0.693 | 1.814 | 2.69 |
| 2.969 | 2.631 | 2.552 | 1.458 | 2.908 | 2.746 | 2.041 |
| 2.765 | 2.681 | 2.646 | 2.39 | 2.933 | 1.391 | 2.476 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3.056 | 2.426 | 3.116 | 3.1 | 1.55 | 0.874 | 2.827 |
| 2.691 | 2.037 | 2.67 | 0.538 | 1.52 | 3.006 | 2.123 |
| | 2.84 | 2.818 | 2.228 | 2.383 | 2.437 | 1.924 |
| | 2.352 | 3.138 | 2.852 | 1.818 | 2.237 | 2.429 |
| | 2.505 | 3.611 | 0.786 | 2.509 | 2.728 | 2.093 |
| | | 2.88 | 2.944 | 1.838 | 2.294 | 2.963 |
| | | 2.806 | 2.205 | 2.112 | 2.385 | 2.221 |
| | | 3.383 | 2.927 | 1.831 | 1.718 | 2.35 |
| | | 2.951 | 2.457 | 1.853 | 3.417 | 2.04 |
| | | 3.237 | 2.386 | 2.286 | 2.191 | 2.421 |
| | | 3.119 | 2.719 | 2.413 | 2.461 | 1.905 |
| | | 3.061 | 2.942 | 0.832 | 2.621 | 1.66 |
| | | 3.001 | 2.261 | 0.684 | 2.775 | 2.396 |
| | | 2.709 | 0.59 | 0.9 | 0.725 | 2.338 |
| | | 2.609 | 3.123 | 1.807 | 1.048 | 1.473 |
| | | | 2.373 | 1.221 | 2.943 | 2.085 |
| | | | 3.208 | 3.175 | | 1.851 |
| | | | 3.147 | 2.9 | | 2.535 |
| | | | | 1.24 | | 3.049 |
| | | | | 2.061 | | 2.738 |
| | | | | 2.669 | | 1.99 |
| | | | | 2.392 | | 2.194 |
| | | | | 2.603 | | 1.643 |
| | | | | 2.841 | | 2.694 |
| | | | | 0.88 | | |
| | | | | 2.338 | | |
| | | | | 2.816 | | |
| | | | | 2.119 | | |

**Table A1.7:** Measured root length (cm) in isoxaben treatment for seedlings of *Arabidopsis thaliana*. Includes measurements from 3 biological replicates (independent experiments) for genotypes: Col-0 (wildtype), *the1-1* and *the1-4* (THE1 alleles), *ixr1-1* (resistant to isoxaben) and *bzip25, cil1* and *bluejay* (insertion lines). Measurement of seedling roots was performed using ImageJ where the scale bar was set to 1 cm.

