



Influence of climate on hyperactivation of the *Arabidopsis thaliana* immune system

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Degree project/Independent project • 60 credits

Swedish University of Agricultural Sciences, SLU

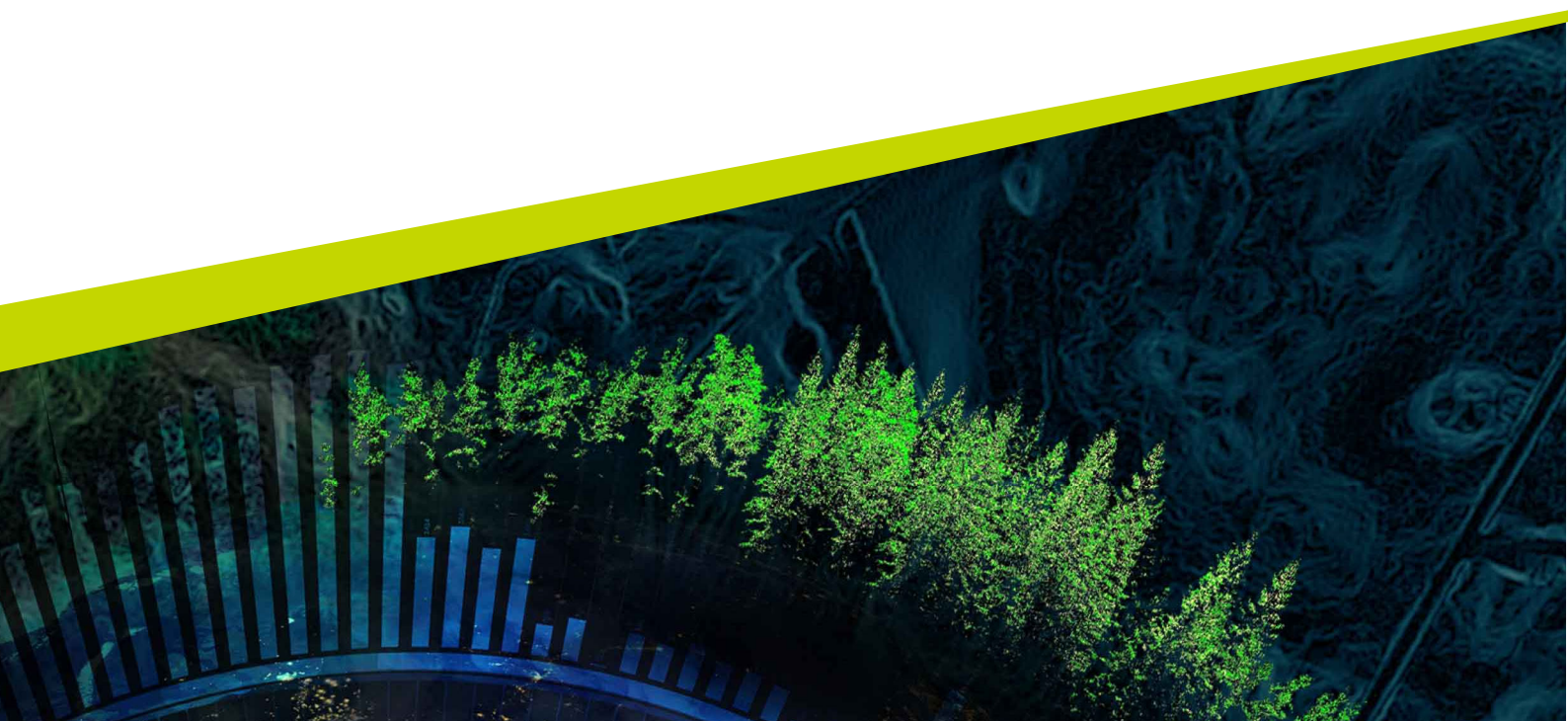
Faculty of Natural Resources and Agricultural Sciences/ Department of Plant Biology

Plant Biology for Sustainable Production - Master's programme - Abiotic and Biotic

Interactions of Cultivated Plants - LM011

Examensarbete/Institutionen för växbiologi, 207

Uppsala, 2026



Influence of climate on hyperactivation of the *Arabidopsis thaliana* immune system

Klimatets inverkan på hyperaktiveringen av immunsystemet hos Arabidopsis thaliana.

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Credits: 60 credits

Level: A2E

Course title: Independent Project in Biology

Course code: EX1001

Programme/education: Plant Biology for Sustainable Production - Master's programme - Abiotic and Biotic Interactions of Cultivated Plants

Course coordinating dept: Department of Plant Biology

Place of publication: Uppsala, Sweden

Year of publication: 2026

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Keywords: Plant immunity, *Arabidopsis thaliana*, *ACD6*, Est-1, Est-1 *acd6* knockout, *RUBY* reporter, *PR1*, environmental regulation, longitudinal sampling, transcriptomics, RNA sequencing

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Abstract

Environmental conditions can strongly influence plant immunity, yet how environmental cues modulate genetically driven immune activation remains poorly understood. The Est-1 accession of *Arabidopsis thaliana* has a hyperactive allele of *ACD6* that leads to constitutive immune activation under controlled conditions, but has a much less pronounced phenotype in the field. This study examined the environmental modulation of *ACD6*-dependent immunity using a PR1:RUBY reporter, molecular analyses, phenotyping and transcriptomic approaches. The PR1:RUBY construct was developed and introduced into Col-0, Est-1, and Est-1 *acd6* knockout backgrounds, and reporter responses were assessed after immune induction with heat-killed *Pseudomonas syringae*. The abundance of the transcripts of PR1 and *RUBY* was significantly higher after treatment, and genotype-dependent differences indicated that the magnitude of the immune response was influenced by the abundance of the transcript of *ACD6*. Visual and image-based analyses of the reporter activity of RUBY were also performed to complement the evidence of immune activation. To examine the environmental effects on the Est-1 phenotype, field-grown plants were sampled longitudinally over growing seasons. An efficient RNA extraction and RNA-sequencing library preparation workflow was successfully optimized to generate RNA and sequencing libraries for these samples suitable for transcriptomic analysis. In summary, the contrasting immune phenotypes observed under controlled and field conditions suggest that environmental context influences immune activation via the *ACD6* pathway, and the developed PR1:RUBY construct and optimized transcriptomic workflow offer valuable experimental tools for further exploration of the relationship between genetic immune activation and environmental context.

Keywords: Plant immunity, *Arabidopsis thaliana*, *ACD6*, Est-1, Est-1 *acd6* knockout, *RUBY* reporter, PR1, environmental regulation, longitudinal sampling, transcriptomics, RNA sequencing

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Abbreviations

Abbreviation	Description
cDNA	Complementary DNA
EDTA	Ethylenediaminetetraacetic acid
ETI	Effector-triggered immunity
KO	Knockout
MES	2-(N-morpholino)ethanesulfonic acid
OD ₆₀₀	Optical density at 600 nm
PCR	Polymerase chain reaction
<i>PR1</i>	Pathogenesis-Related Protein 1
PRR	Pattern recognition receptor
PTI	Pattern-triggered immunity
SA	Salicylic acid

1. Introduction and Literature Review

1.1 Plant Immunity and the Growth-Defense Trade-off

Plants are continuously exposed to a diverse array of microorganisms throughout their life cycle. Soil, leaf surfaces, vascular tissues, and reproductive organs harbor complex microbial communities comprising pathogenic, commensal, and beneficial organisms. Unlike animals, plants lack specialized immune systems capable of generating antibodies. Instead, each plant cell functions as an autonomous immune unit, capable of perceiving environmental signals, recognizing potential threats, and initiating appropriate defense responses while remaining integrated within a coordinated organism wide immune network (Jones & Dangl 2006a; Boller & Felix 2009).

The evolution of such an immune strategy reflects the sessile nature of plants. Unable to escape from pathogens through movement, plants rely on the rapid perception of microbial invasion and the activation of effective defense mechanisms. However, immune activation demands energy and requires the diversion of resources from growth, reproduction, and other physiological processes. Consequently, plants must continuously balance investment in defense with the maintenance of growth and reproductive fitness. This balance, commonly referred to as the growth-defense trade-off, has become a central concept in plant biology and provides an important framework for understanding the regulation of plant immune responses .

The concept of the growth-defense trade-off originated from ecological studies demonstrating that constitutive activation of defense mechanisms often reduces plant growth and reproductive success, even in the absence of pathogen pressure . While these early observations suggested that trade-offs primarily reflected competition for limited metabolic resources, subsequent research has revealed a more complex regulatory landscape. Rather than arising solely from resource allocation, growth-defense trade-offs are now understood to result from extensive interactions among signaling pathways that coordinate development and immunity. Central growth-promoting pathways, including Target of Rapamycin (TOR) signaling, brassinosteroid signaling, and auxin-dependent developmental programs, frequently antagonize immune responses, whereas activation of defense pathways suppresses growth-related processes. Consequently, defense-associated growth inhibition is increasingly viewed as an actively regulated process that enables plants to prioritize survival during pathogen attack while minimizing unnecessary fitness costs under non-stress conditions (Karasov et al. 2017).

1.2 Plant Immune Signaling and Regulation

Plants have an innate immune system, which detects and responds to microbial pathogens. This immune strategy is decentralized, enabling plants to quickly respond to a variety of pathogens and incorporate environmental and developmental data to preserve immune homeostasis.

It is usually believed that plant immunity is a two-layered system, with the first layer referred to as pattern-triggered immunity (PTI) and the second as effector-triggered immunity (ETI) (Jones & Dangl 2006b). PTI is triggered by the recognition of conserved microbial molecules, called pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs), by pattern-recognition receptors (PRRs) at the cell surface. Well-characterized examples are the recognition of bacterial flagellin by FLAGELLIN SENSING 2 (FLS2), elongation factor Tu by ELONGATION FACTOR RECEPTOR (EFR), and fungal chitin by CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) (Gómez-Gómez & Boller 2000; Boller & Felix 2009; Zipfel 2014). Upon PRRs activation, rapid immune signaling events are triggered, such as calcium influx, production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), transcriptional reprogramming and reinforcement of cell walls (Torres et al. 2006).

Pathogens have developed ways to evade the host's immune system by secreting effector proteins that suppress host defence. Plants have evolved in turn a form of immunity known as effector-triggered immunity (ETI), which is principally mediated by intracellular nucleotide-binding leucine-rich repeat (NLR) receptors, which recognize pathogen effectors. ETI activation triggers high levels of immune responses such as hypersensitive response (HR)-related cell death, accumulation of salicylic acid (SA) and induction of defense-related genes .

Initially thought to be separate and sequential immune layers, it is now known that PTI and ETI are very closely connected. The components that are typically linked to PTI elicit responses that are associated with ETI, and components associated with ETI are made more active by PTI-associated responses, including the production of ROS, calcium signaling, and defense gene expression. Based on this, the immune system of plants has been viewed as a unified signaling network instead of a network of individual defense pathways (Yuan et al. 2021; Ngou et al. 2022).

Salicylic acid (SA) is a phytohormone in plants that is a central component of this immune network, and it is important in defense against biotrophic and hemibiotrophic pathogens. Besides the defense function, SA is also used to coordinate immune activation and to balance growth-defense through the integration of pathogen perception and environmental information and physiological status (Vlot et al. 2009; Fu & Dong 2013).

SA signaling is primarily controlled by NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (*NPR1*), which is a significant transcription factor that regulates SA-mediated defense mechanisms. In uninduced cells, *NPR1* is largely oligomeric in the cytoplasm. Once pathogens are recognized and SA is accumulated, the changes in the cellular redox status stimulate the monomerization and nuclear translocation of *NPR1*, which interacts with TGA transcription factors that trigger the expression of defense-related genes (Fu & Dong 2013).

Pathogenesis-related (*PR*) genes are one of the major outputs of SA signaling. Of these, the use of PATHOGENESIS-RELATED 1 (*PR1*) as a molecular marker of immune activation in Arabidopsis plants is widely adopted. *PR1* expression is highly induced after pathogen infection, SA treatment, systemic acquired resistance (SAR) and after constitutive activation of the defense pathways in autoimmune mutants (Uknes et al. 1992; Dong 2004). While *PR1* is mainly recognized as a

marker of immune activation, the *PR1* proteins also play a direct role in the defense by means of antimicrobial activity and the generation of defense-related signaling (Van Loon et al. 2006; Gamir et al. 2017). Because *PR1* expression closely reflects SA-dependent immune activation, the *PR1* promoter provides an effective regulatory element for reporter-based visualization of immune responses in living plants.

Systemic acquired resistance (SAR) is another immune response that depends on SA signaling, and that confers increased resistance against a subsequent attack of the same pathogen. After local pathogen recognition, mobile signals initiate defence responses in remote tissues, which leads to enhanced resistance and elevated expression of SA-responsive genes, such as *PR1* (Durrant & Dong 2004; Fu & Dong 2013).

Immune activation demands a significant investment of plant metabolic resources and thus that plants carefully monitor and control immune signaling to avoid costly growth penalties. Immune homeostasis depends on a large number of interactions between immune pathways and growth-regulatory networks. These regulatory mechanisms can be disrupted and result in autoimmune phenotypes with constitutive activation of defense, increased SA accumulation, spontaneous cell death and decreased growth (Pieterse et al. 2012).

1.3 *ACD6* Regulating Immune Homeostasis and Growth-Defense Trade-offs

Maintaining immune homeostasis is essential for plant survival because both insufficient and excessive immune activation can negatively affect fitness. Low defense responses make an organism more susceptible to pathogens, but high levels of immunity can cause a decrease in growth, development, and reproductive success. Plants have thus developed regulatory systems that allow them to maintain a balance between effective defense and the costs of immune activation. One of the best characterized examples of a gene that regulates immune activation and growth-defense trade-offs in *Arabidopsis thaliana* is ACCELERATED CELL DEATH 6 (*ACD6*) (Lu et al. 2005; Todesco et al. 2010).

ACD6 was first discovered by screening for mutants of *Arabidopsis* that spontaneously undergo cell death and activate defense responses (Rate et al. 1999). The resulting *acd6-1* gain-of-function mutant results in constitutive immune activation, high levels of SA, high expression of defense marker genes, spontaneous lesion formation and severe growth defects (Lu 2003). The encoded protein is a multipass transmembrane protein, which is localized in the plasma membrane and has intracellular ankyrin-repeat domains that mediate protein-protein interactions and immune signaling (Lu et al. 2005). *ACD6* is not a pattern-recognition receptor (*PRR*) that directly recognizes pathogen-derived molecules, but rather a positive regulator that enhances immune signaling following pathogen recognition.

ACD6 is involved in several aspects of plant immunity, such as pattern-triggered immunity (PTI) and salicylic acid (SA)-dependent defense signaling. It has been demonstrated that *ACD6* affects the abundance and activity of immune receptors at the plasma membrane, which in turn increases downstream defense responses after

pathogen perception (Tateda et al. 2014). In addition, higher *ACD6* activity leads to higher levels of SA accumulation and activation of SA-responsive defense genes such as PATHOGENESIS-RELATED 1 (*PR1*) (Lu 2003; Lu et al. 2005). Since *ACD6* activity is linked to SA signaling, over-activation of this regulatory pathway can lead to high levels of defense responses without pathogen infection.

The effects of unchecked *ACD6* activation are especially pronounced in plants with “hyperactive” *ACD6* alleles (Todesco et al. 2014). These phenotypes represent the physiological price of overinvesting in immunity and are a good example of the growth-defense trade-off. The natural variation of *ACD6* activity also illustrates that increased immune activation can increase pathogen resistance but also decrease plant fitness (Todesco et al. 2014).

Studies of natural accessions of *Arabidopsis* revealed the ecological importance of *ACD6* variation. “Todesco et al.” demonstrated that certain accessions carry hyperactive *ACD6* alleles that provide high levels of resistance to a variety of pathogens, but result in high levels of autoimmunity under controlled laboratory conditions (Todesco et al. 2014). The Est-1 accession has a hyperactive *ACD6* allele and shows a higher defense response and higher expression of SA-responsive genes like *PR1*, but also lower growth in the growth chamber. The fitness costs of immune activation may vary greatly across ecological contexts, consistent with the idea that balancing selection is important in maintaining *ACD6* diversity (Todesco et al. 2014; Karasov et al. 2017).

Importantly, the phenotypes associated with *ACD6* are not only dependent on variation at the *ACD6* locus, but also on interactions with other genetic and environmental factors. Genetic modulators of immune signaling networks can modify the severity of *ACD6*-associated autoimmunity, indicating that immune phenotypes are not simply the result of individual genes working alone (Todesco et al. 2010; Chae et al. 2014; Świadek et al. 2017).

Lundberg et al. 2025 recently emphasized the role of environmental context in shaping *ACD6*-related phenotypes (Lundberg et al. 2025). The fitness costs of hyperactive *ACD6* alleles that resulted in severe growth defects under controlled growth-chamber conditions were significantly lower under natural field conditions. The results suggest that environmental factors can play a significant role in the expression of immune-related traits and suggest that this may account for the maintenance of expensive immune variants in natural populations.

Although much research has been conducted on *ACD6*, the mechanisms by which this environmental modulation occurs are still not understood. The mechanisms by which environmental factors inhibit *ACD6*-mediated autoimmunity, their role in plant-microbe interactions and in the activation of *PR1*, and the direct versus indirect role of environmental factors in plant physiology remain unknown. The answers to these questions will be crucial to understanding the interaction between genetic variation and environmental factors in the regulation of immune homeostasis.

1.4 Environmental Regulation of Plant Immunity

Plant immune responses are not genetically predetermined products, but dynamic processes influenced by interactions between genotype and environment. Plants constantly sense abiotic and biotic environments, leading to immune signaling

networks that respond to changes in temperature, humidity, light availability, nutrient status, and microbial communities. Thus, immune phenotypes seen in controlled laboratory settings may be very different from those seen in natural environments. It is therefore crucial to understand how environmental factors modulate immune activation to account for variation in disease resistance, immune homeostasis and growth-defense trade-offs .

Temperature is one of the most critical environmental factors that regulate plant immunity. Temperature affects the accumulation of salicylic acid (SA), nucleotide-binding leucine-rich repeat (NLR)-mediated immunity, and the activation of defense-associated genes. Increased temperatures often inhibit SA-dependent defense responses and diminish autoimmune phenotypes in *Arabidopsis* immune mutants, while cooler temperatures may promote immune activation. These results show that temperature is a key regulator of immune homeostasis, influencing the balance between activation of the immune system and maintenance of growth .

Humidity also plays a role in immune regulation through its influence on pathogen entry, stomatal behaviour and the microenvironment of the leaf. Stomata are important entry points for many bacterial pathogens and the humidity of the atmosphere affects stomatal responses as well as apoplastic conditions for pathogen colonization. Furthermore, humidity influences plant-microbe interactions and defense-related transcriptional responses, which can lead to differences in disease susceptibility under various environmental conditions (Melotto et al. 2006; Vannier et al. 2019; Xin et al. n.d.).

Another important environmental cue that connects energy status and immune regulation is light availability. Photosynthesis, production of reactive oxygen species (ROS), and defense hormone signaling are affected by light intensity, photoperiod, and spectral composition. Photoreceptors such as phytochromes and cryptochromes are involved in the integration of light signals with immune signaling pathways and in the control of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). Moreover, ultraviolet radiation can induce ROS-dependent signaling pathways that lead to defense activation (Ballaré 2014).

In addition to abiotic factors, interactions with microbial communities are an important aspect of immune regulation. Plants are naturally colonized by a variety of bacteria and fungi that affect their ability to acquire nutrients, withstand stress, and resist disease. Beneficial microorganisms can help maintain immune homeostasis by mechanisms including defense priming, modulation of hormone signaling, and maintenance of basal immune activity, without incurring the costs of constitutive defense activation (Trivedi et al. 2020).

One of the key differences between the lab and the natural environment is the complexity of exposure to microbes. Laboratory-grown plants typically interact with simplified microbial communities, while plants in natural environments are exposed to diverse and dynamic microbial communities. Thus, the immune phenotype differences between controlled and field conditions may be due to differences in microbial context, and microbial interactions should be taken into account when studying environmentally regulated immunity (Trivedi et al. 2020).

These results collectively show that plant immune phenotypes are the result of complex interactions between genetic regulation and environmental context. Environmental modulation of immune signaling offers a useful context for

explaining the potential for significant differences in immune-associated traits, such as those associated with hyperactive *ACD6* alleles, between controlled growth conditions and natural environments.