| **ISOXABEN** | | | | | | |
|---|---|---|---|---|---|---|
| Col-0 | *the1-1* | *the1-4* | *ixr1-1* | *bzip25* | *cil1* | *bluejay* |
| 1.47 | 0.899 | 1.197 | 3.454 | 1.014 | 1.126 | 1.049 |
| 1.2 | 1.168 | 1.366 | 3.527 | 0.871 | 1.061 | 0.788 |
| 1.447 | 1.026 | 1.383 | 2.844 | 1.051 | 0.977 | 0.974 |
| 1.259 | 1.029 | 1.265 | 3.177 | 1.085 | 1 | 1.214 |
| 1.289 | 1.513 | 1.209 | 3.445 | 1.008 | 1.418 | 0.797 |
| 1.085 | 1.295 | 1.254 | 3.349 | 0.861 | 0.969 | 0.764 |
| 1 | 1.06 | 1.185 | 2.727 | 1.011 | 1.195 | 0.44 |
| 1.288 | 0.796 | 1.22 | 2.766 | 0.64 | 0.914 | 0.369 |
| 1.181 | 1.247 | 1.238 | 3.4 | 1.205 | 1.033 | 1.069 |
| 1.305 | 0.9 | 1.075 | 3.564 | 1.077 | 1.274 | 0.834 |
| 1.242 | 1.014 | 1.154 | 3.418 | 1.075 | 1.081 | 1.198 |
| 1.095 | 1.183 | 1.273 | 2.576 | 1.124 | 1.214 | 0.852 |
| 1.392 | 1.143 | 1.603 | 2.7 | 1.095 | 1 | 1.123 |
| 1.404 | 1.073 | 1.143 | 2.697 | 1.203 | 0.769 | 0.781 |
| 1.045 | 1.027 | 1.41 | 3.644 | 0.893 | 0.878 | 0.622 |
| 1.428 | 1.131 | 1.171 | 2.96 | 1.054 | 1.031 | 1.016 |
| 0.954 | 1.269 | 1.296 | 3.345 | 1.114 | 1.038 | 0.907 |
| 1.281 | 1.234 | 1.161 | 3.701 | 1.241 | 0.802 | 0.997 |
| 1.276 | 1.323 | 1.71 | 3.577 | 1.055 | 0.689 | 0.849 |
| 1.112 | 1.438 | 0.95 | 3.041 | 1.11 | 1.059 | 0.892 |
| 1.208 | 1.001 | 1.18 | 3.135 | 0.703 | 0.705 | 0.856 |
| 1.629 | 1.141 | 1.25 | 3.551 | 1.197 | 1.268 | 1.103 |
| 1.237 | 0.939 | 1.486 | 3.849 | 1.077 | 1.072 | 0.872 |
| 0.974 | 1.239 | 0.95 | 3.814 | 1.179 | 0.938 | 0.968 |
| 1.212 | 1.039 | 1.218 | 3.629 | 0.921 | 0.849 | 1.11 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1.188 | 1.001 | 1.167 | 3.431 | 1.176 | 0.785 | 0.757 |
| 1.43 | 1.003 | 1.254 | 3.17 | 0.931 | 1.084 | 0.983 |
| 1.098 | 1.028 | 1.513 | 2.718 | 0.88 | 1 | 0.632 |
| 1.193 | 1.475 | 1.187 | 0.504 | 1.054 | 0.677 | 0.939 |
| 1.251 | 1.419 | 1.7 | 3.831 | 0.735 | 1.298 | 1.068 |
| 1.056 | 0.681 | 1.065 | 2.964 | 1.151 | 0.534 | 0.639 |
| 1.138 | 1.14 | 1.334 | 3.722 | 0.923 | 1.443 | 0.809 |
| 1.463 | 1.181 | 1.349 | 3.433 | 1.283 | 0.777 | 0.797 |
| 1.264 | 1.102 | 1.15 | 3.409 | 1.014 | 1.343 | 0.85 |
| 1.384 | 1.393 | 1.148 | 3.354 | 0.912 | 1.005 | 0.861 |
| 1.49 | 1.322 | 1.465 | 3.026 | 1.172 | 1.046 | 0.739 |
| 1.138 | 1.325 | 1.32 | 2.711 | 1.223 | 0.694 | 0.852 |
| 1.048 | 1.254 | 1.227 | 3.455 | 1.069 | 0.807 | 0.783 |
| 1.227 | 1.57 | 1.168 | 3.006 | 1.131 | 0.926 | 0.931 |
| 1.214 | 1.367 | 1.114 | 3.115 | 1.051 | 1.182 | 0.672 |
| 1.169 | 1.202 | 1.342 | 3.524 | 0.964 | 0.841 | 0.629 |
| 1.449 | 1.138 | 1.125 | 2.636 | 0.675 | 0.815 | 0.755 |
| 1.212 | 1.108 | 1.178 | 2.853 | 1.006 | 0.84 | 0.639 |
| 1.212 | 1.154 | 1.235 | 3.35 | 1.225 | 1.058 | 1.11 |
| 1.387 | 1.364 | 1.076 | 2.472 | 1.127 | 1.011 | 1.023 |
| 1.408 | 1.147 | 1.351 | 2.941 | 1.068 | 0.949 | 0.733 |
| 1.35 | 1.062 | 1.18 | 2.954 | 1.098 | 0.847 | 0.801 |
| 1.193 | 1.508 | 1.121 | 2.667 | 1.06 | 0.744 | 1.198 |
| 1.243 | 1.419 | 1.189 | 2.751 | 1.286 | 0.833 | 0.937 |
| 0.897 | 1.26 | 1.354 | 3.324 | 1.132 | 0.868 | 0.97 |
| 1.181 | 1.358 | 2.072 | 1.045 | 1.044 | 0.906 | 1.077 |
| 0.945 | 1.045 | 1.263 | 3.578 | 1.079 | 1.105 | 0.586 |