1.5 Environmental Modulation of *ACD6*-Mediated Immunity and the Need for Dynamic Immune Monitoring

The plant immune system is highly dynamic and strongly influenced by environmental conditions. Individual immune phenotypes are influenced by interactions of genotype and environment, but genetic variation dictates potential immune response. Environmental parameters (abiotic factors: temperature, humidity, light) and interactions with microbial communities may affect immune signalling pathways and the switch between activation of defense pathways and maintenance of growth. Thus, immune phenotypes in laboratory settings may not be representative of those in natural environments (Huot et al. 2017; Karasov et al. 2017).

Hyperactive *ACD6* alleles in *Arabidopsis* accession Est-1 shows enhanced expression of defence marker genes, spontaneous lesion formation, diminished growth and reproductive fitness in controlled growth and constitutive activation of defence pathways involving salicylic acid (SA) (Todesco et al. 2010). The phenotypes have been traditionally attributed to the physiological costs of the constitutive immune activation and to the growth-defence trade-off.

However, the hyperactive *ACD6* alleles' effects are very context-dependent, as shown by more recent field experiments. Under laboratory conditions, growth is severely affected by strong *ACD6* alleles, while such phenotypes are mostly suppressed in plants grown in the field (Lundberg et al. 2025). Under natural conditions, plants with the same *ACD6* allele exhibit lower levels of autoimmunity and higher growth performance, suggesting a field-laboratory paradox: the environment can have a strong effect on the expression of immune-associated traits.

The mechanisms underlying this environmental modulation is poorly understood. The relative contributions of abiotic and biotic factors to the suppression of *ACD6*-mediated autoimmunity are unclear, as is the possibility that these factors interact to regulate SA-dependent immune pathways. The mechanisms must be understood by approaches that can monitor immune activity dynamically in living plants.

1.6 Reporter Systems for Monitoring Plant Immune Responses and the *RUBY* Reporter System

As a result of traditional techniques, such as reverse transcription quantitative PCR (RT-qPCR), RNA sequencing (RNA-seq), microarray analysis and protein-based assays, a number of essential components of SA signalling have been identified, including *NPRI*, *PR1* and *ACD6* (Tsuda & Somssich 2015). These approaches,

however, are typically destructive and only measure a single time point. They therefore make "snapshots" of immune activity, not changes over time within the same plant.

This restriction is especially relevant for the environmentally regulated immunity in which defence changes in a continuous fashion according to temperature, light, humidity and microbial interactions. Furthermore, immune activation can be localized, starting at the infection site and later spreading via systemic acquired resistance (SAR). Thus, bulk molecular analyses might not reflect the spatial and temporal dynamics of immune responses in individual plants.

To overcome these limitations, reporter-based strategies are an important tool to visualize gene expression of immune-related genes in living plants without destructive sampling. Reporter systems can be used repeatedly to track immune activation in response to environmental changes, and are especially valuable for the study of immunophenotypes regulated by environmental factors, like the *ACD6*-dependent autoimmunity. *RUBY* is a visual reporter that produces a deep red betalain pigment in plant tissues when it is expressed. The accumulation of this pigment provides an easily visible, non-destructive readout of reporter activity, allowing gene expression to be monitored directly in living plants without specialized equipment. The *RUBY* reporter system developed in this study, described in more detail below, provides an in vitro method for directly observing SA-dependent immune activation in genetically defined *Arabidopsis* lines in a controlled laboratory setting.

As outlined in the previous section, there is a need to monitor immune activity dynamically, and reporter gene technologies have been developed that allow gene expression to be visualized in living tissues. Reporter systems are useful tools in plant biology, in which the spatial and temporal patterns of gene activation can be monitored by associating regulatory sequences, like promoters, with a detectable reporter gene. Reporter systems offer the advantage of repeated observations of the same plant over time, which is important for the study of environmentally regulated immune responses, as opposed to conventional molecular techniques which require destructive sampling.

Classical reporter systems like β -glucuronidase (GUS), green fluorescent protein (GFP) and luciferase (LUC) have greatly contributed to the understanding of plant gene regulation. GUS reporters have been used to study immune-related promoters such as *PRI* (Jefferson 1987; Beilmann et al. 1992) and have been employed to make detailed analysis of promoter activity possible by histochemical staining. Fluorescence reporters like GFP could be used to visualize the expression of a gene and the localization of a protein in living tissues without destroying the tissue (Chalfie et al. 1994; Haseloff et al. 1997) and luciferase reporters could be used to continuously monitor the dynamics of transcription over time (Millar et al. 1992). These systems also have drawbacks, such as destroying the sample, needing external substrates or specialized imaging equipment.

To address some of these drawbacks, a visible, substrate-free reporter, the *RUBY* reporter system, was developed based on the biosynthesis of red betalain pigments (He et al. 2020). *RUBY* does not require an enzymatic or fluorescent reporter, but rather results in a red pigmentation phenotype, which is visible by the naked eye. The *RUBY* system is a low background reporter platform for monitoring promoter activity in the model species *Arabidopsis thaliana*, which is not naturally able to

produce betalains (He et al. 2020). The *RUBY* construct is composed of three enzymes involved in betalain biosynthesis (CYP76AD1, DOPA 4,5-dioxygenase (DODA), and cyclo-DOPA 5-O-glucosyltransferase (cDOPA5GT)) that are connected by self-cleaving 2A peptides and can be co-expressed under a single promoter. The activation of the promoter causes the red pigment betalain to be produced in the plant, which is visible as a red coloration, an indication of transcriptional activity.

RUBY has been successfully applied in transformation screening, promoter characterization, genome editing, and functional studies in various plant species since its development (He et al. 2020; Wang et al. 2023; Pramanik et al. 2024). It has many advantages such as it can be directly observed visually, it is compatible with living tissues, and it can be observed repeatedly under changing environmental conditions.

But *RUBY* has its drawbacks as well. The accumulation of pigments is not only dependent on the activity of the promoter, but also on the developmental stage, metabolic state and availability of the betalain precursors. As such, *RUBY* is a cumulative transcriptional output over time and cannot detect fast changes in gene expression, as can fluorescent and luminescent reporters (He et al. 2020; Pramanik et al. 2024).

The PR1:*RUBY* reporter system was employed to track salicylic acid (SA)-dependent immune activation in the model plant *Arabidopsis thaliana* in the present study. This system is a non-destructive method to analyse *ACD6*-associated immune responses in controlled environmental conditions, and complements the field-based RNA-seq method used to study environmental regulation of immunity in natural populations.

1.7 Transcriptional monitoring of *ACD6* and environmental-dependent immune activity

PR1:*RUBY* is a powerful reporter system for studying salicylic acid (SA)-dependent immune activation under controlled laboratory conditions in living plants. Understanding the ecological significance of *ACD6*-mediated immunity, however, will need complementary strategies that can be used to capture the transcriptional response under natural environmental conditions, and which do not rely on genetically-modified plants. Field-grown plants are exposed to constantly shifting combinations of temperature, humidity, light, nutrient availability and exposure to microbes that can have a profound effect on immune signaling and defense-related gene expression (Huot et al. 2017; Karasov et al. 2017).

RNA sequencing (RNA-seq) has emerged as a key tool to explore the genome-wide transcriptomic changes that occur in response to plant-pathogen interactions and environmental conditions. In contrast to reporter systems that provide a measurement of the activity of a single promoter, RNA-seq quantifies the expression of thousands of genes simultaneously and identifies coordinated networks of defense genes, hormone signaling pathways, and stress-responsive transcriptional programs (Wang et al. 2009; Tsuda & Somssich 2015). Transcriptomic analysis has been extensively employed to characterize the SA-

dependent immune response, to identify genes involved in SAR, and to investigate the role of environmental factors in the regulation of immune signaling pathways (Tsuda & Somssich 2015; Ding & Ding 2020).

The use of plants that are sampled repeatedly over time (longitudinal RNA-seq) is especially useful for investigating environmentally controlled immunity as it will capture temporal changes in gene expression as plants encounter natural fluctuations in the environment. Combined with environmental metadata, like temperature, humidity and light intensity, it is possible to determine environmental conditions that activate or suppress *ACD6*-dependent immune pathways, and distinguish transient immune responses from changes in immune homeostasis (Karasov et al. 2017; Lundberg et al. 2025).

But the use of RNA-seq in longitudinal field experiments is challenging and expensive, especially if many samples are to be processed. To overcome these problems, we modified and optimized an RNA extraction protocol that was previously published. The optimized protocol was faster than the previous one and could process more samples at a time, which was more appropriate for the large sample numbers produced in the longitudinal field experiment.

We also developed an in-house Tn5-based library preparation method for RNA-seq as an alternative to commercial library preparation kits. The workflow employed in-house reagents and reverse transcriptase locally sourced from Karolinska Institutet, minimizing the need for commercial kits and decreasing the cost per sample. These optimizations enabled a more rapid, high throughput and cost-effective workflow for processing the high number of samples needed for longitudinal transcriptomic analysis.

This study can examine seasonal variation in immune gene expression in field-grown plants of *Arabidopsis* and how this relates to environmental conditions by measuring changes in RNA-seq expression in plants over time. The PR1:RUBY reporter system and field-based RNA-seq are complementary tools that can be used to study immunity associated with *ACD6*, with the former allowing mechanistic studies under controlled laboratory conditions and the latter allowing for transcriptional studies under natural conditions.

1.8 Aim and Significance of the Study

This study aimed to examine the regulation of immune activation by ACCELERATED CELL DEATH 6 (*ACD6*) under controlled and field conditions using a PR1:RUBY reporter system, and under field conditions by transcriptomic analysis of plants. In this regard, the study aimed to reveal the effect of natural variation in *ACD6* on salicylic acid (SA) immune response under different environments and to uncover environmental factors affecting *ACD6* immune response activation and suppression.

For this purpose, a reporter construct was designed that expresses *RUBY* under the control of a *PR1* promoter and cloned into Col-0, Est-1 and Est-1 *acd6* loss-of-function (KO) background, allowing for non-destructive visualization of immune activation in the living plant. These three genetic backgrounds were particularly selected to differentiate *ACD6*-dependent immune activation from background-specific effects, Col-0 was used as the standard *Arabidopsis thaliana* reference

background. Est-1 is a naturally occurring hyperactive allele of *ACD6* and thus serves as a genetic background with increased immune activation via *ACD6*. The Est-1 *acd6* loss-of-function (KO) line is a genetic control line that contains the *acd6* mutation in the Est-1 background to assess whether the immune phenotypes observed are dependent on functional *ACD6*. These three backgrounds allowed basal immune activity to be compared and the specific contribution of the Est-1 *ACD6* allele to immune activation to be assessed under different conditions. The activity of reporters and endogenous *PR1* expression was then compared in the different genotypes after immune induction under controlled laboratory conditions to assess the impact of *ACD6* on the size of downstream immune reactions. In parallel, an RNA-seq workflow was established for field-grown plants as a foundation for longitudinal analysis of seasonal variation in immune gene expression in relation to environmental factors, including temperature, humidity, and light intensity. The overall aim of the study was to gain a broader understanding of the interaction of genetic variation with the environment in regulating immunity and the trade-offs between defence and growth in *ACD6* associated immunity in *Arabidopsis thaliana*.

2. Materials

2.1 Stock Solutions and Buffers

All chemical and antibiotic stock solutions used in this study are listed in Appendix *Table A2* with detailed preparation procedures. All buffers and their detailed preparation procedures are presented in Appendix *Table A3*.

2.2 Growth Media

LB liquid and solid medium

Luria-Bertani (LB) broth containing tryptone (10 g/L, Oxoid, Hampshire, UK), yeast extract (5 g/L, Oxoid, Hampshire, UK), and sodium chloride (10 g/L, VWR International, Radnor, PA, USA), prepared in distilled water and sterilized by autoclaving at 121°C for 20 min. For solid medium, agar (15 g/L, Sigma-Aldrich, St. Louis, MO, USA), was also added before autoclaving. Appropriate antibiotics were added after cooling to approximately 50-55°C when required.

Low-salt LB medium

Low-salt Luria-Bertani (LB) medium containing peptone (10 g/L), yeast extract (5 g/L), and sodium chloride (1 g/L) was prepared in distilled water. For solid medium, agar (20 g/L) was added before autoclaving. The medium was sterilized by autoclaving at 121°C for 20 min. After autoclaving, the medium was allowed to cool to approximately 50-55°C, after which the appropriate antibiotic was added when required. The medium was then poured into sterile Petri plates.

Floral Dip Medium

Floral dip medium was prepared by dissolving 25 g sucrose in sterile distilled water and making up the final volume to 500 mL (5% w/v). The solution was sterilized and allowed to cool to room temperature before the addition of 500 µL of 100 mM acetosyringone to achieve a final concentration of 100 µM, which induces virulence genes in *Agrobacterium tumefaciens*. Subsequently, 100 µL of Silwet L-77 was added to obtain a final concentration of 0.02% (v/v). The medium was gently mixed and incubated at room temperature for 1 hour to allow sufficient activation of *Agrobacterium* virulence genes by acetosyringone and to ensure proper homogenization of the surfactant, thereby enhancing transformation efficiency. The prepared medium was then used for transformation of flowering *Arabidopsis thaliana* plants.

½ MS Agar Medium

800 mL of ½ MS agar medium was prepared by dissolving ½ MS, 1.76g of basal salts in approximately 700 mL of distilled water, followed by the addition of 1% (w/v) sucrose (8 g) and 0.8% (w/v) agar (6.4 g). The solution was stirred until fully dissolved, and the pH was adjusted to 5.8 using NaOH and HCl. The final volume was made up to 800 mL with distilled water, and the medium was autoclaved at 121 °C for 20 minutes. After sterilization, the medium was allowed to cool to 50-55 °C before the addition of sterile-filtered supplements, including Basta (10 mg/L final concentration; 8 mg total) for selection and, when required, timentin (300 mg/L final concentration; 240 mg total) for *Agrobacterium* suppression. The medium was then poured into sterile Petri dishes under aseptic conditions, allowed to solidify, and dried for 1-2 days under sterile conditions to prevent seed clumping.

2.3 Kits

- pENTR™/D-TOPO™ Cloning Kit, with One Shot™ TOP10 Chemically Competent *E. coli* (#K240020, Thermo Fisher Scientific, Waltham, MA, USA)
- Phusion™ High-Fidelity DNA Polymerase (2 U/μL) (#F530S, Thermo Fisher Scientific, Waltham, MA, USA)
- DreamTaq Green Master Mix (2X) (#K1081, Thermo Fisher Scientific, Waltham, MA, USA)
- GeneJET Plasmid Miniprep Kit (#K0502, Thermo Fisher Scientific, Waltham, MA, USA)
- GeneJET™ Gel Extraction Kit (# K0691, Thermo Fisher Scientific, Waltham, MA, USA)
- NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA; Cat. No. E2621).
- iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA; Cat. No. 1725120).
- Thermo Scientific™ KpnI restriction enzyme (10 U/μL; Thermo Fisher Scientific, Vilnius, Lithuania; Cat. No. ER0521).
- Thermo Scientific™ SacI restriction enzyme (10 U/μL; Thermo Fisher Scientific, Vilnius, Lithuania; Cat. No. ER1131).
- Thermo Scientific™ XhoI restriction enzyme (10 U/μL; Thermo Fisher Scientific, Vilnius, Lithuania; Cat. No. ER0692).
- Phusion™ Hot Start II DNA Polymerases (2 U/μL) Catalog number:F549L
- FastAP Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific, catalog no. EF0651)
- T4 DNA Ligase Buffer (10X) (Thermo Fisher Scientific, catalog no. B69)
- Phusion Hot Start II DNA Polymerase (2 U/μL) (Thermo Fisher Scientific, catalog no. F549L)
- DNA Polymerase I (10 U/μL) (Thermo Fisher Scientific, catalog no. EP0041)

2.4 Plant material

- **Col-0** - reference accession
- **Est-1** - accession carrying the hyperactive *ACD6* allele
- **Est-1 *acd6* KO (KO)** - *acd6* knockout background

2.5 Vectors and bacterial strains

- pENTR/D-TOPO Gateway entry vector (Thermo Fisher Scientific, Waltham, MA, USA)
- pGWB601 Gateway binary destination vector for plant transformation
- *Escherichia coli* TOP10
- *Escherichia coli* CcdB-resistant strain DB3.1
- *Agrobacterium tumefaciens* GV3101

3. Methods

3.1 Genomic DNA extraction from *Arabidopsis thaliana*-(Col-0)

Genomic DNA was isolated from approximately 100 mg of frozen leaf tissue of *Arabidopsis thaliana* ecotype Col-0. Frozen leaf tissue was transferred to a 2 mL screw-cap tube containing an equal mixture of 0.1 mm and 0.5 mm zirconia/silica beads (BioSpec Products Inc., Bartlesville, OK, USA) together with one 5 mm glass bead. Samples were flash-frozen in liquid nitrogen and homogenized using a FastPrep bead-beating system at 6.0 m s^{-1} for 1 minute until a fine powder was obtained. Homogenization under frozen conditions facilitated efficient disruption of the plant cell wall while minimizing nuclease activity.

The powdered tissue was immediately resuspended in 750 μL of DNA lysis buffer containing 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM EDTA. Sodium dodecyl sulfate (SDS) was then added to a final concentration of 1.5%, and the lysate was mixed thoroughly to ensure complete cell lysis. The homogenate was centrifuged at $10,000 \times g$ for 1 minute to pellet insoluble cellular debris.

The clarified supernatant was transferred to a fresh microcentrifuge tube containing 200 μL of 5 M potassium acetate (KAc) and mixed gently by inversion to precipitate SDS, proteins, and other cellular contaminants. Following centrifugation at maximum speed for 1 minute, the supernatant was transferred to a new tube containing 400 μL chloroform, vortexed thoroughly, and centrifuged again at maximum speed for 1 minute to separate the aqueous and organic phases. The upper aqueous phase containing genomic DNA was carefully transferred to a clean microcentrifuge tube while avoiding material at the phase interface.

Genomic DNA was precipitated by adding an equal volume of isopropanol, followed by gentle inversion until DNA precipitation became visible. The samples were centrifuged at maximum speed for 5 minutes to pellet the DNA. The supernatant was discarded, and the DNA pellet was washed with 80% ethanol, briefly air-dried to remove residual ethanol, and resuspended in approximately 100 μL nuclease-free water and stored at -20°C for further use.

3.2 Transformation of 35S::*Ruby* in Top 10 *E. coli*

A glycerol stock of the 35S::RUBY plasmid was used to transform chemically competent Top10 *Escherichia coli* cells following standard heat-shock protocols. Briefly, 50 μL of competent cells were mixed with 1-2 μL of plasmid DNA and incubated on ice for 30 minutes, followed by heat-shock at 42°C for 45 seconds and recovery on ice for 2 minutes. Cells were then added to 1000 μL of LB medium without antibiotics and incubated at 37°C with shaking for 1 hour to allow expression of the antibiotic resistance marker. The transformed cells were plated on LB agar containing the spectinomycin 50ug/ml antibiotic and incubated

overnight at 37°C. Single colonies were picked and cultured in 5 mL of LB medium with spectinomycin (50mg/ml) overnight at 37°C with shaking at 240rpm.

Plasmid DNA was subsequently isolated using a standard miniprep protocol according to the standard lab protocol. The concentration and quality of the isolated plasmid were assessed using the NanoDrop spectrophotometer.

3.3 PCR Amplification and Preparation of Vector and Inserts for Assembly

We designed an initial construct to place the *PR1* promoter upstream of the RUBY reporter, followed by the terminator, and to assemble the complete cassette into the pENTR/D-TOPO entry vector using Gibson Assembly. The *PR1* promoter was amplified from *Arabidopsis thaliana* Col-0 genomic DNA using primers L0368 and L0369. The RUBY reporter was amplified from the 35S::RUBY plasmid as two overlapping fragments using primer pairs L0370/L0371 and L0372/L0373 to facilitate amplification of the large insert. The pENTR/D-TOPO vector backbone was PCR-amplified using outward-facing primers L0374 and L0375, which were designed with 5' overhangs homologous to the adjacent fragments. The overlapping sequences enabled seamless Gibson Assembly of the vector backbone, *PR1* promoter, and *RUBY* fragments into a single construct. All primer sequences, including Gibson overhangs, are provided in Appendix *Table A1*, and the complete construct nucleotide sequence is provided in Appendix 8.1.

PCR reactions were performed in a final volume of 25 µL, containing 1 µL template DNA, 5 µL 5× Phusion HF Buffer, 0.5 µL 10 mM dNTP mix, 1.25 µL each of forward and reverse primers (10 µM), 0.5 µL Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific), and nuclease-free DEPC-treated water to the final volume.

PCR amplification was performed using Phusion High-Fidelity DNA Polymerase under the following cycling conditions: an initial denaturation at 98°C for 3:00 minutes, followed by 36 cycles of 98°C for 10 s, annealing at 60-68°C for 30 s (depending on primer pair), and 72°C for 1 minute per kb of expected product length. A final extension was performed at 72°C for 10 min, followed by a hold at 4°C.

Amplified products were separated on a 1% agarose gel stained with Midori Green Advance (Nippon Genetics Europe, Düren, Germany). DNA fragments of the expected size were excised and purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. DNA concentrations were determined using an Invitrogen™ Qubit™ 4 Fluorometer, and purified DNA fragments were stored at -20°C until Gibson Assembly.

3.4 DNA purification using SPRI magnetic beads

DNA purification was performed using SPRI (Solid Phase Reversible Immobilization) magnetic beads. Prior to use, the beads were thoroughly resuspended by vortexing until a uniform brown suspension was obtained. SPRI

beads were added to the DNA samples at bead-to-sample volume ratios appropriate for each cleanup step (the ratio was adjusted according to the required fragment-size selection) and mixed gently by pipetting or flicking. The mixture was briefly centrifuged and incubated at room temperature for 5 min to allow DNA binding to the magnetic beads. Samples were then placed on a magnetic rack for approximately 2 min until the beads were completely collected, and the supernatant was carefully removed.

The bead pellet was washed twice with 200 μ L of freshly prepared 80% ethanol. After the final wash, residual ethanol was removed, and the beads were briefly air-dried until the pellet lost its glossy appearance. Purified DNA was eluted by adding 20 μ L nuclease-free water and incubating for 5 min at room temperature. The tubes were placed on a magnetic rack, and the purified DNA-containing supernatant was transferred to a new microcentrifuge tube for downstream applications.

3.5 Preparation of Competent *E. coli* Cells

We prepared chemical competent *E. coli* cells following the Inoue method (Sambrook & Russell 2006). A single bacterial colony was picked up and transferred to 25 mL of SOB medium and shaken vigorously at 37°C for 6-8 hours. The starter culture was transferred into three 1 litre flasks with 250 mL of SOB medium, with 10 mL, 4 mL and 2 mL of the starter culture, respectively. The cultures were grown overnight at 18°C under moderate shaking. The next morning the OD₆₀₀ of each culture was determined and the culture that achieved an OD₆₀₀ of 0.55 was selected for further processing, the other cultures were discarded.

The selected culture was allowed to cool on ice for 10 minutes, and the cells were centrifuged at $2500 \times g$ for 10 minutes at 4°C. Medium was discarded, and the tube containing the cell pellet was inverted over a stack of tissue paper for removal of residual culture medium. The cell pellet was carefully resuspended in 80 mL of ice-cold Inoue transformation buffer and centrifuged under the same conditions. Buffer was discarded, and the pellet was then resuspended in 20 mL of ice-cold Inoue transformation buffer and 1.5 mL DMSO was added. The cell suspension was allowed to stand on ice for 10 minutes. Competent cells were aliquoted into cold, sterile tubes, snap-frozen in liquid nitrogen and stored at -80°C for future use.

We assessed the transformation competency of the prepared *E. coli* cells by using pUC plasmid DNA. For the competency test, 1 μ L of pUC plasmid DNA was added to 50 μ L of prepared competent cells, and the cells were transformed using the heat-shock procedure described below. Following recovery, the transformed cells were plated on LB agar containing ampicillin at a final concentration of 50 μ g/mL. The selective medium was prepared by adding 50 μ L of a 100 mg/mL ampicillin stock solution to 100 mL of LB agar. Plates were incubated overnight at 37°C, and transformation competency was assessed based on the formation of ampicillin-resistant colonies.

3.6 Cloning PR1:RUBY using the Gibson method

It was first attempted to clone *PR1* and *RUBY* fragments into a pENTR D-TOPO, which could then be recombined into pGWB601 using the Gateway method. These methods ultimately did not produce PR1:RUBY in pGWB601.

3.6.1 Generating linearized pENTR D-TOPO and assembling Gibson reaction

The purified PCR fragments, including the *PR1* promoter, the two overlapping RUBY fragments (~2 kb each), and the linearized D-TOPO p ENT R vector, were assembled into a complete construct using Gibson assembly. Reactions were set up by using New England Biolabs Gibson Assembly Master Mix, which contains exonuclease, DNA polymerase, and ligase activities to facilitate seamless joining of overlapping DNA fragments in a single-step isothermal reaction. The DNA fragments were combined in equimolar ratios with the master mix and incubated at 50°C for 15 minutes to allow directional assembly of the *PR1* promoter upstream of the full-length RUBY coding sequence within the D-TOPO backbone. The assembled products were subsequently used for transformation into chemically competent Top10 *Escherichia coli* cells for propagation.

3.6.2 Transformation and Colony Screening in *E. coli*

The putatively assembled PR1-RUBY-D-TOPO construct from the Gibson assembly reaction was subsequently purified using SPRI magnetic beads to remove salts and enzymes prior to transformation. It was then transformed into chemically competent Top10 *Escherichia coli* cells following standard heat-shock protocols. Transformed cells were plated on LB agar containing kanamycin to select for successful uptake of the plasmid. Plates were incubated overnight at 37°C, and colonies appeared the following day. To confirm the presence of the *PR1* insert, individual colonies were screened by colony PCR using primers specific for the *PR1* promoter. Despite multiple colonies being tested, all colonies were negative for the *PR1* insert, indicating that the assembled construct was not present or that the Gibson assembly had failed.

3.6.3 Optimizing Gibson Assembly to Prevent Vector Self-Ligation

The initial negative results from colony PCR indicated that the linearized D-TOPO vector had a tendency to self-ligate during the Gibson assembly, preventing successful incorporation of the *PR1* and *RUBY* fragments. To overcome this, the vector was amplified in two separate fragments to ensure complete linearization and reduce the likelihood of self-ligation. The first vector fragment was amplified using the forward primer L0374 and the midway reverse primer L0384, while the

second fragment was amplified using the midway forward primer L0385 and the reverse primer L0375. This modification resulted in a total of five DNA fragments: the two vector fragments, the *PRI* promoter, and the two overlapping *RUBY* fragments. The fragments were combined in equimolar ratios and subjected to Gibson assembly using New England Biolabs Gibson Assembly Master Mix, incubating at 50°C for 60 minutes, which is recommended for assemblies involving more than two fragments. The assembled reaction was subsequently purified using SPRI magnetic beads to remove salts and enzymes prior to transformation. Despite these optimizations, transformation into chemically competent Top10 *E. coli* cells did not yield any colonies, indicating that further troubleshooting would be required to successfully assemble the five-fragment construct.

3.6.4 Optimization Attempts for Gibson Assembly

To improve the efficiency of the assembly and reduce vector self-ligation, the *RUBY* coding sequence was re-amplified as a single fragment rather than in two overlapping fragments. The assembly reaction was then set up with four fragments: the *PRI* promoter, the full-length *RUBY* fragment, and the two PCR-amplified vector fragments. Equimolar ratios of the fragments were carefully adjusted (0.1 pmol), and the Gibson assembly was performed under standard conditions (50 °C for 60 minutes) followed by purification using SPRI magnetic beads to remove salts and enzymes prior to transformation. The purified assembly product was used to transform chemically competent Top10 *E. coli* cells. Despite these optimizations, no colonies were obtained. Additional attempts were made using different equimolar ratios (0.15 pmol, 2 pmol) of *PRI* and *RUBY* fragments to improve assembly efficiency, but these variations also failed to produce colonies. These results suggested that the assembly of multiple fragments into the D-TOPO backbone was inefficient under the conditions used and highlighted the need for an alternative cloning or assembly strategy.

3.7 Cloning PR1:RUBY into pGWB601 using restriction enzymes

As an alternative cloning strategy, the *PRI* construct was assembled using the Gateway-compatible binary vector pGWB601, which contains a *ccdB* selection cassette flanked by *attR1* and *attR2* recombination sites. These methods successfully produced PR1:RUBY in pGWB601.

3.7.1 pGWB601 plasmid Isolation

The pGWB601 plasmid was transformed into *ccdB*-tolerant *Escherichia coli* competent cells and transformants were selected on LB agar plates supplemented with spectinomycin (50 mg L⁻¹). A single colony was inoculated into liquid LB

medium containing spectinomycin (50 mg L^{-1}) and incubated overnight at 37°C with shaking at 250 rpm. Plasmid DNA was isolated from overnight bacterial cultures using the alkaline lysis method with minor modifications based on the protocol described by (Sambrook and Russell 2006a). Briefly, 1.5 mL of overnight bacterial culture was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 14,000 rpm for 1 minute to pellet the cells. The supernatant was discarded, and the centrifugation step was repeated until a compact bacterial pellet was obtained. The pellet was resuspended in 150 μL of resuspension buffer by vortexing. Subsequently, 200 μL of freshly prepared alkaline lysis solution was added and mixed gently by inverting the tube 4-6 times until the solution became viscous. Then, 300 μL of neutralization solution was added and mixed by inversion. The mixture was centrifuged at 14,000 rpm for 5 minutes to separate cellular debris. The supernatant containing plasmid DNA was transferred to a new microcentrifuge tube, and an equal volume of isopropanol was added to precipitate the DNA. The mixture was incubated at -80°C for 30 minutes followed by centrifugation at 14,000 rpm for 5 minutes. The supernatant was discarded and the pellet was washed with 600 μL of 70% ethanol and centrifuged again for 5 minutes. After removing the ethanol, the DNA pellet was air-dried for 10-30 minutes and resuspended in a 20-50 μL TE buffer containing RNase A (pH 8.0). The isolated plasmid DNA was verified by agarose gel electrophoresis.

3.7.2 Plasmid Restriction Digest and Gel purification of cut fragments

Purified pGWB601 plasmid DNA was digested with SacI restriction enzyme and XhoI restriction enzyme using Thermo Scientific FastDigest restriction enzymes according to the manufacturer's instructions. The 20 μL reaction contained approximately 1 μg of plasmid DNA, 1 μL of each enzyme, 2 μL of $10\times$ FastDigest buffer, and nuclease-free water to the final volume. The mixture was incubated at 37°C for 5 minutes.

The digestion products were separated by agarose gel electrophoresis. The 1.5 kb fragment containing the *ccdB* cassette and the linearized 9.2 kb plasmid backbone were visualized on the gel. The backbone was excised for downstream ligation and cloning.

3.7.3 PCR Amplification of *PR1* and RUBY Fragments with new primers

The *PR1* promoter fragment was amplified from approximately 30 ng of *Arabidopsis thaliana* Col-0 genomic DNA using primer pair L0439 and L0440. The primers were designed to introduce XhoI and KpnI restriction sites at the 5' and 3' ends of the amplified fragment, respectively, for directional cloning into the pGWB601 vector. PCR reactions were prepared in a final volume of 25 μL

containing 5 μL of 5 $\times$ HF buffer, 1.25 μL of Phusion High-Fidelity DNA Polymerase, 1.25 μL each of forward and reverse primers, template DNA, and nuclease-free water. Amplification was performed using an annealing temperature of 68°C, an extension time of 1 min 50 s, and 36 cycles, followed by a final extension at 72°C for 10 min.

The *RUBY* reporter gene fragment was amplified from plasmid DNA containing the *RUBY* sequence using primer pair L0441 and L0442. These primers introduced KpnI and SacI restriction sites to enable directional cloning of the reporter fragment into the pGWB601 vector downstream of the *PR1* promoter. PCR reactions were prepared using the same Phusion High-Fidelity DNA Polymerase system described above. Amplification was performed using an annealing temperature of 69°C, an extension time of 4 min 30 s, and 36 cycles, followed by a final extension at 72°C for 10 min.

3.7.4 Restriction digestion and purification of PCR fragments

Purified PCR-amplified fragments of the *PR1* promoter and *RUBY* reporter gene were digested using Thermo Scientific FastDigest restriction enzymes according to the manufacturer's instructions. The *PR1* promoter fragment was digested with XhoI and KpnI, while the *RUBY* fragment was digested with SacI and KpnI. Each digestion reaction contained 1 μL of each enzyme, the appropriate FastDigest buffer, and the DNA fragment in a total volume adjusted with nuclease-free water, and was incubated at 37 °C for 5 minutes.

3.7.5 Ligation with T4 Ligase

After purification of the digested *PR1* promoter, *RUBY* reporter gene fragments, and plasmid backbone, ligation reactions were assembled to facilitate the insertion of both fragments into the vector. Two ligation setups were prepared with different vector-to-insert ratios to optimize cloning efficiency. In the first reaction, a 1:3 vector-to-insert ratio was used, consisting of 6 μL of digested vector (12 pmol), 1 μL of *PR1* insert (37.5 pmol), 2.3 μL of *RUBY* insert (19.1 pmol), 4 μL of 5 $\times$ T4 DNA ligase buffer, 1 μL of T4 DNA ligase enzyme, and 5.7 μL of nuclease-free water, resulting in a total volume of 20 μL . In the second reaction, a 1:4:4 vector-to-*PR1*-to-*RUBY* ratio was prepared using 4 μL of digested vector, 0.85 μL of *PR1* insert, 3.8 μL of *RUBY* insert, 2 μL of 5 $\times$ T4 DNA ligase buffer, 0.5 μL of T4 DNA ligase, and nuclease-free water to a final volume of 11 μL . Both ligation reactions were incubated at 22 °C for 1 hour to allow formation of recombinant plasmids.

3.7.6 Transformation

Following ligation, the reaction mixtures were used directly for transformation. The ligation products were first diluted to 1:3 and 1:4 to improve transformation efficiency, and 1 μL from each dilution was used to transform chemically competent *Escherichia coli* cells, including both commercially available cells and

laboratory-prepared TOP10 cells, using a standard chemical transformation protocol. The transformed cells were then plated on appropriate selective media and incubated to allow colony formation.

3.7.7 Screening with Colony PCR

Individual colonies obtained from transformation plates were used as templates for colony PCR. The PCR reactions were set up in a total volume of 25 μL , containing 5 μL of 5 $\times$ HF buffer, 0.5 μL of 10 mM dNTPs, 1.25 μL of Karolinska Phusion DNA polymerase, 1.25 μL of each primer (L439 and L440), 8 μL of DEPC-treated water, and a bacterial colony as the DNA template. The PCR cycling conditions followed standard Phusion polymerase recommendations, including initial denaturation, followed by repeated cycles of denaturation, annealing, and extension, with a final extension step to ensure complete amplification.

3.7.8 Plasmid isolation of positive colonies

Plasmid isolation was performed from the three PCR-positive colonies following overnight culture. Each positive colony was inoculated into LB medium supplemented with spectinomycin (50 $\mu\text{g}/\text{mL}$) and incubated overnight with shaking to allow plasmid amplification. Plasmid DNA was then extracted using the Thermo Fisher GeneJET Plasmid Miniprep Kit according to the manufacturer's instructions. The purified plasmid DNA was eluted in 50 μL of DEPC-treated water to obtain concentrated samples for downstream analysis.

The concentration and purity of the isolated plasmid DNA were measured using the Qubit high-sensitivity double-stranded DNA assay. The DNA concentrations of the three samples were determined to be 85 $\text{ng}/\mu\text{L}$ for the first colony, 73 $\text{ng}/\mu\text{L}$ for the second colony, and 253 $\text{ng}/\mu\text{L}$ for the third colony. These purified plasmids were subsequently used for further verification and downstream applications.

3.7.9 Gel purification of the uncut pGWB601 vector

A single *Escherichia coli* colony containing the pGWB601 vector was cultured in 100 mL of LB medium containing kanamycin (50 $\mu\text{g}/\text{mL}$) for overnight (ca. 16 h) shaking to generate enough plasmid DNA for further cloning applications.

The plasmid DNA was isolated from the overnight culture by an in-house alkaline lysis maxiprep protocol. Cells were harvested by centrifugation at 4500 rpm using a bench-top centrifuge (model) to obtain a bacterial pellet. The pellet was resuspended in 4 mL of Solution I, followed by the addition of 8 mL freshly prepared Solution II for alkaline lysis. Subsequently, 6 mL of Solution III was added for neutralisation. The mixture was gently inverted to ensure homogeneity, briefly spun down, and filtered through microcloth to remove cellular debris and genomic DNA contaminants.

The clarified lysate was then subjected to nucleic acid precipitation by adding an equal volume (18 mL) of isopropanol. The mixture was gently inverted, incubated on ice for 10 minutes, and centrifuged at 4500 rpm to pellet the nucleic

acids. The resulting DNA pellet was washed with 80% ethanol, briefly air-dried, and resuspended in 1 mL of nuclease-free water. The dissolved DNA was transferred into a 2 mL microcentrifuge tube, and 400 μ L of chloroform was added. The mixture was vortexed, mixed thoroughly, and centrifuged to separate the phases, with chloroform facilitating removal of contaminants and resulting in a cleaner aqueous phase. The aqueous phase was recovered and further purified by adding 1/9 volume of 3 M sodium acetate (e.g., 111 μ L for 1 mL aqueous phase) and 8/9 volume of isopropanol (e.g., 888 μ L for 1 mL aqueous phase). The solution was mixed and centrifuged again to re-pellet the DNA. This second pellet was cleaner due to the chloroform extraction step. The pellet was washed again with 80% ethanol, briefly air-dried, and resuspended in 1 mL of autoclaved water.

RNase A (10 μ L) was then added, and the sample was incubated at room temperature for approximately 20 minutes after complete dissolution of the DNA to remove residual bacterial RNA. A bead-based cleanup was subsequently performed using SPRI magnetic beads at a DNA-to-bead ratio of 1:0.7, by adding 700 μ L of beads to the sample. After bead purification, the DNA was eluted again in 1 mL of autoclaved water. Plasmid DNA concentration and purity were assessed using a NanoDrop spectrophotometer, yielding a concentration of 3489 ng/ μ L, with A260/280 and A260/230 ratios of 2.06 and 2.20, respectively, indicating high-purity DNA with minimal protein and organic contamination. To avoid *Escherichia coli* genomic DNA contamination observed in earlier preparations, the maxiprep-isolated pGWB601 vector was subjected to gel purification. A total of 200 μ L of plasmid DNA was loaded onto a 0.8% agarose gel and electrophoresed at 100 V for 45 minutes to separate the supercoiled plasmid DNA from any high-molecular-weight genomic DNA contamination.

3.7.10 Double Digestion and Dephosphorylation of Gel-Purified pGWB601

The gel-purified pGWB601 vector was subjected to double digestion and dephosphorylation in a 20 μ L reaction to prepare it for ligation and prevent self-ligation. The reaction mixture contained 15 μ L (approximately 700 ng) of purified plasmid DNA, 2 μ L of FastDigest buffer, 1 μ L of XhoI (Thermo Fisher), 1 μ L of SacI (Thermo Fisher), 0.5 μ L of alkaline phosphatase enzyme, and 1.5 μ L of nuclease-free water, bringing the final volume to 20 μ L.

The reaction was incubated at 37 °C for 15 minutes to ensure complete restriction digestion and dephosphorylation of the vector ends. Following incubation, the digested product was purified using SPRI magnetic beads at a 1:1 bead-to-sample ratio. The DNA was then eluted in 10 μ L of DEPC-treated water. The final DNA concentration was measured using a Qubit fluorometer and was recorded as 23 ng/ μ L, confirming successful purification of the digested and dephosphorylated vector for downstream ligation steps.

3.7.11 Ligation of Digested Vector and Inserts

Ligation reactions were assembled using the double-digested and dephosphorylated pGWB601 vector together with the digested *PR1* promoter and *RUBY* insert

fragments at a vector-to-insert molar ratio of 1:3:3. The reaction was prepared in a total volume of 20 μ L, containing 4.4 μ L (approximately 100 ng) of vector DNA, 6 μ L (approximately 300 ng) of *PR1* insert, and 5.5 μ L (approximately 300 ng) of *RUBY* insert. To this mixture, 4 μ L of 5 $\times$ T4 DNA ligase buffer, 1 μ L of T4 DNA ligase enzyme, and nuclease-free water were added to adjust the final volume to 20 μ L.

The ligation mixture was incubated at 22 $^{\circ}$ C for 1 hour and subsequently used for transformation into chemically competent *Escherichia coli* cells. However, following overnight incubation on selective media.