| 1.075 | 1.188 | 1.321 | 3.32 | 1.049 | 0.945 | 0.981 |
| 1.257 | 1.204 | 1.328 | 3.194 | 1.19 | 1.002 | 0.991 |
| 1.026 | 1.542 | 1.28 | 2.858 | 1.188 | 1.171 | 0.783 |
| 1.192 | 1.019 | 1.469 | 0.856 | 0.773 | 1.066 | 0.695 |
| 0.668 | 0.778 | 1.112 | 2.304 | 1.251 | 1.112 | 0.965 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1.252 | 0.952 | 1.348 | 2.153 | 1.097 | 1.306 | 1.316 |
| 0.539 | 0.878 | 1.11 | 2.409 | 1.135 | 0.634 | 1.793 |
| 0.848 | 0.727 | 1.29 | 2.769 | 1.058 | 0.694 | 0.973 |
| 1.188 | 0.579 | 1.3 | 3.039 | 0.704 | 0.592 | 0.513 |
| 0.717 | 0.728 | 1.819 | 2.763 | 0.982 | 0.568 | 0.824 |
| 1.011 | 0.855 | 1.12 | 2.6 | 1.258 | 0.65 | 0.667 |
| 0.846 | 0.806 | 1.047 | 2.484 | 0.998 | 0.898 | 0.553 |
| 0.885 | 1.127 | 1.128 | 0.98 | 0.922 | 0.769 | 1.28 |
| 0.631 | 0.931 | 1.107 | 2.691 | 0.915 | 0.885 | 0.823 |
| 1.129 | 0.618 | 1.627 | 3.138 | 1.124 | 0.925 | 0.556 |
| 0.762 | 1.048 | 0.829 | 2.527 | 1.918 | 0.882 | 0.793 |
| 0.825 | 0.891 | 1.033 | 2.584 | 1.163 | 0.944 | 0.781 |
| 0.734 | 0.891 | 0.789 | 0.813 | 0.942 | 0.791 | 0.62 |
| 1.179 | 0.684 | 1.35 | 2.511 | 1.246 | 0.908 | 0.667 |
| 0.736 | 1.025 | 1.365 | 2.992 | 0.955 | 0.818 | 0.865 |
| 0.896 | 0.634 | 0.882 | 2.639 | 0.438 | 0.913 | 1.191 |
| 0.963 | 1.178 | 1.144 | 2.727 | 1.198 | 0.976 | 0.915 |
| 1.019 | 0.957 | 0.9 | 2.259 | 1.309 | 0.945 | 0.991 |
| 1.175 | 1.217 | 1.587 | 2.368 | 1.297 | 1.156 | 0.757 |
| 0.904 | 0.772 | 1.104 | 2.198 | 0.854 | 0.819 | 0.951 |
| 0.878 | 1.093 | 1.209 | 2.094 | 1.18 | 0.94 | 0.856 |
| 0.581 | 1.146 | 1.007 | 1.227 | 1.181 | 1.339 | 0.843 |
| 0.974 | 0.706 | 1.375 | 2.31 | 1.326 | 0.762 | 0.81 |
| 0.241 | 0.687 | 1.388 | 2.62 | 1.317 | 1.038 | 1.18 |
| 0.868 | 0.69 | 1.149 | 2.485 | 1.456 | 0.867 | 1.578 |
| 1.083 | 0.798 | 0.844 | 2.613 | 1.129 | 0.968 | 1.267 |
| 0.479 | 0.768 | 0.93 | 2.283 | 1.374 | 1.481 | 0.621 |
| 0.85 | 0.951 | 0.71 | 2.486 | 1.205 | 0.908 | 0.812 |
| 0.907 | 0.604 | 1.076 | 2.108 | 1.248 | 0.722 | 0.786 |
| 0.972 | 0.594 | 0.916 | 2.489 | 1.451 | 0.93 | 1.514 |
| 0.855 | 1.06 | 1.204 | 2.604 | 1.001 | 0.834 | 1.091 |
| 0.762 | 0.64 | 0.405 | 2.115 | 1.014 | 0.707 | 0.903 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.791 | 0.639 | 0.815 | 0.714 | 1.426 | 1.185 | 0.919 |
| 0.785 | 0.698 | 1.119 | 1.098 | 1.161 | 1.03 | 0.83 |
| 0.812 | 0.655 | 1.154 | 2.826 | 1.019 | 0.905 | 0.709 |
| 0.773 | 0.81 | 1.518 | 2.276 | 0.614 | 0.78 | 0.915 |
| 1.229 | 0.911 | 1.186 | 2.548 | 1.39 | 0.999 | 1.061 |
| 0.982 | 0.761 | 1.026 | 2.595 | 1.05 | 0.706 | 1.304 |
| 0.861 | 0.609 | 1.188 | 1.997 | 1.33 | 1.142 | 0.958 |
| 1.605 | 1.011 | 1.459 | 2.808 | 1.404 | 0.735 | 0.839 |
| 0.954 | 0.678 | 1.24 | 2.625 | 1.415 | 1.258 | 0.86 |
| 0.939 | 0.823 | 1.195 | 2.682 | 1.085 | 0.67 | 0.749 |
| 0.865 | 0.905 | 1.404 | 2.254 | 1.219 | 0.92 | 0.899 |
| 0.933 | 0.802 | 1.105 | 2.389 | 1.406 | 0.683 | 0.724 |
| 1.001 | 0.832 | 1.501 | 2.453 | 1.394 | 0.866 | 0.713 |
| 0.838 | 0.87 | 1.354 | 0.576 | 1.181 | 1.093 | 0.74 |
| 0.896 | 0.742 | 1.409 | 2.029 | 1.481 | 0.514 | 0.748 |
| 0.825 | 0.748 | 1.191 | 2.295 | 0.893 | 0.877 | 0.83 |