3.7.12 Assembly of Recombinant Constructs by Cyclic Ligation Method

A three-fragment ligation was performed using the pGWB601 binary vector, *PR1*, and *RUBY* inserts. DNA amounts were calculated based on fragment size and concentration to achieve a molar ratio of 1:3:3 (vector:PR1:RUBY). Reactions were assembled on ice in a total volume of 20 μ L. The experimental reaction contained 4.35 μ L (100 ng) of pGWB601 vector, 0.87 μ L (43 ng) of *AtPR1* insert, and 2.54 μ L (137 ng) of *RUBY* insert. In addition, 2.00 μ L of 10 $\times$ T4 DNA ligase buffer and 1.00 μ L of T4 DNA ligase (5 U/ μ L) were added, and the volume was adjusted to 20 μ L with 9.24 μ L of nuclease-free water.

A negative control was included with no-ligase control containing 4.35 μ L of vector, 0.87 μ L of *AtPR1* insert, 2.54 μ L of *RUBY* insert, 2.00 μ L of ligase buffer, and 10.24 μ L of nuclease-free water.

Ligation reactions were carried out using a cycling program consisting of 99 cycles of 10 $^{\circ}$ C for 30 seconds and 30 $^{\circ}$ C for 30 seconds, followed by a final incubation at 16 $^{\circ}$ C for 2 hours. The ligase was subsequently heat-inactivated at 65 $^{\circ}$ C for 10 minutes.

For transformation, 2 μ L of each ligation mixture was added to 50 μ L of chemically competent *Escherichia coli* TOP10 cells. Cells were allowed to recover in LB medium at 37 $^{\circ}$ C for 60 minutes with shaking before plating on LB agar supplemented with streptomycin for selection. Control transformations were performed in parallel to assess background ligation and transformation efficiency.

3.8 Transformation of pGWB601-PR1::RUBY plasmid DNA in *Agrobacterium*

Agrobacterium tumefaciens GV3101 was transformed with the binary plasmid construct using the freeze-thaw method. Briefly, 1 and 2 μ L (approximately 60-100ng) of plasmid DNA was added to 50 μ L of competent GV3101 cells and incubated on ice for 5 min. The mixture was frozen in liquid nitrogen for 5 min and subsequently thawed at 37 $^{\circ}$ C for 5 min. After thawing, 500 μ L of LB medium was added, and the cells were incubated at 28 $^{\circ}$ C with shaking for 2-3 h for recovery. The transformation mixture was spin-down at 4000rpm for 5 minutes 850ul of the

supernatant was discarded and bacterial pellet was suspended in remaining 100ul LB by pipetting up and down. This whole 100ul suspended pallet was plated on LB agar supplemented with rifampicin (25 µg/mL), gentamicin (30 µg/mL), and the (50ug/ml) spectinomycin for selection of the binary vector pGWB601. Plates were incubated at 28°C for 2 days until colonies appeared.

3.9 Floral Dip Transformation of *Arabidopsis thaliana*

Transformation of *Arabidopsis thaliana* was performed using the floral dip method with *Agrobacterium tumefaciens* strain GV3101 harboring the recombinant construct. Plants were grown in soil under controlled conditions (22°C, 16 h light/8 h dark photoperiod) and used for transformation at 4 weeks of age. At the time of dipping, plants were at the optimal developmental stage characterized by the presence of numerous young unopened floral buds, with minimal mature siliques. Prior to transformation, mature flowers and siliques (>5 mm) were removed to promote the formation of new, transformable floral tissues.

For transformation, *Agrobacterium* cultures were grown to an optical density (OD600) of approximately 1.0, harvested by centrifugation (4000 × g, 10 min, room temperature), and resuspended in freshly prepared floral dip medium. Aerial parts of plants were inverted and the inflorescences were submerged in the *Agrobacterium* suspension. Gentle agitation was applied for 20-30 seconds to ensure thorough wetting of all floral tissues. Following dipping, plants were placed horizontally and covered with a transparent plastic dome, covered with a black plastic sheet to maintain high humidity and placed in the greenhouse for 30 hours.

After incubation, covers were removed and plants were allowed to grow under standard growth conditions in the greenhouse. Temporary wilting was observed but plants typically recovered within 2-3 days. Seeds (T1 generation) were harvested 4 weeks post-transformation when siliques were fully matured and dried, followed by drying at room temperature for 2 days prior to storage or selection.

3.9.1 Seed Sterilization

Arabidopsis thaliana seeds were surface sterilized prior to sowing to prevent microbial contamination. Dry seeds were first treated with 2% (v/v) sodium hypochlorite solution and incubated for an appropriate duration with gentle mixing to ensure effective sterilization. The bleach solution was then completely removed, and the seeds were subsequently washed with 70% (v/v) ethanol to further eliminate surface contaminants. Following ethanol treatment, seeds were rinsed thoroughly 5 times with sterile distilled water to remove any residual sterilizing agents. After the final wash, seeds were carefully transferred onto sterile filter paper and allowed to air dry under aseptic conditions in a laminar flow hood before further use.

3.9.2 Selection of Transgenic Seedlings on ½ MS Medium Supplemented with Basta

Selection of transformed *Arabidopsis thaliana* seedlings was carried out on ½ MS medium supplemented with 5 mg/L Basta (glufosinate-ammonium). Surface sterilized seeds of all lines, including *Arabidopsis thaliana* EST1, KO and Col-0, were plated and stratified at 4°C in the dark for 2 days to ensure uniform germination. Following transfer to growth conditions, selection was monitored over 7 days. Sensitive seedlings exhibited chlorosis and growth arrest, whereas resistant transformants remained green, developed true leaves, and showed normal root elongation. In parallel, seeds of the same lines were grown on ½ MS medium without Basta as a control to confirm normal germination and growth.

3.9.3 Basta Spray Selection on Soil-Grown Plants

After initial selection, healthy and resistant seedlings of all three transformed lines (*Arabidopsis thaliana* EST1, Est-1 *acd6* knockout mutant and Col-0) were transferred to soil for further growth. Seven days after transplantation, plants were subjected to a secondary selection by foliar application of Basta at a concentration of 250 mg/L. Prior to spraying, watering was withheld for two days to reduce surface moisture and facilitate effective uptake of the herbicide. Plants were evenly sprayed with the Basta solution and covered overnight to maintain humidity. The following day, covers were removed and normal watering was resumed. Non-transformed *Arabidopsis thaliana* (Col-0) plants were included as a control to verify the effectiveness of the selection.

3.10 Bacterial Inoculation for Activation of PAMP-Triggered Immune Responses (*PR1* Activation)

Pseudomonas syringae pv. tomato DC3000 was revived from glycerol stock and streaked onto low-salt LB agar plates supplemented with nitrofurantoin (100 µg/mL). Plates were incubated at 28°C in the dark for 2 days until colonies appeared. A single colony was selected and inoculated into 250 mL of liquid low-salt LB medium containing nitrofurantoin (100 µg/mL), followed by incubation at 28°C in the dark with shaking at 250 rpm. The bacterial culture was grown to an optical density (OD₆₀₀) of approximately 2. Cells were harvested by centrifugation at 4500 rpm for 10 minutes, and the pellet was washed with autoclaved water to remove residual media components. The bacterial suspension was then resuspended in autoclaved water and diluted to a final OD₆₀₀ of 1. The suspension was subsequently heat-killed by incubation at 65°C for 1 hour.

Initially, heat-killed bacterial suspensions were applied to leaves using an air-spray device to induce PAMP-triggered immune responses. However, no visible RUBY pigmentation was observed 48 h after treatment. Therefore, a leaf

infiltration method was subsequently adopted to improve delivery of the bacterial suspension into the leaf tissue. Individual leaves were gently pricked with a sterile toothpick, and the heat-killed bacterial suspension was infiltrated into the leaf tissue using a syringe.

Forty-eight hours after bacterial treatment, leaf samples were harvested for visualization of PR1:RUBY reporter activity. One treated leaf from each plant was collected and processed for ethanol clearing to visualize betalain pigmentation.

A second treated leaf from the same plant was harvested immediately after sampling, flash-frozen in liquid nitrogen, and stored at -80°C until RNA isolation for subsequent gene expression analysis.

3.11 Field experiment and sample collection

Field experiments were carried out in natural environmental conditions for two successive years of growth. In the first field season, field plants of *Arabidopsis thaliana* were harvested from the Fyrisån River region close to the SLU campus from 30 March to 10 April, temperature and humidity were monitored. Ten plants were sampled each day at the same time after sunrise, to minimize variation due to diurnal expression changes. During this sampling period, a total of 120 plants were collected.

During the second field season a new field experiment was set up at the SLU Weather Station. Col-0 and Est-1 plants were planted at the beginning of winter and then harvested in spring from 13th March to 13th April, at regular time intervals. The samples from both of the field experiments were taken for the longitudinal transcriptomic analysis.

Plant tissue was collected and placed immediately into 1.5 mL screw-cap tubes and rapidly frozen in liquid nitrogen to ensure the integrity and minimize the transcriptional changes of the collected plant tissue during handling. Samples were kept at -80°C for RNA extraction.

3.12 RNA isolation

Total RNA was isolated from frozen *Arabidopsis thaliana* leaf tissue using a plasmid DNA spin column-based method adapted for RNA purification (Yaffe et al. 2012). Frozen leaf tissue was manually ground using sterile glass beads. The powdered tissue was transferred to a 1.5 mL screw-cap tube containing one sterile glass bead and 500 µL of equally mixed 2.0 mm zirconia-silica/ceramic beads and 0.5 mm zirconia-silica beads. Subsequently, 500 µL of guanidine HCl extraction buffer was added, and the samples were homogenized for 1 min using a FastPrep-24™ 5G Homogenizer (MP Biomedicals, Irvine, CA, USA). The homogenates were centrifuged at 13,000 rpm for 20 min at 4°C to pellet cellular debris.

The clarified supernatant was carefully transferred to a fresh 1.5 mL microcentrifuge tube and mixed with an equal volume of 96% ethanol by brief vortexing. The mixture was immediately loaded onto a plasmid DNA spin column and centrifuged at $8,000 \times g$ for 45 s. The flow-through was discarded, and the column was washed sequentially with 450 µL of 3 M sodium acetate and 320 µL

of 70% ethanol, with centrifugation at $8,000 \times g$ for 45 s after each wash. Following the final wash, the column was centrifuged at maximum speed for 2 min to completely dry the silica membrane.

RNA was eluted by applying 40 μL DEPC-treated water directly onto the membrane, followed by incubation at room temperature for 2 min and centrifugation at $8,000 \times g$ for 2 min. When required, a second elution was performed to improve RNA recovery.

The concentration and purity of the isolated RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was assessed by agarose gel electrophoresis. RNA was stored at -80°C before use for further downstream analysis.

3.13 DNase treatment and cDNA synthesis

The isolated RNA samples were treated with RNase-free DNase I to remove the residual genomic DNA contamination before cDNA synthesis. The same DNase treatment procedure was used for all samples, with the amount of RNA used as the starting material adjusted according to the downstream application. 1 μg of total RNA was used for RNA-sequencing library preparation and 100 ng of total RNA was used for qPCR. The RNA was treated with 1 μL of the $10\times$ DNase reaction buffer and 1 μL (1 U) of DNase I for each sample in a 10 μL reaction mixture. The reaction was then incubated for 30 min at 37°C in a thermocycler. DNase activity was then stopped by adding 1 μL of 25mM EDTA and incubating at 65°C for 5 minutes.

First-strand cDNA synthesis was performed using DNase-treated RNA as the template. One microliter of oligo(dT) primer was added to the DNase-treated RNA, and the mixture was incubated at 65°C for 10 min to facilitate primer annealing. The reaction mixture was then supplemented with 4 μL of $10\times$ cDNA synthesis buffer, 0.5 μL of 100 μM dithiothreitol (DTT), 0.5 μL of RNase inhibitor, 1 μL of dNTP mix, and 1.25 μL of Karolinska reverse transcriptase (1:5 dilution), resulting in a final reaction volume of 20.75 μL . Reverse transcription was performed at 42°C for 1 h, followed by enzyme inactivation at 85°C for 5 min. The synthesized cDNA was stored at -20°C until further use in downstream gene expression analyses.

3.14 RNA sequencing library preparation

3.14.1 Second-strand cDNA synthesis

Double-stranded cDNA (ds-cDNA) was synthesized from first-strand cDNA using DNA Polymerase I and RNase H. For each reaction, a 35 μL master mix was prepared containing 5 μL DNA Polymerase I buffer, 3 μL of 10 mM dNTP mix, 0.4 μL RNase H, 5 μL DNA Polymerase I, and 21.6 μL nuclease-free water. The master mix was combined with 15 μL of first-strand cDNA to obtain a final reaction volume of 50 μL . Reactions were incubated at 16°C for 1 h to allow second-strand synthesis.

Following incubation, the ds-cDNA was purified using SPRI magnetic beads at a 1.8× bead-to-sample ratio by adding 90 µL SPRI beads to each 50 µL reaction. The bead suspension was mixed thoroughly, incubated to allow DNA binding, washed according to the manufacturer's recommendations, and the purified DNA was eluted in 20 µL nuclease-free water. The resulting purified ds-cDNA was used directly for subsequent Tn5-based library preparation.

3.14.2 Tn5 based library preparation

Tn5-based library preparation was performed using a non-magnetic bead protocol adapted from Stern et al. (2016). Adapter duplexes were first prepared by annealing complementary oligonucleotides. Duplex A was generated by mixing 9 µL of 100 µM Tn5ME_A (L0361) with 9 µL of 100 µM Tn5ME_rev (L0026) and 2 µL of annealing buffer. Duplex B was prepared by mixing 9 µL of 100 µM Tn5ME_B (L0360) with 9 µL of 100 µM Tn5ME_rev (L0026) and 2 µL of annealing buffer. Annealing reactions were heated to 98°C for 3 min and cooled to 23°C at a ramp rate of 0.1°C s⁻¹ (approximately 20 min). Equal volumes of duplex A and duplex B were subsequently mixed to generate the adapter duplex mixture.

For Tn5 loading, 1 µL of Karolinska Tn5 transposase was mixed with 16 µL of Tn5 dilution buffer, followed by the addition of 3 µL of the mixed adapter duplex. The reaction was mixed by gentle flicking, briefly centrifuged, and incubated at room temperature for 30 min to allow formation of the loaded Tn5 transposome.

Tagmentation reactions were assembled in a total volume of 10 µL containing approximately 10 ng of double-stranded cDNA, 0.2 µL of assembled Tn5 transposome, 2 µL of 5× TAPS-DMF-MgCl₂ buffer, and nuclease-free water to volume. Reactions were incubated at 55°C for 7 min. Tn5 activity was terminated by adding 2.5 µL of 0.2% SDS, followed by an additional incubation at 55°C for 7 min to ensure complete enzyme inactivation.

Tagmented DNA was amplified by PCR in a final reaction volume of 25 µL containing 1.5 µL of inactivated fragmented DNA, 5 µL of 5× HF Phusion buffer, 0.5 µL of 10 mM dNTP mix, 1.5 µL of primer mix, 1.25 µL of diluted Karolinska Phusion polymerase, and 14.25 µL of nuclease-free water. PCR amplification was performed using the following cycling conditions: 72°C for 5 min; 98°C for 2 min; 18 cycles of 98°C for 30 s, 60°C for 20 s, and 72°C for 30 s; followed by a final extension at 72°C for 5 min and a hold at 12°C.

The amplified libraries were assessed by agarose gel electrophoresis. Approximately 5 µL of PCR product was loaded onto the gel, and successful library amplification was assessed based on the presence of a DNA smear with a fragment distribution centered at approximately 500 bp.

3.15 Quantitative reverse transcription PCR (RT-qPCR)

Complementary DNA (cDNA) samples from all three biological replicates of both uninoculated (control) and inoculated plants were used for quantitative real-time PCR (RT-qPCR) analysis. Each cDNA sample was analyzed using two technical replicates. RT-qPCR reactions were prepared in a final volume of 10 µL each

reaction in a 96-well PCR plate. Each reaction contained 5 μ L of Bio-Rad SYBR Green PCR Master Mix, 3 μ L nuclease-free water, 1 μ L primer mix, and 1 μ L cDNA template. The reaction setup was performed on ice before loading the plate into the real-time PCR instrument BIORAD CFU reverse transcription RCR system.

Gene-specific primers were used to quantify transcript levels of *ACTIN*, *PR1*, and *RUBY*. The primer pair L0326 forward/L0327 reverse was used for amplification of *ACTIN*, which served as the housekeeping reference gene. The L0330 forward/L0331 reverse primer pair was used for *PR1* amplification, and L0491 forward/L0492 reverse primers were used for *RUBY* amplification. *ACTIN* expression was used for normalization.

The qPCR amplification program consisted of an initial denaturation step at 95°C for 1 min, followed by 40 cycles of amplification consisting of denaturation at 95°C for 10 s and primer annealing/extension at 60°C for 30 s, with fluorescence measurement after each cycle. A melting curve analysis was performed after amplification from 65°C to 95°C with a temperature increment of 0.5°C for 5 s followed by fluorescence acquisition to confirm amplification specificity.

3.16 Ethanol clearing and image-based quantification of PR1:RUBY pigmentation

Leaves collected 48 hours post inoculation were used for visualization of *RUBY* pigmentation. One inoculated leaf from each individual plant (Col0-1, Col0-2, Col0-3, Est-1, Est-2, Est-3, KO-1, KO-2, and KO-3) was harvested and placed individually in separate small plastic Petri dishes containing 98% absolute ethanol. Petri dishes were Parafilm-sealed to prevent ethanol evaporation and left to incubate overnight at room temperature to remove chlorophyll and clear leaf tissue. Following the clearing, leaves were very carefully washed with water, on a clean glass plate and scanned using an Epson scanner (Perfection V850 Pro) at a resolution of 1200 dpi against a black background.

Images of leaves were taken after ethanol clearing and processed using Fiji/ImageJ. The Color Threshold function of HSB (hue, saturation, and value) color space was applied to quantify the *RUBY* pigmentation. All images were processed with a fixed threshold (Hue: 223-254, Saturation: 31-107, Brightness: 98-223) to find *RUBY*-positive areas. The processed images were transformed into binary masks with *RUBY*-positive areas being white. The number of *RUBY*-positive pixels was counted and divided by the total number of pixels in the leaf to calculate the percentage of the *RUBY*-positive area.

3.17 Crossing of transgenic *Arabidopsis thaliana* lines

Manual crosses were performed between independent T1 PR1:RUBY transgenic *Arabidopsis thaliana* lines in the Est-1 and Est-1 *acd6* KO genetic backgrounds. For crossing, flowers were emasculated by removing the immature anthers before anthesis, followed by transfer of pollen from the selected donor plant onto the

receptive stigma of the recipient flower. Crossed flowers were labelled and monitored under standard growth conditions. Developing siliques were allowed to mature on the plants, after which the seeds were harvested and stored for subsequent propagation. The resulting F1 plants were grown and self-pollinated to generate F2 progeny for segregation and selection of plants carrying the desired genetic combination.

3.18 Tagmentation-based insertion Mapping (TagTIM)

The genomic insertion sites of the *PR1* transgene were identified using tagmentation-based insertion mapping (TagTIM), adapted from (Mehmetoğlu Boz et al. 2026). Genomic DNA was extracted from transgenic *Arabidopsis thaliana* plants and subjected to Tn5-mediated tagmentation, which simultaneously fragments genomic DNA and ligates adapter sequences to the fragment ends. This approach enables selective amplification of genomic DNA flanking the transgene insertion site using an adapter-specific primer together with a transgene-specific primer.

Tn5 adapters were generated by annealing complementary oligonucleotides and assembled with purified Tn5 transposase according to the published protocol. For each reaction, 10 ng of genomic DNA was mixed with 0.2 µL assembled Tn5 transposase, 2 µL of 5× TAPS-DMF-MgCl₂ buffer, and nuclease-free water to a final reaction volume of 10 µL. Tagmentation was performed at 55°C for 7 min. The reaction was terminated by adding 2.5 µL of 0.2% SDS, followed by incubation at 55°C for a further 7 min to inactivate the transposase.

The tagmented DNA was amplified in a first PCR using 1.5 µL of the tagmentation product as template. Each 25 µL reaction contained 1.5 µL primer mix consisting of the adapter-specific primer L0417 and transgene-specific primer L0512, 0.25 µL Thermo Fisher Phusion Hot Start DNA Polymerase, 5 µL of 5× HF buffer, 0.5 µL dNTP mix, and 7.75 µL nuclease-free water. The DNA template diluted in water was initially heated at 98°C for 3 min before addition of the PCR master mix. Amplification was performed with an initial denaturation at 98°C for 3 min, followed by 24 cycles of 98°C for 30 s, 68°C for 15 s, and 72°C for 50 s, with a final extension at 72°C for 5 min. PCR products were purified using 0.6× SPRI magnetic beads.

A second PCR was performed using 1 µL of the purified first PCR product as template to incorporate sequencing barcodes. Each 25 µL reaction contained 1.5 µL TruSeq primer mix (L0429-L0432 and L0701 and L0702), 1.25 µL Karolinska Phusion DNA Polymerase, 5 µL of 5× HF buffer, 0.5 µL dNTP mix, and 15.75 µL nuclease-free water. Amplification consisted of an initial denaturation at 98°C for 3 min, followed by 11 cycles of 98°C for 30 s, 68°C for 15 s, and 72°C for 50 s, with a final extension at 72°C for 5 min.

For sample indexing, barcode primer L0702 was used for the Est-1 transgenic lines (Est1-Est4), whereas barcode primer L0701 was used for the Est-1 *acd6* knockout lines (KO1-KO4). Both sample groups were amplified using TruSeq indexing primers L0429-L0432.

Following the second PCR, amplification products were purified using 0.65× SPRI magnetic beads and eluted in 20 µL nuclease-free water. To verify successful tagmentation and library preparation, 5 µL of each purified PCR product was analyzed by agarose gel electrophoresis. Successful libraries produced the characteristic smear expected for segmented DNA fragments.

Equal volumes of the purified PCR products from Est1-Est4 were pooled to generate a single Est library (final volume 10 µL), while products from KO1-KO4 were pooled separately to generate a KO library (final volume 10 µL). The pooled libraries were submitted to Plasmidsaurus for Oxford Nanopore long-read sequencing. Sequencing reads were aligned to the *PR1* reference construct and the *Arabidopsis thaliana* reference genome.

3.19 Sequencing and bioinformatic analysis

In silico construct design and primer analysis

Plasmid constructs were designed and analyzed using SnapGene (Dotmatics), which was used for construct assembly, sequence visualization, sequence alignment, and primer design. Primer properties were evaluated using the Thermo Fisher Scientific Primer Analyzer.

RNA-seq data analysis

Sequencing and bioinformatic analyses were performed using platform-specific workflows for Illumina and Oxford Nanopore sequencing. For Illumina RNA sequencing, paired-end reads were quality assessed, adapter-trimmed, and filtered using fastp, followed by alignment to the *Arabidopsis thaliana* reference genome using STAR. The resulting alignment files were visualized using Integrative Genomics Viewer (IGV) to examine read coverage, transcript alignment, and exon-exon splice junctions.

Nanopore Sequencing Data Analysis and Identification of T-DNA Insertion Sites

Nanopore sequencing reads generated from TagTIM libraries were subjected to quality assessment and filtering prior to downstream analysis. Filtered reads were mapped independently against the *Arabidopsis thaliana* TAIR10 reference genome and the pGWB601-PR1:RUBY construct sequence using minimap2 with Oxford Nanopore read mapping parameters. SAM/BAM files were generated, sorted, and indexed using samtools. Command-line text-processing utilities, including (grep), (sed) and (awk), were used for filtering and processing sequencing and alignment outputs. Reads aligning to the *Arabidopsis* genome were analyzed to identify genomic regions flanking T-DNA insertion sites, while reads mapping to the pGWB601

3.20 Statistical Analysis

The statistical analysis was carried out with R software (version 4.5.2). Relative expression values generated from RT-qPCR analysis were analyzed with the R packages tidyverse for data manipulation, ggplot2 for data visualization, and emmeans for estimated marginal means and post hoc pairwise comparisons. Two-way analysis of variance (ANOVA) was used to assess the influence of genotype, treatment and interaction on *PR1* and *RUBY* transcript accumulation. Post hoc pairwise comparisons with multiple testing corrections were used to further examine significant effects. A p-value of < 0.05 was considered to be statistically significant.

Images of ethanol-cleared leaves were analyzed in Fiji/ImageJ for quantification of PR1:RUBY reporter activity. The percentage of the leaf area with *RUBY* was calculated from the thresholded images with the color threshold method HSB (Hue, Saturation, Brightness). A threshold was set for each image and the percentage of *RUBY*-positive pixels to the total leaf area was calculated. One-way ANOVA was used to compare *RUBY*-positive area values among the genotypes in R. A significant ANOVA was followed by pairwise comparisons made using Tukey's honestly significant difference (HSD) test. Data are expressed as mean $\pm$ SD (standard deviation) of three biological replicates.

4. Results

4.1 Amplification and Purification of *PR1* Promoter and *RUBY* Reporter Fragments

We extracted the genomic DNA from *Arabidopsis thaliana* leaves, and quantified its concentration with a Qubit fluorometer (90 ng/μL). Then we used this genomic DNA as a template to PCR amplify the *PR1* promoter. A DNA fragment of the expected size of 1.3 kb was obtained.

We amplified the *RUBY* reporter sequence from the 35S::RUBY plasmid, isolated from transformed *Escherichia coli*. The coding region of *RUBY* was amplified as two overlapping fragments of about 2106 bp and 2120 bp, respectively, because of its large size. Both fragments were successfully amplified, with the expected sizes.

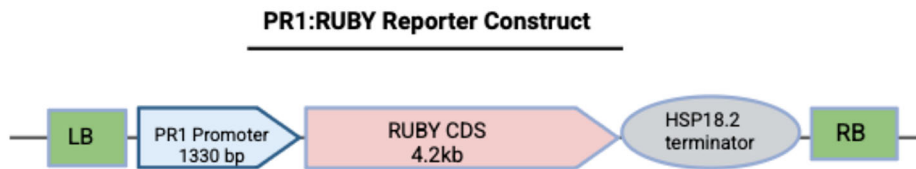


Figure 1: Schematic representation of the *PR1*:*RUBY* reporter construct (Created with BioRender.com). The *PR1* promoter drives expression of the *RUBY* reporter cassette, followed by the *HSP18.2* terminator. The construct was assembled within the T-DNA region of the pGWB601 DTOPO pENTR vector between the left border (LB) and right border (RB) sequences.

To optimize the amplification of pENTR/D-TOPO vector backbone we attempted multiple annealing temperatures, but this did not work. No PCR product was obtained of the expected vector backbone size 2500 bp. We repeated the PCR with Taq DNA polymerase, it successfully amplified the backbone of the pENTR/D-TOPO vector and generated an expected-size DNA fragment for downstream cloning.

Successful amplification of the *PR1* promoter, both *RUBY* fragments, and the pENTR/D-TOPO vector backbone was confirmed by agarose gel electrophoresis. We gel-purified all the PCR products using a commercial gel extraction kit, and the DNA concentrations were measured with a Qubit fluorometer prior to subsequent cloning experiments. The purified *PR1* promoter fragment had a concentration of 26.5 ng/μL, the first *RUBY* fragment 25.7 ng/μL, the second *RUBY* fragment 46.8 ng/μL, and the pENTR/D-TOPO vector backbone 8.0 ng/μL.

4.2 Gibson Assembly, Transformation, and Colony PCR Screening

We assessed the transformation competency of the prepared chemically competent *E. coli* (TOP 10) cells by using pUC plasmid DNA. Transformation resulted in colony formation on ampicillin-containing LB agar, confirming that the prepared cells were competent for subsequent transformation.

We assembled the *PR1* promoter fragment (1,330 bp), *RUBY* 1st half, *RUBY* 2nd half, and the vector backbone using the Gibson Assembly method. We calculated equimolar amounts of each DNA fragment and combined them in a Gibson Assembly reaction to promote balanced incorporation of all fragments into the final construct. Following assembly, the reaction mixture was purified using SPRI magnetic bead cleanup to remove enzymes, salts, and other components of the Gibson Assembly Master Mix that could interfere with downstream applications.

Then we transformed the purified assembly product into chemically competent *Escherichia coli* cells by heat-shock transformation. After a recovery period, the transformed cells were plated on LB agar supplemented with kanamycin (50 mg/L) and incubated overnight at 37°C to allow selection of colonies carrying the assembled plasmid (Figure 2).

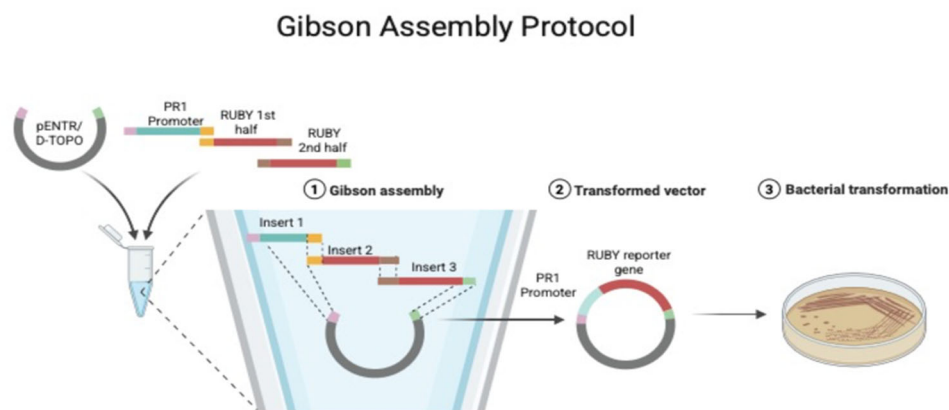


*Figure 2: Schematic presentation of Gibson assembly strategy for construction of the *PR1*:*RUBY* reporter cassette (Created with BioRender). The *PR1* promoter and two *RUBY* fragments were assembled into the linearized pENTR/D-TOPO vector using Gibson assembly, generating the complete ***PR1*:*RUBY*** construct, which was subsequently transformed into *E. coli* for plasmid propagation and screening.*

The following day, we screened all individual kanamycin-resistant colonies by colony PCR using primers specific for the *PR1* promoter to verify successful assembly of the construct. Despite screening multiple colonies, none produced the expected PCR product corresponding to the *PR1* promoter fragment. These results indicated that the Gibson Assembly was unsuccessful and that the desired construct had not been generated.

4.3 Optimization of Gibson Assembly and Cloning Strategy

To reduce vector self-ligation, which was suspected to contribute to the high frequency of empty colonies, we amplified the pENTR/D-TOPO vector backbone as two separate PCR fragments. Together with the *PR1* promoter and the two overlapping *RUBY* fragments, we performed a five-fragment Gibson assembly, but this yielded no colonies upon transformation.

Another optimization strategy was evaluated in which we amplified the *RUBY* coding sequence as a single fragment, reducing the assembly from five fragments to four while retaining the two PCR-amplified vector fragments. Despite repeated Gibson assembly reactions and optimization of fragment molar ratios, we again could not obtain any colonies, suggesting that efficient assembly of the construct into the pENTR/D-TOPO backbone could not be achieved under the conditions tested.

4.4 Evaluation and Optimization of PR1-RUBY Gibson Assembly

To verify the assembly of the *PR1* promoter and *RUBY* coding sequence prior to vector cloning, we performed a Gibson assembly reaction containing only these two DNA fragments. Agarose gel electrophoresis of the reaction products revealed a faint DNA band of approximately 5.6 kb, consistent with the expected size of the assembled PR1:RUBY fragment (Fig 3). However, the band intensity was very low. Following gel purification, the recovered DNA concentration was approximately 1 ng/ μ L, which was insufficient for subsequent cloning. Attempts to amplify the purified fragment using Phusion High-Fidelity DNA Polymerase were unsuccessful.

Analysis of the agarose gel also showed a strong band corresponding to the *RUBY* fragment, whereas the *PR1* promoter fragment was not clearly visible, suggesting inefficient incorporation of the *PR1* fragment during assembly. To improve assembly efficiency, the molar ratio of the input fragments was adjusted by increasing the amount of the *PR1* promoter relative to the *RUBY* fragment (0.75 pmol *PR1* and 0.25 pmol *RUBY*). Despite this optimization, we obtained no successful assembly, indicating that altering the fragment ratio did not improve ligation efficiency.

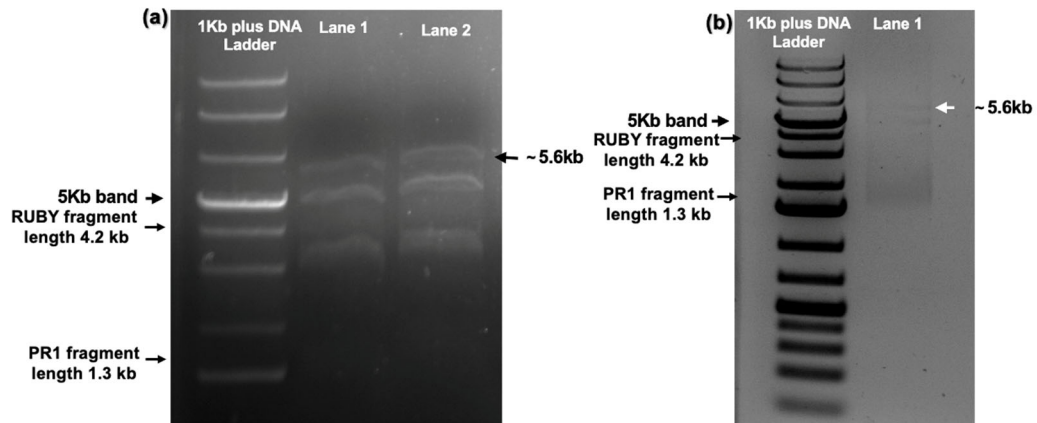


Figure 3: Gibson assembly of the *PR1:RUBY* construct. **(a)** Agarose gel electrophoresis of the Gibson assembly reaction (0.1 pmol). The expected assembled ***PR1:RUBY*** product (~5.6 kb) is indicated by the arrow and appears as a faint band. Additional DNA bands of varying sizes are also visible, representing unassembled fragments and/or incomplete assembly products generated during the Gibson assembly reaction. **(b)** Agarose gel electrophoresis of the Gibson assembly reaction (0.025pmol *RUBY*+0.075pmol *PR1*) containing the *PR1* promoter and *RUBY* construct. The arrow indicates the expected Gibson assembly product of approximately 5.6 kb, corresponding to the assembled *PR1:RUBY* fragment.

4.5 Alternative Cloning Strategy Using the pGWB601 Binary Vector

Following the unsuccessful Gibson assembly attempts, we implemented an alternative restriction-based cloning strategy using the binary vector pGWB601 (Figure 4). The *in silico* map of the resulting recombinant pGWB601 plasmid containing the *PR1:RUBY* construct is provided in Appendix *Figure A1*.

Restriction-Ligation Cloning Workflow for pGWB601-PR1:RUBY Construct

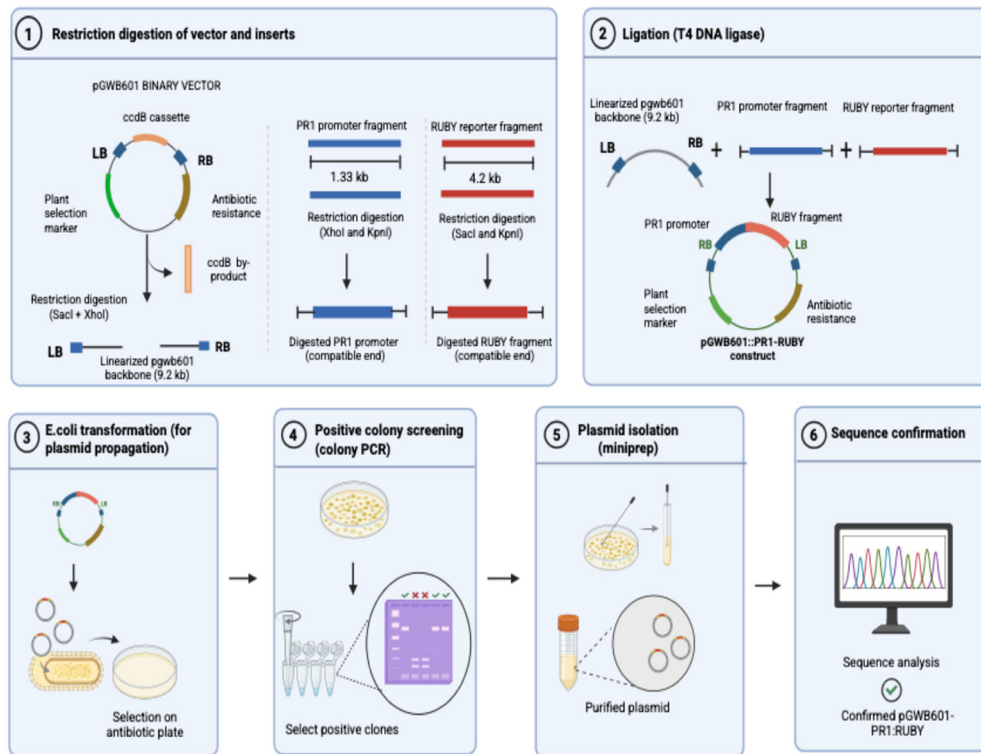


Figure 4: Cloning and transformation workflow for generation of *PR1:RUBY* transgenic *Arabidopsis* lines (created with bioRender). The pGWB601 binary vector was linearized and ligated with the *PR1* promoter (1.33 kb) and *RUBY* reporter (4.2 kb) fragments to generate the **pGWB601:PR1:RUBY** construct. The construct was transformed into *E. coli* for propagation, verified by colony PCR and sequencing.

Restriction digestion of the pGWB601 plasmid with SacI and XhoI generated the expected DNA fragments corresponding to the linearized vector backbone (9.2 kb) and the excised *ccdB* cassette (1.5 kb), suggesting successful preparation of the vector for restriction ligation cloning (Figure 5 a). To facilitate restriction-based ligation, we re-amplified *PR1* promoter and *RUBY* reporter genes using redesigned primers containing the appropriate restriction enzyme recognition sites. PCR amplification produced single DNA bands of the expected sizes (1.3 kb for *PR1* and 4.2 kb for *RUBY*) with no detectable non-specific amplification (Figure 5 b). The amplified fragments were gel-purified to obtain clean DNA suitable for downstream cloning.

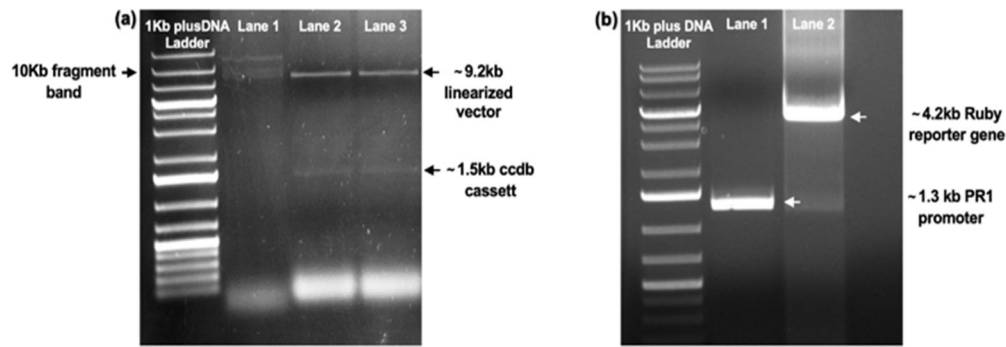


Figure 5: Restriction digestion of pGWB601 and PCR amplification of PR1 and RUBY cloning fragments. **(a)** Agarose gel electrophoresis of the pGWB601 plasmid following restriction digestion with *SacI* and *XhoI*. **Lane 1** contains the undigested pGWB601 plasmid used as a control. **Lanes 2 and 3** contain the restriction-digested plasmid, showing the expected 9.2 kb linearized vector backbone and the 1.5 kb excised *ccdB* cassette, confirming successful digestion and preparation of the vector for directional cloning. **(b)** Agarose gel electrophoresis of PCR-amplified PR1 promoter and RUBY reporter fragments. **Lane 1** shows amplification of the PR1 promoter fragment using primer pair L0439 + L0440, producing a single DNA band of the expected size (1.3 kb). **Lane 2** shows amplification of the RUBY reporter fragment using primer pair L0441 + L0442, producing a single DNA band corresponding to the expected fragment size (4.2 kb). The specific amplification of both fragments confirmed successful generation of DNA inserts for subsequent restriction-based cloning.

We digested the purified PR1 fragment with *XhoI* and *KpnI* and RUBY fragment digested with *SacI* and *KpnI*, generating compatible cohesive ends for directional cloning into the prepared pGWB601 vector. Following restriction digestion, the vector backbone and both insert fragments were purified using SPRI magnetic beads, which removed residual restriction enzymes, small DNA fragments, and other reaction components. The purified DNA fragments were subsequently used for ligation, providing high-quality vector and insert DNA for assembly of the PR1:RUBY construct.