| 0.973 | 0.872 | 1.165 | 2.132 | 0.937 | 1.407 | 1.661 |
| 1.195 | 1.412 | 0.94 | 3.122 | 1.319 | 1.234 | 1.076 |
| 1.126 | 1.298 | 1 | 2.816 | 1.154 | 1.156 | 1.245 |
| 1.005 | 1.086 | 1.166 | 2.995 | 1.245 | 0.647 | 1.554 |
| 1.058 | 1.205 | 1.618 | 3.606 | 0.654 | 0.628 | 1.176 |
| 1.215 | 0.815 | 3.373 | 3.885 | 1.201 | 1.261 | 0.785 |
| 0.915 | 0.603 | 2.739 | 3.098 | 0.981 | 0.882 | 1.483 |
| 1.069 | 0.818 | 1.467 | 2.623 | 1.458 | 1.024 | 1.796 |
| 0.977 | 1.187 | 2.968 | 2.858 | 1.226 | 0.958 | 1.522 |
| 1.154 | 0.85 | 1.231 | 3.081 | 1.435 | 1.158 | 1.32 |
| 1.427 | 0.982 | 1.158 | 0.982 | 0.816 | 0.929 | 1.856 |
| 1.073 | 0.976 | 1.364 | 3.248 | 1.329 | 0.985 | 1.366 |
| 1.085 | 1.183 | 2.427 | 2.93 | 1.142 | 0.976 | 2.53 |
| 1.127 | 1.124 | 1.489 | 2.617 | 1.261 | 0.797 | 0.81 |
| 1.125 | 0.948 | 1.605 | 0.595 | 1.162 | 1.209 | 1.829 |
| 0.968 | 0.954 | 1.622 | 2.644 | 1.002 | 0.924 | 0.756 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.928 | 1.071 | 1.061 | 3.542 | 1.387 | 1.34 | 2.481 |
| 0.919 | 0.971 | 1.207 | 0.919 | 1.049 | 1.117 | 2.148 |
| 1.003 | 1.067 | 1.666 | 2.492 | 1.144 | 1.854 | 2.518 |
| 0.979 | 1.186 | 1.496 | 0.92 | 1.076 | 0.885 | 2.123 |
| 1.027 | 1.089 | 1.922 | 2.827 | 1.27 | 0.878 | 1.618 |
| 1.03 | 1.472 | 1.373 | 0.547 | 1.121 | 1.042 | |
| 1.026 | 1.138 | 1.23 | 2.541 | 1.236 | 0.907 | |
| 0.823 | 1.14 | 1.462 | 1.825 | 0.886 | 0.944 | |
| 0.991 | 1.164 | 1.772 | 2.852 | 1.024 | 0.864 | |
| 1.059 | 1.116 | 1.507 | 2.797 | 0.815 | 0.971 | |
| 0.967 | 1.169 | 1.419 | 3.492 | 1.211 | 1.031 | |
| 1.005 | 1.06 | 1.537 | 0.694 | 1.272 | 1.116 | |
| 1.039 | 1.137 | 1.338 | 3.108 | 0.904 | 0.757 | |
| 0.954 | 1.198 | 1.291 | 3.094 | 1.295 | 1.024 | |
| 1.138 | 1.08 | 1.415 | 2.635 | 1.425 | 0.909 | |
| 0.928 | 0.985 | 1.38 | 2.793 | 1.05 | 1.145 | |
| 1.467 | 1.048 | 1.219 | 2.56 | 0.988 | 1.059 | |
| 1.276 | 1.253 | 2.832 | 3.646 | 1.228 | 1.131 | |
| 1.184 | 1.08 | 1.781 | 2.752 | 1.173 | 0.763 | |
| 1.119 | 1.496 | 1.591 | 2.869 | 1.087 | 1.014 | |
| 1.091 | 1.07 | 1.331 | 3.427 | 0.849 | 1.064 | |
| 1.181 | 1.149 | 1.469 | 3.2 | 1.074 | 0.802 | |
| 1.052 | 0.766 | 1.659 | 3.417 | 0.66 | 1.06 | |
| 1.077 | 1.188 | 1.19 | 2.642 | 1.026 | 1.028 | |
| 1.139 | 0.919 | 1.374 | 0.55 | 1.083 | 0.966 | |
| 1.195 | 1.39 | 2.041 | 1.188 | 1.191 | 1.191 | |
| 1.178 | 1.353 | 1.323 | 3.054 | 1.162 | 1.3 | |
| 1.079 | 0.815 | 1.76 | 2.177 | 0.928 | 1.066 | |
| 1.156 | 1.182 | 1.539 | 3.052 | 1.218 | 1.405 | |
| 1.139 | 1.004 | | 2.202 | 1.152 | 0.811 | |
| 1.323 | 1.275 | | 3.038 | 1.171 | 0.818 | |
| 0.915 | 0.934 | | 3.382 | 1.095 | 0.949 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1.111 | 0.989 | | 2.429 | 1.284 | 1.393 | |
| 0.902 | 1.147 | | | 0.986 | 0.865 | |
| 0.994 | 1.299 | | | 1.173 | 0.927 | |
| | 1.224 | | | 1.382 | 0.825 | |
| | 0.759 | | | 1.093 | | |
| | 1.042 | | | 1.223 | | |
| | 0.84 | | | 1.302 | | |
| | 1.215 | | | 1.098 | | |
| | 0.597 | | | | | |
| | 1.077 | | | | | |
| | 1.184 | | | | | |
| | 1.195 | | | | | |
| | 0.65 | | | | | |
| | 1.119 | | | | | |
| | 1.179 | | | | | |
| | 1.095 | | | | | |

**Table A1.8:** Measured root length (cm) in sorbitol treatment for seedlings of *Arabidopsis thaliana*. Includes measurements from 3 biological replicates (independent experiments) for genotypes: Col-0 (wildtype), *the1-1* and *the1-4* (THE1 alleles), *ixr1-1* (resistant to isoxaben) and *bzip25, cil1* and *bluejay* (insertion lines). Measurement of seedling roots was performed using ImageJ where the scale bar was set to 1 cm.

| **SORBITOL** | | | | | | |
|---|---|---|---|---|---|---|
| Col-0 | *the1-1* | *the1-4* | *ixr1-1* | *bzip25* | *cil1* | *bluejay* |
| 0.293 | 0.243 | 0.288 | 0.559 | 0.285 | 0.795 | 0.594 |
| 0.102 | 0.71 | 0.8 | 0.867 | 0.665 | 0.293 | 0.371 |
| 0.856 | 0.671 | 0.697 | 0.572 | 0.784 | 1.712 | 0.462 |
| 0.482 | 0.406 | 0.932 | 0.81 | 0.51 | 0.318 | 0.602 |
| 0.759 | 0.376 | 0.832 | 0.92 | 0.244 | 0.615 | 0.621 |
| 0.631 | 0.509 | 0.981 | 0.737 | 1.031 | 0.181 | 1.156 |
| 1.38 | 0.43 | 0.78 | 0.72 | 0.704 | 0.222 | 0.644 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1.13 | 0.375 | 0.526 | 1.029 | 0.613 | 0.189 | 0.241 |
| 0.699 | 0.451 | 0.785 | 1.545 | 0.764 | 0.222 | 0.148 |
| 1.897 | 0.499 | 0.805 | 0.553 | 0.314 | 0.423 | 1.071 |
| 1.068 | 0.478 | 0.455 | 0.747 | 0.724 | 0.538 | 1.273 |
| 0.893 | 0.575 | 0.99 | 1.06 | 0.914 | 0.928 | 0.59 |
| 0.751 | 0.34 | 1.039 | 0.697 | 0.934 | 0.458 | 0.463 |
| 1.509 | 0.562 | 0.759 | 0.732 | 0.749 | 0.549 | 0.416 |
| 1.28 | 0.401 | 0.757 | 0.479 | 0.212 | 0.764 | 0.946 |
| 1.022 | 0.428 | 1.107 | 0.854 | 0.484 | 0.832 | 1.199 |
| 0.476 | 0.63 | 0.485 | 1.148 | 0.223 | 1.252 | 0.403 |
| 0.881 | 0.446 | 0.739 | 0.203 | 1.208 | 0.957 | 0.425 |
| 1.509 | 0.408 | 0.781 | 0.327 | 0.271 | 0.311 | 0.619 |
| 1.471 | 0.62 | 0.844 | 0.738 | 0.388 | 1.198 | 0.318 |
| 1.123 | 0.363 | 0.871 | 1.393 | 0.365 | 0.39 | 0.765 |
| 1.411 | 0.682 | 0.769 | 0.78 | 0.456 | 0.223 | 0.289 |
| 0.945 | 0.411 | 0.577 | 0.393 | 0.305 | 1.13 | 0.637 |
| 0.258 | 0.373 | 0.742 | 0.494 | 0.391 | 0.544 | 0.446 |
| 1.207 | 0.562 | 0.634 | 0.639 | 0.571 | 0.971 | 0.491 |
| 0.677 | 0.238 | 0.538 | 0.552 | 0.268 | 0.972 | 0.622 |
| 0.417 | 0.507 | 0.758 | 1.129 | 0.385 | 0.265 | 0.369 |
| 0.663 | 0.509 | 0.712 | 0.654 | 1.086 | 0.399 | 0.15 |
| 0.655 | 0.5 | 0.846 | 0.353 | 0.526 | 0.456 | 0.468 |
| 1.103 | 0.426 | 0.228 | 0.601 | 0.776 | 0.504 | 0.66 |
| 1.574 | 0.415 | 0.614 | 0.755 | 0.746 | 1.086 | 0.761 |
| 0.696 | 0.403 | 0.567 | 1.294 | 0.802 | 0.526 | 0.137 |
| 0.792 | 0.567 | 0.743 | 1.128 | 0.723 | 0.537 | 0.196 |
| 0.261 | 0.347 | 0.32 | 1.369 | 0.479 | 0.441 | 0.476 |
| 1.029 | 0.741 | 0.525 | 0.66 | 0.618 | 0.4 | 0.975 |
| 0.525 | 0.373 | 0.977 | 1.194 | 0.469 | 0.693 | 1.16 |
| 0.384 | 0.52 | 0.337 | 0.803 | 0.322 | 0.354 | 0.663 |
| 0.781 | 0.517 | 0.853 | 1.055 | 0.421 | 0.469 | 0.22 |
| 0.475 | 0.563 | 0.908 | 0.694 | 0.624 | 1.092 | 0.488 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 0.713 | 0.316 | 0.837 | 1.111 | 0.499 | 0.373 | 0.337 |
| 0.349 | 0.696 | 0.6 | 0.471 | 0.526 | 0.539 | 0.833 |
| 1.14 | 0.408 | 0.636 | 0.597 | 0.618 | 0.824 | 0.313 |
| 0.601 | 0.578 | 0.493 | 0.655 | 0.139 | 0.403 | 0.169 |
| 0.464 | 0.257 | 0.53 | 0.649 | 0.495 | 0.895 | 0.188 |
| 0.968 | 0.428 | 0.258 | 0.76 | 0.514 | 0.689 | 0.581 |
| 0.866 | 0.3 | 0.659 | 0.868 | 0.422 | 1.06 | 0.842 |
| 1.02 | 0.294 | 0.473 | 0.72 | 0.794 | 0.844 | 0.558 |
| 1.029 | 0.954 | 0.616 | 0.554 | 1.051 | 0.317 | 0.406 |
| 0.542 | 0.327 | 0.763 | 0.74 | 0.6 | 0.93 | 0.49 |
| 1.112 | 0.577 | 0.508 | 0.736 | 0.648 | 0.717 | 0.56 |
| 0.264 | 0.42 | 0.706 | 0.98 | 0.332 | 0.573 | 0.501 |
| 1.056 | 0.45 | 0.485 | 0.54 | 0.642 | 0.168 | 0.513 |
| 0.558 | 0.192 | 0.665 | 0.32 | 0.121 | 0.153 | 0.334 |
| 2.125 | 0.47 | 0.282 | 0.507 | 1.505 | 1.276 | 0.716 |
| 1.548 | 0.49 | 0.469 | 0.581 | 0.97 | 1.543 | 1.253 |
| 1.147 | 0.439 | 0.204 | 0.668 | 1.59 | 0.47 | 0.729 |
| 1.299 | 0.47 | 0.335 | 0.377 | 1.307 | 0.52 | 1.583 |
| 1.794 | 0.333 | 0.256 | 0.437 | 1.483 | 1.383 | 1.483 |
| 1.334 | 0.473 | 0.644 | 0.505 | 0.943 | 0.802 | 0.912 |
| 1.31 | 0.37 | 0.154 | 0.356 | 1.156 | 0.666 | 1.44 |
| 1.533 | 0.282 | 0.485 | 0.523 | 1.379 | 1.224 | 0.992 |
| 1.632 | 0.494 | 0.552 | 1.116 | 0.597 | 1.171 | 1.631 |
| 1.753 | 0.741 | 0.345 | 0.182 | 1.76 | 1.123 | 1.856 |
| 1.177 | 0.56 | 0.311 | 0.713 | 1.529 | 1.041 | 1.786 |
| 1.845 | 0.504 | 0.485 | 0.665 | 1.468 | 0.561 | 1.422 |
| 0.81 | 0.321 | 0.708 | 0.55 | 1.398 | 0.526 | 1.501 |
| 1.774 | 0.602 | 0.573 | 1.12 | 1.323 | 0.684 | 1.786 |
| 1.072 | 0.419 | 0.509 | 0.561 | 1.129 | 0.96 | 1.533 |
| 2.092 | 0.266 | 0.382 | 0.813 | 1.34 | 1.187 | 1.378 |