Transformation of the ligation products into chemically competent *Escherichia coli* produced numerous colonies on selective medium, indicating successful uptake of plasmid DNA. We screened the resulting colonies by colony PCR using PR1-specific primers and identified several colonies producing the expected 1.3 kb amplicon. Col-0 genomic DNA was included as a positive control and produced the expected amplification product. Based on the colony PCR results, we selected three putative positive colonies for plasmid isolation and sequencing. Plasmid DNA was successfully isolated from all three PCR-positive colonies. Quantification using the Qubit High-Sensitivity dsDNA Assay showed DNA concentrations of 85 ng/ μ L, 73 ng/ μ L, and 253 ng/ μ L for colonies 1, 2, and 3, respectively. The recovered plasmid DNA was of sufficient quantity for Plasmidsaurus nanopore sequencing and subsequent downstream applications.

Unexpectedly, sequencing demonstrated that none of the selected clones contained the intended PR1:RUBY construct. Sequence analysis showed that the pGWB601 backbone was identical to the designed vector sequence; however, an approximately 7 kb DNA fragment had been inserted between the *SacI* and *XhoI*

cloning sites instead of the *PR1* promoter and *RUBY* reporter gene. BLAST analysis of the inserted fragment against the NCBI nucleotide database identified it as *Escherichia coli* genomic DNA with essentially complete sequence identity to the corresponding chromosomal region.

Further examination of the sequence revealed that the contaminating genomic DNA fragment contained *SacI* and *XhoI* restriction sites compatible with those used to linearize the pGWB601 vector. Consequently, digestion of contaminating genomic DNA generated cohesive ends that were compatible with the linearized vector, allowing unintended ligation of the bacterial genomic DNA during the cloning reaction. These results indicate that genomic DNA contamination was present in the plasmid preparation and was incorporated into the vector in place of the intended *PR1* and *RUBY* inserts.

4.6 Troubleshooting of Genomic DNA Contamination

To remove high-molecular-weight *Escherichia coli* genomic DNA contamination, we performed a large-scale plasmid preparation, followed by SPRI magnetic bead cleanup. The purified plasmid DNA was then separated on a 0.8% agarose gel, which enabled separation of the 10.7 kb pGWB601 plasmid from the high-molecular-weight genomic DNA. We excised the plasmid band and purified it by gel extraction, yielding contaminant-free vector DNA with a concentration of 47 ng/μL, as determined by Qubit fluorometry. The purified vector backbone was subsequently redigested and dephosphorylated to prevent vector self-ligation. Following a second SPRI magnetic bead cleanup to remove restriction enzymes, phosphatase, and small DNA fragments, we ligated the vector backbone with the purified *PR1* promoter and *RUBY* reporter fragments at equimolar insert-to-vector ratios. Despite these additional purification and cloning steps, transformation of the ligation products into chemically competent *Escherichia coli* did not produce any colonies on selective medium, indicating that the conventional restriction-ligation cloning strategy remained unsuccessful.

4.7 Assembly of *PR1* Construct Using Cyclic Ligation Strategy

Following the unsuccessful conventional restriction-ligation approaches, we explored a cyclic ligation strategy as an alternative method for assembling the *PR1*:*RUBY* construct into the pGWB601 binary vector. This strategy was selected to improve the efficiency of multi-fragment assembly and overcome the difficulties encountered during previous cloning attempts. Following transformation and overnight incubation, we observed a total of 25 colonies on the experimental plate, whereas only 3 colonies appeared on the control plate, indicating low background and successful ligation. We screened the resulting colonies by colony PCR using the primer set targeting the T-DNA and *PR1* junction region amplification, yielding an expected fragment size of approximately 509 bp. Nearly all colonies

from the experimental plate produced the expected amplicon, confirming efficient insertion of the *PR1* fragment into the vector (Figure 6a).

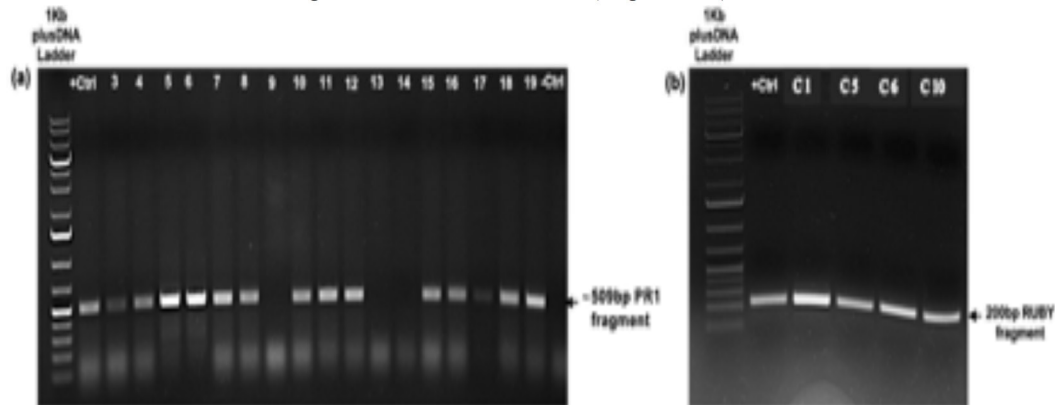


Figure 6: Colony PCR screening of *PR1*:pGWB601 recombinant clones. **(a)** Colony PCR was performed using M13 forward and 466 reverse primers to amplify the junction between the pGWB601 vector backbone and the *AtPR1* promoter insert, generating an expected fragment of approximately 509 bp. Lane 1: 1kb plus DNA ladder; Lane 2: positive control (+EV control); Lanes 3–19: colony PCR products from transformant colonies; Lane 20: negative control (no template). **(b)** PCR screening of selected recombinant colonies using *RUBY*-specific primers L0441 and L0465. Lane 1: 1kb plus DNA ladder; Lane 2: positive control containing *RUBY* plasmid DNA; Lanes 3–6: colony PCR products from colonies 1, 5, 6, and 10, respectively. All tested colonies produced the expected ~200 bp *RUBY*-specific amplicon, confirming the presence of the *RUBY* insert.

Based on colony PCR results, four representative colonies (1, 5, 6 and 10) were selected for further validation of the second insert. We screened these colonies using the *RUBY*-specific colony PCR primer pair described in the methods section, which was expected to produce an amplicon of approximately 200 bp. A positive control containing 35:S *RUBY* plasmid DNA (+ev control) was included to validate the amplification. All selected colonies showed the expected *RUBY*-specific band, confirming the successful incorporation of both *PR1* and *RUBY* fragments (Figure 6b). Overall, the cyclic ligation strategy was highly efficient, yielding a high proportion of correctly assembled constructs with minimal background from control reactions.

4.8 Plasmid Isolation and Restriction for Validation of Recombinant Constructs

We isolated Plasmid DNA from selected positive colonies (1, 5, 6, and 10). The concentration of purified plasmid DNA were 67 ng/μL, 66 ng/μL, 62 ng/μL, and 60 ng/μL for colonies 1, 5, 6, and 10, respectively. The relatively consistent DNA yields across samples indicate uniform plasmid recovery and suggest stable propagation of the recombinant constructs within *E. coli*.

For further validation, I subjected plasmid DNA from colonies 1 and 5 to restriction digestion using the same enzymes employed during the cloning strategy.

The empty pGWB601 vector was included as a control. Agarose gel electrophoresis of the digested products revealed distinct bands corresponding to the expected sizes of the vector backbone and inserted fragments in colonies 1 and 5, whereas the empty vector control displayed only the linearized vector backbone (Figure 7). These results were consistent with successful integration of the PR1:RUBY construct into pGWB601. Together with the colony PCR results, the restriction digestion confirmed colonies 1 and 5 as putative positive recombinant clones, which were selected for subsequent sequencing analysis.

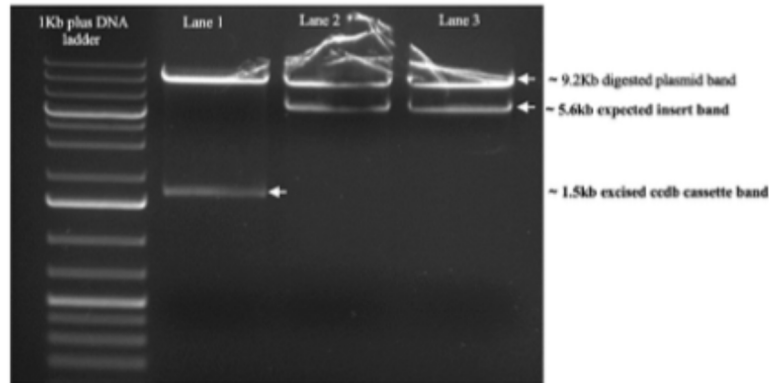


Figure 7: : Restriction digestion analysis of pGWB601 vector and recombinant PR1:RUBY construct. Agarose gel electrophoresis of *SacI* and *XhoI*-digested plasmids showing the release of expected fragments. **Lane 1:** undigested/empty pGWB601 vector digested with *SacI* and *XhoI*, producing the expected 9.2 kb vector backbone and 1.5 kb *ccdB* cassette fragments. **Lane 2 & 3:** Recombinant plasmid containing the PR1:RUBY construct digested with *SacI* and *XhoI*, producing the 9.2 kb vector backbone and an approximately 5.6 kb insert-containing fragment, corresponding to successful replacement of the *ccdB* cassette with the PR1:RUBY construct.

4.9 Sequence Validation of Recombinant Constructs

The assembled construct was verified by Plasmidsaurus Nanopore sequencing of plasmid DNA from two selected recombinant *E. coli* colonies (colonies 1 and 5). The generated sequences were analyzed in SnapGene and aligned against the in silico-designed reference construct with the pGWB601 backbone, the *Arabidopsis thaliana* PR1 promoter (*AtPR1*) and the *RUBY* reporter cassette (Appendix Figure A2).

The sequenced plasmids were in high concordance with the expected construct sequence for both. The reference sequence was aligned to plasmid 1 (PR1-RUBY-pGWB061.fasta1), which had 14,620 bp aligned with four mismatches and five gap events, and to plasmid 5 (PR1-RUBY-pGWB061.fasta2), which had 14,619 bp aligned with four mismatches and nine gap events. Although there were minor differences, the overall organization of both recombinant constructs was similar to that in silico generated reference, with the pGWB601 backbone and the reporter cassette containing *AtPR1* and *RUBY* in the correct orientation.

These results led to the selection of the recombinant plasmids for further transformation into *Arabidopsis* and functional studies. The recombinant *E. coli* clones were frozen as glycerol stocks and kept at -80 °C for further research.

4.10 Transformation and Confirmation of the PR1:RUBY Binary Construct in *Agrobacterium tumefaciens* GV3101

We introduced the confirmed PR1:RUBY binary plasmid construct into *Agrobacterium tumefaciens* GV3101 for subsequent plant transformation experiments. Following selection on antibiotic-containing medium, transformed *Agrobacterium* colonies were obtained, indicating successful recovery of bacterial transformants carrying the pGWB601-based binary vector (Figure 8).

To verify the presence of the PR1:RUBY construct in the selected *Agrobacterium* colonies, I screening the resulting colonies by colony PCR using construct-specific primer pair as described in method section, and *RUBY* plasmid DNA was used as positive control (+ev control). PCR amplification produced the expected ~200 bp amplicon corresponding to the target region, confirming the presence of the PR1:RUBY binary construct in the transformed *Agrobacterium* colonies (Figure 8). Confirmed colonies were selected for subsequent *Arabidopsis thaliana* transformation experiments.

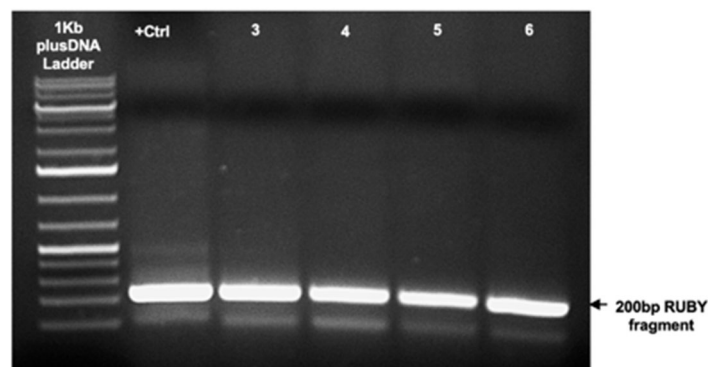


Figure 8: Colony PCR confirmation of the PR1:RUBY construct in *Agrobacterium tumefaciens* GV3101 transformants. Colony PCR was performed using primers L0441 (forward) and L0465 (reverse), which amplify an approximately 200 bp fragment of the *RUBY* insert. **Lane 1:** 1 kb Plus DNA ladder; **Lane 2:** positive control (+ev control, *RUBY* plasmid DNA); **Lanes 3-6:** representative *Agrobacterium* colonies used directly as PCR templates. The presence of the expected ~200 bp amplicon in lanes 3-6 confirmed successful transformation of *Agrobacterium* with the pGWB601-PR1:RUBY binary vector.

4.11 Floral Dip Transformation of *Arabidopsis thaliana*

We introduced the PR1:RUBY binary construct into three different lines of *Arabidopsis thaliana* Col-0, Est-1, and Est-1 *acd6* knockout (KO) by floral dip transformation with *Agrobacterium tumefaciens* GV3101 harboring the PR1:RUBY binary vector. After transformation, plants showed temporary wilting but recovered within 2-3 days in the greenhouse. Siliques were matured about 4 weeks after transformation and viable T1 seeds were produced from all three

genotypes. We harvested the individual transformed plants separately and the T1 seed populations were used for further selection and molecular confirmation of PR1:RUBY integration. The recovery of T1 seeds from Col-0, Est-1 and KO plants showed that floral dip transformation was successful and resulted in the production of transgenic plants for further phenotypic and molecular studies (Figure 9).

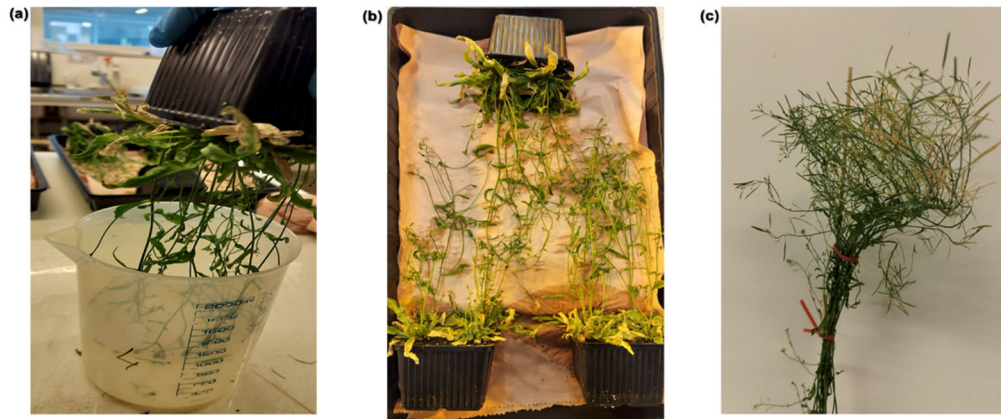


Figure 9: Floral dip transformation of *Arabidopsis* plants. **(a)** *Arabidopsis* plants were immersed in the *Agrobacterium*-containing floral dip solution to facilitate transformation. **(b)** Plants were laid horizontally in trays following floral dipping to maintain contact with the transformation solution. **(c)** Transformed plants were allowed to recover and develop siliques for subsequent seed collection.

4.12 Selection of PR1:RUBY Transgenic *Arabidopsis thaliana* Plants

T1 seeds obtained from transformed *Arabidopsis thaliana* Col-0, Est-1, and KO plants carrying the PR1:RUBY construct were subjected to Basta selection. Seeds of all three transformed lines after surface sterilization were sown on $\frac{1}{2}$ MS medium without Basta and $\frac{1}{2}$ MS medium with Basta. Seedlings on $\frac{1}{2}$ MS medium without Basta were used as non-selective controls and had normal germination and green growth in all three genetic backgrounds.

Putative Basta-resistant seedlings were identified based on their ability to maintain green tissue and continue growth on Basta-containing medium, whereas susceptible seedlings became chlorotic and showed arrested growth. Surviving seedlings from the Col-0, Est-1, and KO populations were transferred to soil for further selection and molecular validation.

Additional selection was performed on soil by spraying the transplanted seedlings with a high concentration of Basta solution (250 mg L^{-1}). Seedlings that survived herbicide treatment and were green and healthy were kept for additional studies. The same concentration of Basta was applied to non-transformed Col-0 seedlings at the same developmental stage in soil and used as negative controls. These control plants were very susceptible to the herbicide and did not survive, thus demonstrating that the resistance to the herbicide in the surviving plants was linked to the introduced PR1:RUBY T-DNA construct.

4.13 PR1:RUBY Reporter Activation Following Immune Induction

The functionality of PR1:RUBY reporter system was observed in transgenic *Arabidopsis thaliana* transgenic lines: Col-0, Est-1 and KO cultivated under short-day conditions. Basal *RUBY* reporter activity varied among transformed plants. Red pigmentation of leaves was observed in some plants from early developmental stages, even in the absence of experimental immune induction, which may be due to environmental conditions or microbial exposure, leading to spontaneous activation of *PR1* promoter. Other transformed plants, however, did not exhibit *RUBY* expression and remained green.

In the immune induction experiment we chose three biological replicates from each genetic background that did not exhibit any basal red pigmentation for treatment with heat-killed *Pseudomonas syringae* pv. tomato DC3000 (Pst), and three biological replicates were kept as control. Initially I sprayed the plants with heat-killed *Pseudomonas* but no *RUBY* pigmentation was seen after the spray treatment. Therefore, we chose leaf infiltration as an alternative treatment and infiltrated heat-killed Pst into selected leaves of the same plants. Control plants were left uninoculated.

No signs of red colouration were observed 24 h post-infiltration (hpi). Red pigmentation was observed at the infiltration sites after 48 hpi, while the non-infiltrated leaves were still green (Figure 10). The intensity of pigmentation was found to be different between the three genetic backgrounds.

Inoculated leaves from each plant were harvested for subsequent molecular and visual analyses. We used the leaves for RNA extraction and RT-qPCR analysis, and other leaves were ethanol-cleared and used for visualisation and quantitative image analysis of *RUBY* pigmentation. Treatment resulted in the appearance of red pigmentation at the infiltration sites, indicating reporter activation of inducible PR1:RUBY.