| 1.515 | 0.464 | 0.408 | 0.488 | 1.438 | 0.702 | 1.311 |
| 1.513 | 0.522 | 0.379 | 1.097 | 1.408 | 0.198 | 1.404 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1.851 | 0.648 | 0.283 | 0.718 | 1.399 | 1.014 | 1.625 |
| 1.07 | 0.622 | 0.523 | 0.393 | 1.826 | 1.186 | 1.601 |
| 1.236 | 0.36 | 0.501 | 0.777 | 1.078 | 0.595 | 1.71 |
| 1.26 | 0.244 | 0.348 | 0.6 | 1.603 | 1.079 | 0.61 |
| 0.628 | 0.449 | 0.597 | 0.263 | 1.655 | 1.175 | 1.143 |
| 1.613 | 0.434 | 0.236 | 0.265 | 1.532 | 1.518 | 1.011 |
| 1.482 | 0.62 | 0.407 | 0.704 | 1.254 | 0.47 | 1.029 |
| 1.514 | 0.421 | 0.67 | 0.636 | 1.553 | 1.018 | 1.455 |
| 0.887 | 0.309 | 0.353 | 0.372 | 1.193 | 1.48 | 1.229 |
| 1.877 | 0.599 | 0.181 | 0.429 | 1.008 | 1.356 | 1.182 |
| 1.452 | 0.361 | 0.25 | 0.604 | 1.138 | 1.15 | 1.746 |
| 1.734 | 0.429 | 0.191 | 0.628 | 1.359 | 1.5 | 0.709 |
| 1.319 | 0.706 | 0.668 | 0.72 | 1.211 | 1.466 | 1.283 |
| 0.665 | 0.425 | 0.724 | 1.123 | 1.563 | 1.07 | 1.192 |
| 1.014 | 0.193 | 0.477 | 0.556 | 1.523 | 1.123 | 1.404 |
| 1.329 | 0.4 | 0.207 | 0.434 | 1.199 | 0.614 | 1.878 |
| 1.203 | 0.384 | 0.458 | 0.98 | 1.373 | 0.447 | 1.671 |
| 1.445 | 0.442 | 0.206 | 0.872 | 1.523 | 1.215 | 1.657 |
| 2.257 | 0.361 | 0.188 | 0.59 | 0.731 | 1.056 | 1.169 |
| 1.688 | 0.464 | 0.635 | 0.647 | 1.308 | 1.45 | 1.322 |
| 1.695 | 0.432 | 0.472 | 0.54 | 1.592 | 0.529 | 1.032 |
| 1.302 | 0.513 | 0.279 | 0.549 | 1.356 | 0.975 | 0.964 |
| 1.662 | 0.485 | 0.446 | 0.166 | 1.372 | 1.38 | 1.46 |
| 2.089 | 0.464 | 0.2 | 0.65 | 0.785 | 0.821 | 1.541 |
| 1.414 | 0.283 | 0.435 | 0.749 | 1.468 | 1.631 | 1.534 |
| 1.173 | 0.492 | 0.43 | 0.553 | 1.333 | 0.322 | 0.574 |
| 0.97 | 0.502 | 0.359 | 0.73 | 1.285 | 0.985 | 1.49 |
| 1.418 | 0.22 | 0.612 | 1.358 | 1.793 | 0.922 | 1.159 |
| 1.145 | 0.326 | 0.3 | 0.335 | 1.168 | 0.908 | 0.8 |
| 1.614 | 0.564 | 0.44 | 0.419 | 1.626 | 0.813 | 1.216 |
| 1.646 | 1.705 | 0.58 | 0.333 | 1.18 | 1.349 | 1.339 |
| 1.734 | 1.659 | 1.454 | 0.511 | 1.699 | 1.229 | 1.298 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 1.541 | 1.726 | 1.542 | 1.425 | 1.412 | 1.639 | 1.949 |
| 1.554 | 0.648 | 1.48 | 1.449 | 1.711 | 1.646 | |
| 1.752 | 1.316 | 1.535 | 1.404 | 0.692 | 1.363 | |
| 1.471 | 1.364 | 1.405 | 1.406 | 0.642 | 1.179 | |
| 1.866 | 1.831 | 1.545 | 1.604 | 1.796 | 1.825 | |
| 1.73 | 1.79 | 0.784 | 1.288 | 1.581 | 1.244 | |
| 1.439 | 1.806 | 1.431 | 1.615 | 1.662 | 1.58 | |
| 1.671 | 1.534 | 1.415 | 1.616 | 1.719 | 1.339 | |
| 1.734 | 0.541 | 1.439 | 1.808 | 1.696 | 2.082 | |
| 1.99 | 1.438 | 2.252 | 1.744 | 1.364 | 1.735 | |
| 2.189 | 1.205 | 1.413 | 1.797 | 0.742 | 1.392 | |
| 2.329 | 1.333 | 1.58 | 1.404 | 1.476 | 1.283 | |
| 1.929 | 1.636 | 1.806 | 1.304 | 1.196 | 1.213 | |
| 1.865 | 1.64 | 1.446 | 1.585 | 1.342 | 1.396 | |
| 2.047 | 1.11 | 0.7 | 1.535 | 1.239 | 1.412 | |
| 1.858 | 1.81 | 1.058 | 0.642 | 0.822 | 1.373 | |
| 1.986 | 1.819 | 1.58 | 0.621 | 1.386 | 1.453 | |
| 1.879 | 1.841 | 1.701 | 1.617 | 1.547 | 1.278 | |
| 2.095 | 1.144 | 0.546 | 2.125 | 1.58 | 1.486 | |
| 1.783 | 1.605 | 0.788 | 1.323 | 1.59 | 1.472 | |
| 1.978 | 0.946 | 1.203 | 1.328 | 1.248 | 1.117 | |
| 2.192 | 1.772 | 1.508 | 1.093 | 1.588 | 1.02 | |
| 1.593 | 1.17 | 1.833 | 1.397 | 1.225 | 1.552 | |
| 2.099 | 1.33 | 1.205 | 1.515 | 1.313 | 1.844 | |
| 2.079 | 1.544 | 1.352 | 1.669 | 1.41 | 1.402 | |
| 2 | 0.541 | 1.486 | 1.372 | 1.74 | 1.168 | |
| 1.589 | 1.731 | 0.578 | 1.691 | 1.377 | 1.075 | |
| 1.83 | 0.779 | 0.49 | 1.514 | 1.363 | 1.914 | |
| 2.331 | 1.606 | 0.501 | 1.518 | 1.577 | 1.592 | |
| 2.014 | 0.796 | 1.294 | 1.588 | 1.349 | 1.83 | |
| 1.907 | 1.393 | 1.415 | 1.27 | 1.566 | 1.617 | |
| 1.9 | 0.917 | 1.747 | 0.835 | 1.025 | 0.999 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2.024 | 0.666 | 0.532 | 1.724 | 1.57 | 1.49 | |
| 1.959 | 1.838 | 1.069 | 1.638 | 1.659 | 1.589 | |
| 1.626 | 1.262 | 1.417 | 1.694 | 1.571 | 1.861 | |
| 2.044 | 0.501 | 0.805 | 1.313 | 1.575 | | |
| 1.615 | 0.649 | 1.705 | 1.337 | 0.661 | | |
| 2.1 | 1.363 | 0.574 | 1.195 | 1.349 | | |
| 1.941 | 1.277 | 1.586 | 1.444 | 1.586 | | |
| 1.483 | 1.331 | 0.979 | 0.689 | 1.227 | | |
| 1.77 | 0.692 | 1.543 | 0.797 | 0.672 | | |
| 1.667 | 1.671 | 1.702 | 1.679 | 1.72 | | |
| 1.805 | 1.169 | 1.949 | 0.613 | 1.426 | | |
| 2.005 | 1.358 | 1.723 | 0.889 | 1.637 | | |
| 1.76 | 1.552 | 1.763 | 1.682 | | | |
| 2.418 | 1.691 | 1.523 | 1.619 | | | |
| 1.846 | | 1.014 | 1.44 | | | |
| 1.733 | | 1.627 | 1.482 | | | |
| 2.122 | | 1.708 | | | | |
| 1.975 | | 1.064 | | | | |
| 2.104 | | | | | | |
| 2.17 | | | | | | |
| 2.254 | | | | | | |
| 2.087 | | | | | | |
| 2.331 | | | | | | |
| 2.019 | | | | | | |

# Appendix 2 – Statistics

A one-way ANOVA and post hoc Tukey HSD test was conducted to compare seedling root length between genotypes (Col-0, *the1-1, the1-4, bzip25, cil1* and *bluejay*) and treatments (mock, isoxaben and sorbitol), and p-values presented in Table A2.1. A student's t-test was used in the gene expression and the SA biosensor analysis, and p-values are included in Table A2.2 and Table A2.3, respectively. Additionally, a one-way ANOVA and post hoc Tukey HSD test was performed on the dataset form SA biosensor analysis (Table A2.4).

**Table A2.1:** A post hoc Tukey HSD test was performed among genotypes: Col-0, *the1-1, the1-4, bzip25, cil1* and *bluejay,* with mock, isoxaben (ISX) and sorbitol treatments. Different lowercase letter indicates significant difference ($p < 0.05$). Provided by L. A. Baez.

| | **Mock** | **ISX** | **Sorbitol** |
|---|---|---|---|
| **Col-0** | bc | g | e |
| ***the1-1*** | d | gh | i |
| ***the1-4*** | a | ef | hi |
| ***ixr1-1*** | c | ab | ghi |
| ***bzip25*** | d | fg | g |
| ***cil1*** | d | ghi | ghi |
| ***bluejay*** | d | gh | ghi |

**Table A2.2:** P-values found for relative expression of genes of interest (*BZIP25, CIL1* and *BLUEJAY*) between insertion lines (*bzip25, cil1* and *bluejay*) and wildtype Col-0 using a student's t-test in excel. Green cells indicate statistical significance.

| | ***bzip25*** | ***cil1*** | ***bluejay*** |
|---|---|---|---|
| **t-test** | $5.52014\times10^{-08}$ | 0.000117 | 0.000243 |

**Table A2.3:** P-values found for amount of detected luminescence between *the1-1, the1-4, bzip25, cil1* and *bluejay,* and Col-0 isoxaben-treated seedlings using a student's t-test in excel. Green cells indicate statistical significance.