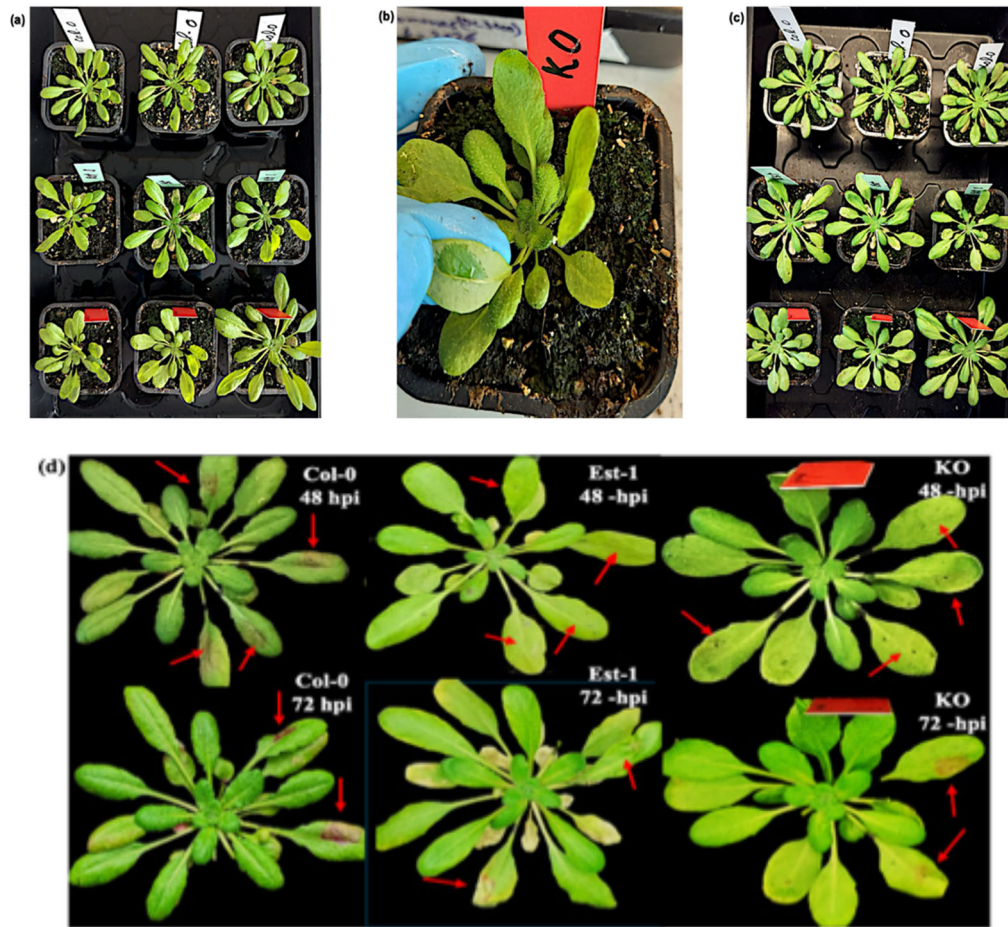


Figure 10: *PR1*:*RUBY* reporter activation following immune induction with heat-killed *Pseudomonas syringae* pv. tomato DC3000. **(a)** Transgenic *Arabidopsis thaliana* Col-0, *Est-1*, and KO plants carrying the *PR1*:*RUBY* reporter construct before immune induction. Three biological replicates from each genotype were selected for the experiment and showed no visible basal *RUBY*-associated red pigmentation. **(b)** Infiltration of heat-killed *P. syringae* pv. tomato DC3000 into marked leaves to induce PAMP-triggered immunity. **(c)** Plants 48 hours after infiltration, showing *RUBY*-mediated red betalain accumulation in infiltrated leaves as a result of *PR1* promoter activation. Representative images of three biological replicates per genotype are shown. **(d)** Close-up views of infiltrated leaves at 48 and 72 hpi. Red arrows indicate the infiltration sites, showing variable *RUBY* pigment production among leaves.

4.14 Salicylic Acid-Induced Activation of the *PR1* Reporter

To confirm the responsiveness of the *PR1* reporter system to salicylic acid (SA) signaling, we sprayed one plant of each transgenic genotype (Col-0, *Est-1*, and KO) with 500 μ M salicylic acid to confirm the responsiveness of *PR1*:*RUBY* reporter system to salicylic acid (SA) signaling. After SA treatment, red pigmentation first appeared in the midribs of younger *Est-1* leaves approximately 24 h after treatment, coinciding with activation of the *PR1* promoter and expression of the *RUBY*

reporter. The pigmentation became more prominent after 48 h. All three genotypes were found to be activated by SA, but the intensity of the pigmentation varied. The Est-1 background showed greater red pigmentation than Col-0 and KO plants, indicating higher activation of the *PR1* promoter and higher SA-responsive signaling in the Est-1 genotype. These observations validated the PR1:RUBY reporter construct and its sensitivity to exogenous SA treatment (Figure 11).

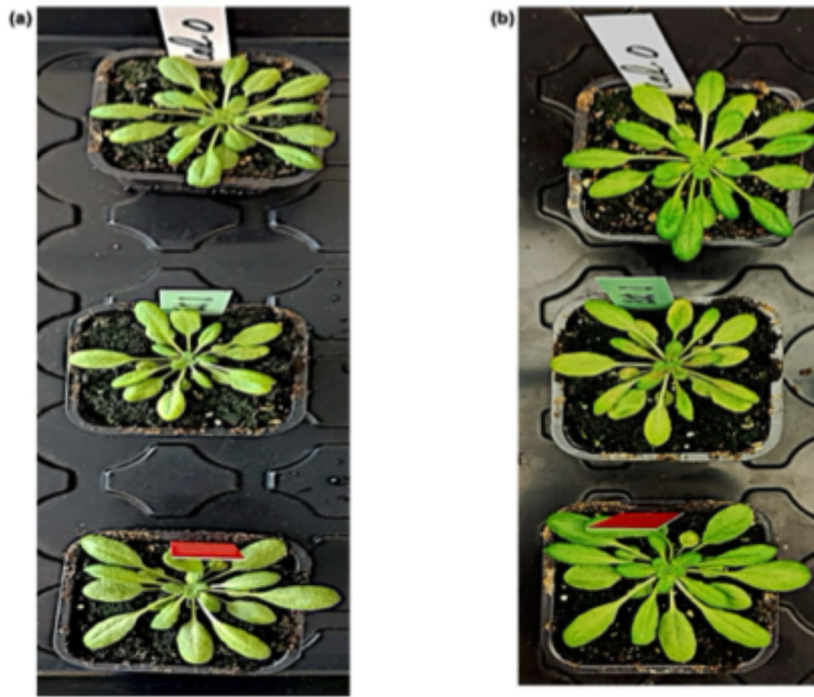


Figure 11: Salicylic acid-induced activation of the PR1:RUBY reporter in transgenic Arabidopsis thaliana lines. (a) Col-0, Est-1, and KO plants carrying the PR1:RUBY construct before salicylic acid treatment, showing the absence of visible RUBY-associated red pigmentation. (b) Same plants 48 hours after spraying with 500 μ M salicylic acid, showing induction of red betalain pigmentation. Est-1 plants exhibited stronger red pigmentation compared with Col-0 and KO plants. Representative images of one plant per genotype are shown.

4.15 Visual Validation of Reporter Expression after Immune Induction

To quantify the activation of the PR1:RUBY reporter, we analyzed ethanol-cleared scanned leaves using Fiji (ImageJ) (Figure 12). Clear differences in RUBY-positive leaf area were observed among Col-0, Est-1, and KO plants. The percentage of RUBY-positive leaf area was highest in Col-0 plants ($30.58 \pm 3.54\%$) while very limited RUBY-positive area was observed in Est-1 plants ($0.009 \pm 0.015\%$). The RUBY-positive area of the KO plants was intermediate ($3.09 \pm 4.19\%$) which was higher than that of Est-1 but lower than that of Col-0.

One-way ANOVA showed a significant effect on *RUBY*-positive leaf area ($F(2,6) = 84.76$, $P < 0.001$). Tukey's HSD post hoc analysis showed that Col-0 had significantly greater *RUBY*-positive leaf area than both Est-1 and KO. However, Est-1 and KO did not differ significantly from each other. Accordingly, Col-0 was assigned significance group a, while Est-1 and KO were assigned group b (Table 1).

These results indicate that *PR1* reporter activation, as reflected by *RUBY*-positive leaf area, was significantly higher in Col-0 than in the Est-1 and KO backgrounds under the conditions tested. Although the mean *RUBY*-positive area was higher in KO than in Est-1, this difference was not statistically significant (Figure 13).

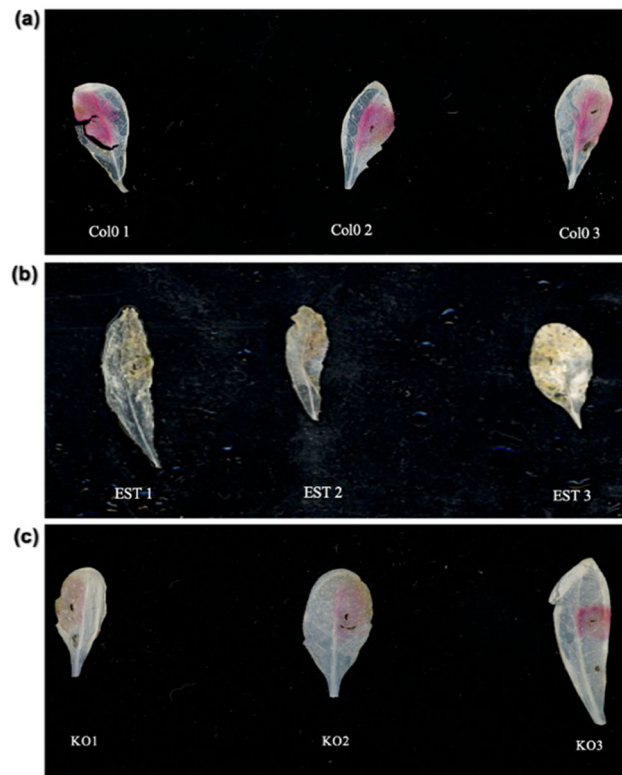


Figure 12: Visualization of *PR1:RUBY* reporter activity in ethanol-cleared leaves at 48 hpi. (a) Col-0, (b) Est, and (c) Est *acd6* KO ethanol-cleared leaves after scanning. The pink coloration represents *RUBY*-derived betalain accumulation. Images were acquired under identical scanning conditions.

Table 1: Quantification of *PR1*:RUBY reporter activity in ethanol-cleared leaves (48 hpi).

Genotype	Mean RUBY-positive area (%)	SD	Significance group
<i>Col-0</i>	30.58	3.54	a
<i>Est</i>	0.009	0.015	b
<i>Est acd6</i> KO	3.09	4.19	b

Table 1: Quantification of RUBY-positive leaf area in ethanol-cleared leaves of *Col-0*, *Est*, and *Est acd6* KO plants. Total leaf area and RUBY-positive area were measured from scanned ethanol-cleared leaf images using Fiji (ImageJ). The percentage of RUBY-positive area was calculated as the RUBY-positive area divided by the total leaf area $\times 100$, thereby normalizing reporter activity to leaf size.. Values are presented as mean $\pm$ SD from three biological replicates. Different letters indicate statistically significant differences between genotypes according to Tukey's HSD post hoc test ($P < 0.05$).

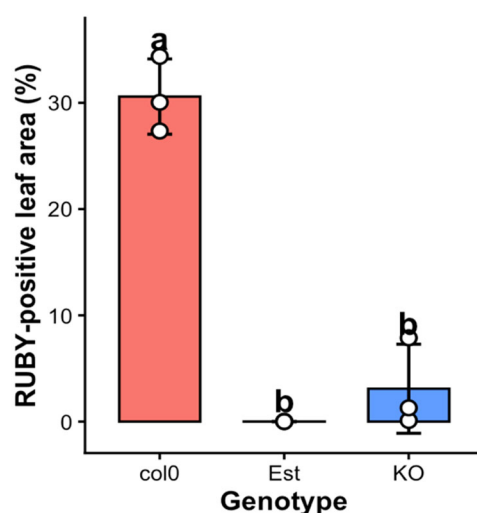


Figure 13: : Quantification of *PR1*:RUBY reporter activity based on RUBY-positive leaf area (48 hpi). The percentage of RUBY-positive leaf area was quantified from ethanol-cleared leaves of *Col-0*, *Est*, and KO plants using Fiji (ImageJ). Bars represent mean values $\pm$ SD from three biological replicates. Individual biological replicate values are shown as points. Different letters indicate significant differences between genotypes determined by Tukey's HSD post hoc test following one-way ANOVA ($F(2,6) = 84.76$, $P < 0.001$). Different letters indicate statistically significant differences between genotypes according to Tukey's HSD post hoc test.

4.16 Molecular Analysis of *PR1*:RUBY Transformants Following PAMP-Induced Immune Activation

We quantified endogenous *PR1* transcript accumulation in heat-killed Pst-treated and untreated *PR1* transformants from the *Col-0*, *Est-1*, and KO backgrounds using RT-qPCR. We normalized *PR1* transcript abundance to Actin as the housekeeping gene and calculated relative expression using the $2^{-\Delta C_t}$ method to assess activation of SA-associated immune signaling.

In all three genetic backgrounds, transcript levels of *PR1* were higher when treated with Pst compared to their respective uninoculated controls (Figure 14). Mean relative *PR1* expression in inoculated plants was highest in Est-1 (17.0 ± 9.49), followed by Col-0 (14.8 ± 4.64) and KO (4.05 ± 0.981) (Table 2). In comparison, mean *PR1* expression in uninoculated plants was substantially lower, with values of 0.0877 ± 0.150 in Col-0, 0.390 ± 0.354 in Est-1, and 0.0000242 ± 0.0000183 in KO. Thus, heat-killed Pst treatment induced *PR1* transcript accumulation in all three genetic backgrounds, but to different extents in the different genotypes.

Two-way ANOVA performed on ΔCt values showed significant effects of treatment, genotype, and their interaction (Table 2). Genotype significantly affected *PR1* expression ($F_{2,12} = 18.973$, $P = 0.000192$), while treatment had the strongest overall effect ($F_{1,12} = 124.062$, $P = 1.10 \times 10^{-7}$). A significant genotype $\times$ treatment interaction ($F_{2,12} = 10.462$, $P = 0.002344$) was observed, indicating that the response to Pst treatment differed among the genetic backgrounds.

Tukey's post hoc analysis of the genotype main effect showed no significant difference between Col-0 and Est-1 ($P = 0.111$). In contrast, the KO background differed significantly from both Col-0 (adjusted $P = 0.00574$) and Est-1 (adjusted $P = 0.000150$). Overall, heat-killed Pst treatment strongly induced endogenous *PR1* transcript accumulation in all three genetic backgrounds, while the significant genotype $\times$ treatment interaction indicated that the magnitude of this response was genotype-dependent.

Table 2: Relative expression of *PR1* transcripts in *PR1::RUBY* transgenic *Arabidopsis* lines under control and immune-induced conditions.

Genotype	Treatment	Mean relative <i>PR1</i> expression ($2^{-\Delta Ct}$)	SD	n
Col-0	Uninoculated	0.0877	0.150	3
Col-0	Inoculated	14.8	4.64	3
Est-1	Uninoculated	0.390	0.354	3
Est-1	Inoculated	17.0	9.49	3
KO	Uninoculated	0.0000242	0.0000183	3
KO	Inoculated	4.05	0.981	3

Values represent mean relative *PR1* expression calculated using the $2^{-\Delta Ct}$ method $\pm$ standard deviation (SD) from three biological replicates ($n = 3$). Technical replicate Ct values were averaged before calculation of ΔCt .

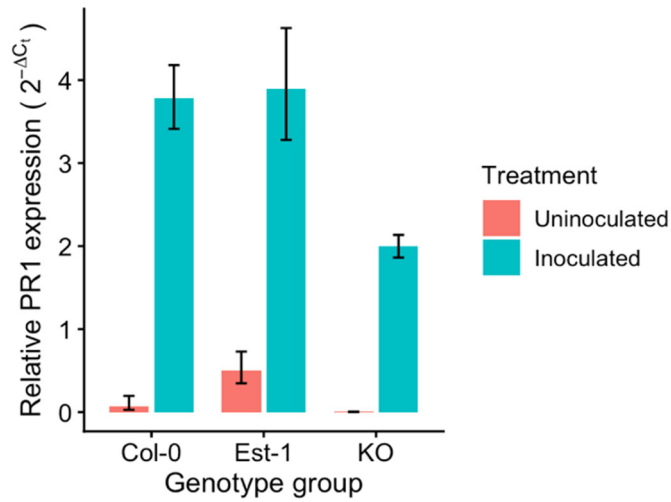


Figure 14: Relative expression of *PR1* in *PR1:RUBY* transgenic *Arabidopsis* lines following immune induction. *PR1* transcript abundance was determined by RT-qPCR and normalized against the *Actin* reference gene. Relative expression values were calculated using the $2^{-\Delta C_t}$ method and square-root transformed for visualization. Bars represent mean square-root transformed relative expression values $\pm$ standard deviation (SD) from three biological replicates ($n = 3$). Uninoculated and inoculated plants were compared across Col-0, Est-1, and KO genotypes.

Table 3: Two-way ANOVA analysis of *PR1* expression

Gene	Source of variation	Df	F value	p value
<i>PR1</i>	Genotype	2	18.973	0.000192
<i>PR1</i>	Treatment	1	124.062	1.10×10^{-7}
<i>PR1</i>	Genotype $\times$ Treatment	2	10.462	0.002344
<i>PR1</i>	Residual	12		

Table footnote: Two-way ANOVA was performed on ΔC_t values with genotype, treatment, and genotype $\times$ treatment as fixed factors ($n = 3$ biological replicates per genotype $\times$ treatment combination).

Consistent with endogenous *PR1* activation, *RUBY* transcript abundance increased following immune induction in all transformed genetic backgrounds (Figure 15). Relative to untreated controls, mean relative *RUBY* expression ($2^{-\Delta C_t}$) increased from 0.00439 to 0.949 in Col-0, from 0.00442 to 0.0663 in Est-1, and from 0.000636 to 0.0864 in KO plants (Table 4).

Table 4: Relative expression of *RUBY* in *PR1:RUBY* transgenic *Arabidopsis* lines following immune induction.

Genotype	Treatment	Mean relative expression ($2^{-\Delta Ct}$)	SD	n
Col-0	Control	0.00439	0.00665	3
Col-0	Inoculated	0.949	0.755	3
Est-1	Control	0.00442	0.00215	3
Est-1	Inoculated	0.0663	0.0404	3
KO	Control	0.000636	0.000154	3
KO	Inoculated	0.0864	0.124	3

Values represent mean relative expression calculated using the $2^{-\Delta Ct}$ method and normalized against the Actin reference gene. Data are presented as mean $\pm$ standard deviation (SD) from three biological replicates (n = 3).

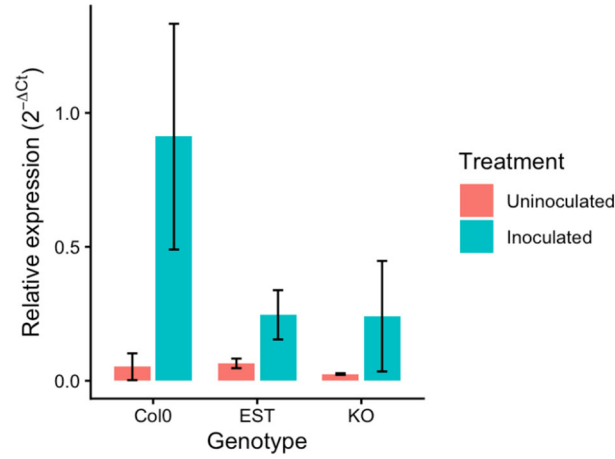


Figure 15: : Relative expression of *RUBY* reporter transcripts in *PR1:RUBY* transgenic *Arabidopsis* lines following PAMP-induced immune activation. *RUBY* transcript accumulation was analyzed in Col-0, Est-1, and KO plants under control and heat-killed *Pseudomonas syringae* pv. tomato DC3000 treatment conditions. Total RNA was extracted 48 h after treatment, and *RUBY* expression was quantified by RT-qPCR. Expression levels were normalized against the Actin reference gene and calculated using the $2^{-\Delta Ct}$ method. Bars represent square-root transformed relative expression values $\pm$ standard deviation (SD) from three biological replicates (n = 3).

Two-way ANOVA of ΔCt values revealed a significant effect of genotype on *RUBY* expression ($F_{2,12} = 4.153$, $P = 0.0426$) and a highly significant effect of treatment ($F_{1,12} = 61.385$, $P = 4.65 \times 10^{-6}$), whereas the genotype $\times$ treatment interaction was not statistically significant ($F_{2,12} = 3.508$, $P = 0.0631$) (Table 5). Tukey-adjusted post-hoc comparisons confirmed that *RUBY* expression was significantly higher following inoculation in Col-0 ($P = 2.97 \times 10^{-5}$), Est-1 ($P = 0.0172$), and KO ($P = 0.0010$). Together, these results demonstrate that immune induction significantly increased *RUBY* expression across all three genetic backgrounds, while there was no statistically significant evidence that the magnitude of the treatment response differed among genotypes.

Table 5: . Two-way ANOVA analysis of *RUBY* expression.

<i>Gene</i>	<i>Source of Variation/Factor</i>	<i>Df</i>	<i>F value</i>	<i>p value</i>
<i>RUBY</i>	<i>Genotype</i>	2	4.153	0.0426
<i>RUBY</i>	<i>Treatment</i>	1	61.385	4.65×10^{-6}
<i>RUBY</i>	<i>Genotype $\times$ Treatment</i>	2	3.508	0.063135
<i>RUBY</i>	<i>Residual</i>	12		

Comparison of endogenous *PR1* and *PR1* promoter-driven *RUBY* transcript accumulation showed a consistent induction in response to Pst treatment across all three genetic backgrounds. Both endogenous *PR1* and *RUBY* transcripts increased substantially in treated plants compared with their respective untreated controls. For endogenous *PR1*, the highest mean relative transcript accumulation following Pst treatment was observed in Est-1, which was not significantly different from Col-0 but was significantly higher than the KO. In contrast, *PR1* promoter-driven *RUBY* transcript accumulation was highest in Col-0, followed by KO and Est-1. Thus, although both endogenous *PR1* and *RUBY* showed clear treatment-induced transcriptional responses, their genotype-specific expression patterns were not identical.