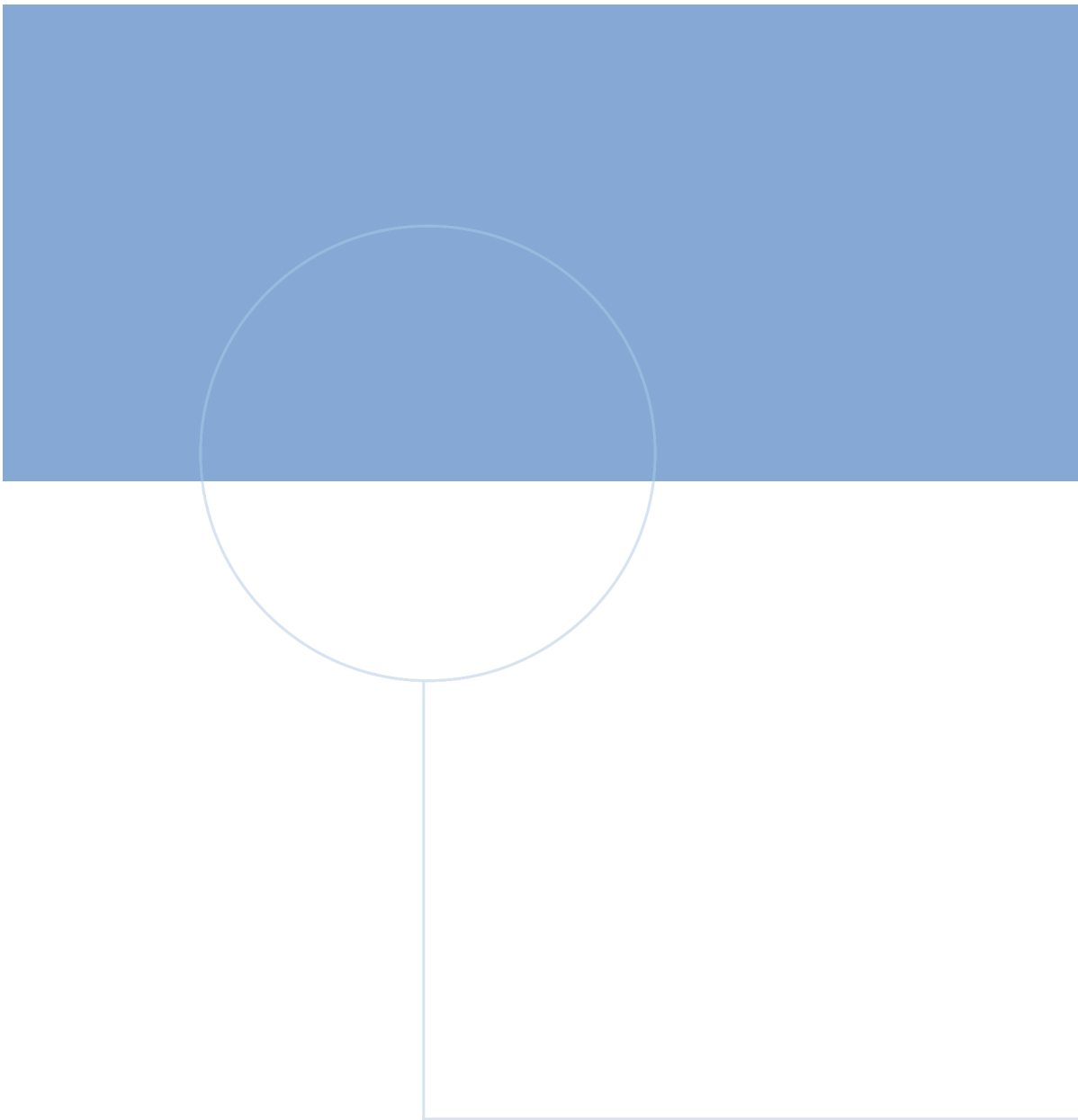
| | ***the1-1*** | ***the1-4*** | ***bzip25*** | ***cil1*** | ***bluejay*** |
|---|---|---|---|---|---|
| **t-test** | $2{,}1555\times10^{-9}$ | $4{,}7689\times10^{-13}$ | 0,71376 | 0,41539 | 0,03721 |

**From SA biosensor analysis**

One-way ANOVA p-value: $1.1102\times10^{-16}$***

**Table A2.4:** P-values found amount of detected luminescence between *the1-1, the1-4, bzip25, cil1* and *bluejay,* and Col-0 isoxaben-treated seedlings using a post hoc Tukey HSD test. Green cells indicate statistical significance.

| | ***the1-1*** | ***the1-4*** | ***bzip25*** | ***cil1*** | ***bluejay*** |
|---|---|---|---|---|---|
| **Col-0** | 0.0010053 | 0.0010053 | 0.8999947 | 0.8999947 | 0.2525254 |
| ***the1-1*** | | 0.0010053 | 0.0010053 | 0.0010053 | 0.0635566 |
| ***the1-4*** | | | 0.0010053 | 0.0010053 | 0.0010053 |
| ***bzip25*** | | | | 0.8999947 | 0.1568870 |
| ***cil1*** | | | | | 0.0697034 |



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