4.17 Mapping of PR1 T-DNA Insertion Sites in T1 Transformants

The T-DNA insertion site was investigated using the Tn5-tagmented sequencing data by mapping reads against the pGWB601-PR1:*RUBY* construct and the *Arabidopsis thaliana* genome. However, the available reads were predominantly mapped to the construct sequence and did not provide sufficient coverage across the T-DNA-genome junction to clearly define the insertion site. The observed alignment pattern could be due to the insertion having been extended with extra vector backbone sequence or due to the available reads not covering the genomic junction.

4.18 Generation of Progeny Through Manual Crossing and Self-Pollination

Manual crosses were performed between independent T1 PR1:*RUBY* transgenic *Arabidopsis thaliana* lines in the Est-1 and Est-1 *acd6* KO genetic backgrounds. Following manual pollination, the crossed flowers remained viable and developed elongated, swollen siliques within approximately one week (Figure 16), indicating successful fertilization and seed development. The siliques continued to develop under standard growth conditions and were allowed to mature on the plants. Mature

seeds were subsequently collected from the crosses for propagation and further generation of progeny.

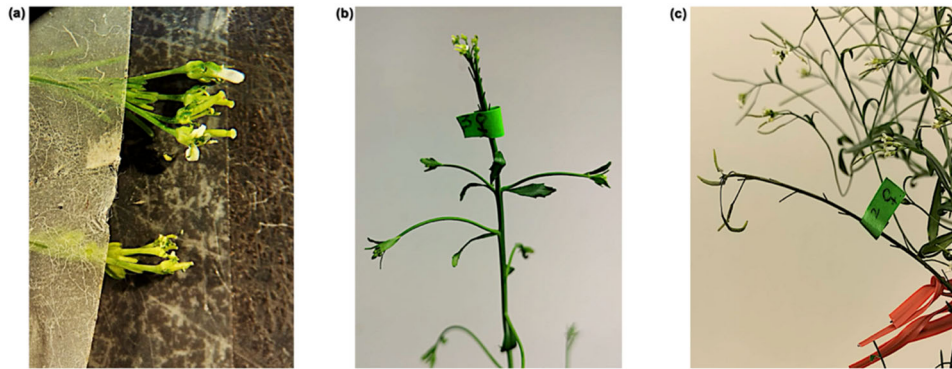


Figure 16: Manual crossing procedure used to generate *F1* progeny. **(a)** Removal of anthers from flowers using a stereomicroscope to expose the stigma. **(b)** Pollen from the male parent was transferred to the exposed stigma using a fine brush. **(c)** Siliques formed following successful pollination.

4.19 Longitudinal transcriptomic analysis

To investigate temporal variation in the transcriptomic response of *Arabidopsis thaliana* under field conditions, we used samples collected from two consecutive field experiments. In particular, we extracted total RNA from 120 plants collected from the Fyrisån River area using a LogSpin-based RNA extraction method that we optimized during this study. The resulting RNA samples were quantified and assessed for concentration, purity, and integrity prior to downstream transcriptomic analysis.

To evaluate and standardize transcriptomic workflow, we selected one plant RNA sample for sequence library preparation. The extracted RNA was used for first-strand cDNA synthesis, and then second-strand cDNA synthesis was performed to obtain double-stranded cDNA. The concentration of the resulting ds-cDNA was 3 ng/μL. The ds-cDNA was then tagged with Tn5 transposase and sequencing adapters and sample-specific barcodes were added following the library preparation protocol.

The amplified library was subjected to agarose gel electrophoresis, which resulted in a visible smear of DNA, confirming successful fragmentation and amplification of the sequencing library. The chosen library was then sequenced using paired-end Illumina sequencing.

Sequencing of the RNA sample from the single selected plant yielded 20.27 million read pairs (40.53 million reads) with an initial read length 150 bp. After quality filtering and adapter trimming, 15.98 million read pairs (about 31.95 million individual reads) were kept for further analysis. The retained reads had high sequence quality, with 97.98% of bases in read 1 and 99.20% of bases in read 2 with a Phred quality score ≥ 20 . Similarly, 87.45% of read 1 bases and 95.72% of read 2 bases had a Phred quality score ≥ 30 .

After alignment to the reference genome of *Arabidopsis thaliana*, 57.78% of reads aligned uniquely and 20.98% aligned to multiple genomic locations. There were 763,921 splice junctions detected, 749,204 of which were annotated splice junctions, and 750,845 were canonical GT/AG junctions. Processed reads were mapped to the reference genome of *Arabidopsis thaliana* using STAR, and the resulting alignments were visualized in IGV. The reads showed strong coverage across highly expressed photosynthesis-related genes, including AT1G67090, which encodes a small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Figure 17). Many reads also spanned annotated exon-exon junctions. The observed alignment patterns, the detection of exon-exon junctions, and the representation of highly expressed plant genes were consistent with successful sequencing of predominantly transcript-derived RNA.

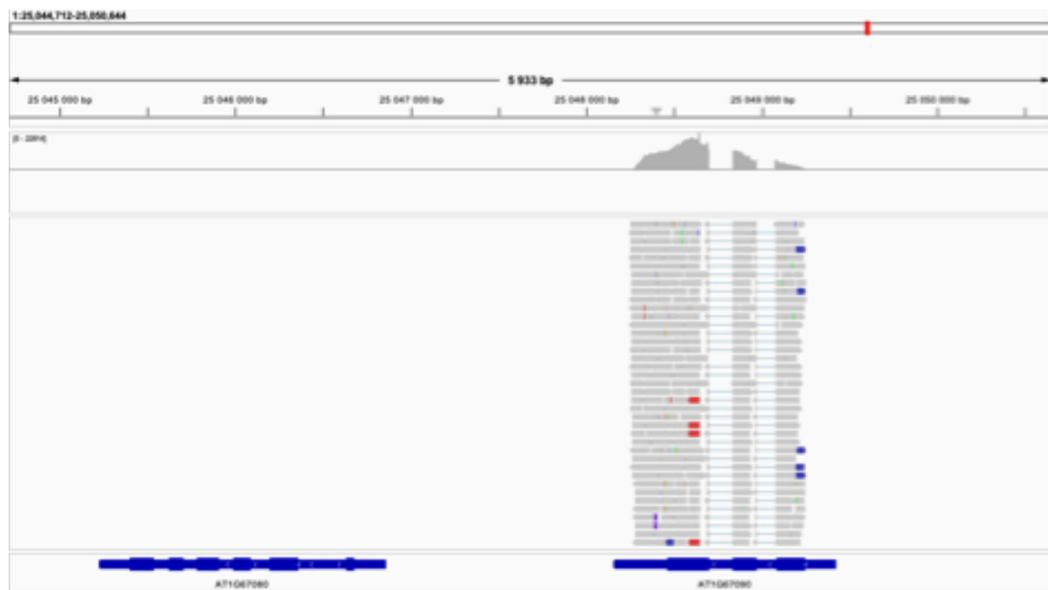


Figure 17: IGV visualization of RNA-seq reads aligned to the *Arabidopsis thaliana* reference genome, showing coverage across the RBCS gene and reads spanning annotated exon-exon junctions.

5. Discussion

The aim of this work was to develop and validate a *PR1* promoter-driven *RUBY* reporter system for investigating immune activation in different *Arabidopsis thaliana* genetic backgrounds in relation to salicylic acid (SA)-associated signaling. The reporter construct was assembled from the *PR1* promoter, the large *RUBY* coding sequence, and the pGWB601 binary vector backbone, which was technically challenging and necessitated testing multiple cloning strategies.

Although fragment ratios, vectors and assembly conditions were optimized, Gibson assembly did not yield a functional PR1:*RUBY* construct under the conditions tested. The large size of the *RUBY* coding sequence, along with the need to assemble several pieces of DNA with homologous overlapping regions, may have reduced assembly efficiency. A faint DNA fragment that matched the predicted PR1:*RUBY* product was found in the two-fragment assembly experiment, but the product of the assembly was recovered at very low levels and was not successfully amplified for use in subsequent cloning steps.

Since the Gibson assembly attempts were unsuccessful, a restriction-based cloning strategy was pursued with the pGWB601 binary vector. The pGWB601 vector has a *ccdB* negative selection cassette in the Gateway cloning region. *ccdB* counterselection works because an intact vector containing functional *ccdB* cannot propagate in a *ccdB*-sensitive *E. coli* strain. The *ccdB* cassette was removed from the pGWB601 vector to create the compatible cloning sites for insertion of the *PR1* promoter and the *RUBY* reporter fragments. Multiple transformants were recovered but initial screening and further sequencing revealed that the chosen clones were not carrying the desired PR1:*RUBY* construct. Instead, an approximately 7 kb fragment of *E. coli* genomic DNA was inserted into the vector. This was likely due to contamination with genomic DNA with compatible restriction sites, which ligated into the pGWB601 linearized vector during cloning. The finding highlighted the importance of careful vector preparation and sequence-level validation, because colony PCR does not distinguish between the desired recombinant plasmid and a plasmid with an unwanted insert. Sequencing thus allowed for the identification of the cloning artefact and the structure of the recovered plasmids. Further troubleshooting and optimization were then required to obtain the desired PR1:*RUBY* construct.

Following further optimization, the final PR1:*RUBY* construct was successfully generated by cyclic ligation strategy. After multiple unsuccessful cloning attempts, the recovery of the right recombinant clones proved to be very efficient with a high percentage of correctly assembled constructs and little background from the control reactions. Cyclic ligation involves multiple rounds of heating and cooling to allow for repeated opportunities for fragment annealing and ligase-mediated joining. The repeated temperature cycling may provide multiple opportunities for complementary DNA ends to anneal and for ligase-mediated joining to occur, potentially increasing the probability of productive multi-fragment assembly. Based on the success of this strategy, cyclic ligation represents a potentially useful alternative for constructing complex DNA assemblies when other cloning strategies are inefficient or unsuccessful.

Colony PCR, restriction digestion, and DNA sequencing confirmed that both the *PR1* promoter and the *RUBY* reporter cassette were correctly incorporated, oriented, and intact within the final construct. The validated PR1:*RUBY* binary vector therefore provided a reliable reporter platform for investigating *PR1* promoter activation and immune responses in different *Arabidopsis thaliana* genetic backgrounds. Introduction of the construct into the Col-0, Est-1, and Est-1 *acd6* knockout (KO) backgrounds by *Agrobacterium*-mediated floral dip transformation showed that the reporter system could be efficiently transferred into diverse genetic contexts. Recovery of Basta-resistant T1 transformants and subsequent molecular validation confirmed the presence of the PR1:*RUBY* T-DNA, providing transgenic lines suitable for functional analysis of immune-responsive reporter activation.

Recovery of Basta-resistant seedlings appeared to vary among the three genetic backgrounds, with Col-0 showing a higher recovery rate than Est-1 or KO. These differences could be due to variation in the transformation efficiency, seed germination, seedling vigor, or tolerance to Basta selection. Seed numbers and survival rates were not quantified, however, so it wasn't possible to determine if the differences observed were due to variation in transformation efficiencies or due to later seedling survival.

Interestingly, before experimental induction of immunity, independent transformants of Est-1 PR1:*RUBY* were found to be heterogeneous in terms of reporter activity. Some of the transformants of Est-1 showed visible red color during the initial developmental stages, while others were green under the same conditions. The spontaneous pigmentation may indicate elevated basal activity of the *PR1* promoter in some transformants without experimental PAMP treatment. This is consistent with the constitutive immune activation associated with the hyperactive *ACD6* allele in the Est-1 background and its elevated endogenous *PR1* expression. Basal *RUBY* pigmentation, however, may also vary between independent transformants due to environmental factors, exposure to microbes, developmental stage, or differences in the expression of transgenes due to independent T-DNA insertion events. Only transformants that did not show any pigmentation were used for the subsequent Pst infiltration experiments to reduce the variation in basal reporter expression.

Even though there was no visible *RUBY* pigmentation prior to infiltration, these selected green Est-1 transformants showed significantly less pigmentation after Pst infiltration compared to Col-0 and the Est-1 *acd6* knockout lines. This was supported by RT-qPCR analysis 48 h after immune induction, revealing much lower transcript levels of *RUBY* in Est-1 than in Col-0. By contrast, endogenous *PR1* expression was highly induced in Est-1 and was significantly higher than in both Col-0 and the KO. This difference suggests that the endogenous *PR1* transcript accumulation in the Est-1 T1 transformants analyzed in this study was not completely captured by the PR1:*RUBY* reporter. The endogenous *PR1* and the *PR1* promoter-driven *RUBY* showed an increase in transcript accumulation after Pst treatment, but to different extents. The lower *RUBY* transcript accumulation and pigmentation despite strong induction of endogenous *PR1* may be linked to line-specific differences in transgene expression, such as T-DNA insertion site, transgene copy number, chromatin environment, or transcript stability. The observed pigmentation differences may also be caused by differences in the production or accumulation of downstream *RUBY*.

Therefore, the relationship between endogenous *PR1* and PR1:RUBY expression should be interpreted specifically for the independent T1 transformants analyzed in this study. Whether this difference persists in independently selected stable T2 or T3 lines would need to be tested. Nevertheless, the consistent increase in *RUBY* transcript abundance after Pst treatment in all three genetic backgrounds suggests that the reporter responded to immune induction in the T1 transformants examined. Thus, the PR1:RUBY construct provides a convenient reporter system for monitoring *PR1* promoter-associated immune activation, although quantitative comparisons should ideally be performed using genetically stable lines.

The heterogeneous *RUBY* expression observed among independent T1 transformants raised the possibility that differences in reporter activity could be influenced by the genomic position of T-DNA insertion. To investigate this possibility, Tn5-based sequencing was performed on selected Est-1 and KO transformants to identify the genomic regions surrounding the integrated PR1:RUBY construct. The analysis indicated that the examined transformants carried the construct at different genomic positions, providing a possible explanation for variation in reporter expression among independent T1 lines. However, because only a subset of transformants was analyzed and reporter expression was not directly compared with insertion position across a larger number of independent lines, the contribution of insertion-site effects could not be conclusively established.

The variation observed among independent T1 PR1:RUBY transformants highlights limitations associated with using an integrated reporter construct for quantitative comparisons. A way to minimize this variation would be to create stable homozygous reporter lines and compare independent lines with the reporter at different genomic locations. This would minimize the variation due to segregation of the transgenes and give more uniform genetic material for further experiments. In stable lines, however, position effects due to differences in genomic insertion sites and copy number may still need to be taken into account, even in stable lines.

Other reporter systems might offer other means to address some of these limitations. Direct visualization of promoter activity in living plants, and repeated measurements over time without destructive sampling, may be possible using fluorescent reporters like GFP or mCherry. This may be especially helpful for the study of dynamic changes in immune activation in response to changing environmental conditions. Another quantitative method might be luciferase-based reporters, which can be measured sensitively over a wide range of expression levels and can be monitored repeatedly in living plants. Fluorescent and luminescent reporters, therefore, may be more appropriate for detecting relatively small or transient changes in promoter activity, as compared to *RUBY*, which generates a visible and relatively stable pigmentation signal.

Alternatively, a combination of immune-responsive promoter reporters could be employed instead of just *PR1*. For instance, pairing a *PR1* reporter with reporters of other salicylic acid- or defense-related genes may be useful to distinguish between changes in *PR1* regulation and changes in immune signaling. The use of reporter measurements in combination with endogenous gene-expression analysis would also be useful to confirm the validity of the assumption that reporter activity is a good indicator of the transcriptional state of the endogenous gene.

These methods may offer complementary information on immune activation and may help to differentiate biological differences in promoter activity from differences due to transgene integration or reporter-specific properties. While the PR1:RUBY system is still useful for visualizing immune activation, a combination of genetically stable reporter lines with other quantitative reporter systems may offer a more comprehensive framework for future studies of environmentally regulated immunity.

In parallel, genetic crosses were started to generate stable PR1:RUBY lines in Est-1 and KO backgrounds which were to be used in the subsequent experiments. Independent T1 PR1:RUBY transformants in the Est-1 and Est-1 *acd6* KO backgrounds were intercrossed to generate F1 progeny combining the two genetic backgrounds. F1 seeds were harvested from the resulting crosses and will be grown to obtain F1 plants, which will be self-pollinated to produce the F2 population. In the F2 generation, segregation will enable the identification of plants carrying the desired PR1:RUBY genotype, including homozygous individuals, which can subsequently be propagated to establish stable reporter lines. The development of homozygous lines should reduce variation associated with transgene segregation and provide more uniform material for future phenotypic and molecular analyses.

The contrasting behavior of the hyperactive *ACD6* allele under controlled and field conditions suggests that environmental factors strongly influence the resulting immune phenotype. To explore the molecular basis of this environmental modulation, transcriptomic analysis of field-grown plants was initiated. The RNA-seq workflow established in this study provides a practical basis for processing a larger number of field samples, combining an optimized RNA extraction procedure with an in-house Tn5-based library preparation approach. Initial Illumina sequencing confirmed the presence of plant-derived transcripts, supporting the suitability of the samples for transcriptomic analysis. Further analysis of these field transcriptomes, together with comparison with transcriptomic data from controlled conditions, may help identify biological processes associated with the different *ACD6*-dependent responses observed across environments. Such comparative analysis could provide insight into how environmental conditions modulate immune signaling associated with the hyperactive *ACD6* allele.

An important technical outcome of this study was the optimization of the RNA extraction workflow previously used in the laboratory. The optimized workflow is more reagent-efficient and faster than the previous method, which needed more reagents and was more time-consuming, and can process multiple samples at once. This decreased the hands-on time and increased the cost-effectiveness of the workflow for processing large sample numbers. Importantly, the optimized method yielded RNA of similar concentration and quality as the previous method, suggesting that the improved processing efficiency did not affect the quality or yield of the RNA. This optimized workflow is especially well suited for experiments with many samples, like the longitudinal field study, and offers a practical workflow that can be applied to future transcriptomic experiments in the lab.

Taken together, the results highlight the complexity of interpreting *PR1* promoter activity using an integrated reporter system in independent T1 transformants. Although the PR1:RUBY reporter responded to immune induction, the differences between endogenous *PR1* and *RUBY* expression suggest that transgene-specific effects may influence reporter output. Establishing genetically

stable lines will therefore be important for determining whether the observed differences persist across generations and for enabling more reliable quantitative comparisons. In parallel, analysis of field-grown transcriptomes may help identify the molecular processes underlying the contrasting *ACD6*-dependent immune phenotypes observed under different environmental conditions.

6. Conclusion

Overall, this work established a PR1:RUBY reporter platform for monitoring immune-associated *PR1* promoter activity in different *Arabidopsis* genetic backgrounds. The reporter responded to immune induction, while variation among independent T1 transformants highlighted the potential influence of transgene-specific factors and the importance of establishing stable lines for quantitative comparisons. The contrasting endogenous *PR1* and *RUBY* responses further demonstrate the need for careful interpretation of reporter output from individual T1 insertion events. Together with the ongoing transcriptomic analysis of field-grown plants, these findings provide a foundation for further investigating how environmental conditions modulate immune activation associated with the hyperactive *ACD6* allele.

7. Future Perspectives

Although this study developed and characterized the PR1:RUBY reporter system as a tool for studying immune activation under controlled laboratory conditions, additional research is needed to elucidate how natural variation in *ACD6* interacts with environmental factors to regulate immunity. Following stabilization of the reporter lines, homozygous T2/T3 PR1:RUBY lines in the Est-1 and *acd6* KO backgrounds could be used for more reproducible controlled-environment experiments.

The existing PR1:RUBY lines could be utilized to study the effects of specific environmental factors on immune activation in controlled settings. Experiments that change the temperature, humidity, photoperiod, light intensity, UV exposure, and microbial conditions may help identify the environmental factors that modify the immune phenotype of the hyperactive *ACD6* allele. Time-course experiments after immune induction could further clarify whether the effect of *ACD6* is not only on the amplitude of *PR1* promoter activation, but also on the timing and duration of the response. Comparing stable reporter lines under these conditions would also be useful to determine if the differences seen between Est-1, Col-0 and the *acd6* KO are maintained across generations.

The field transcriptomic work provides a complementary method to investigate *ACD6*-dependent immune responses in natural environmental conditions. The field RNA-seq data, along with the transcriptomic data obtained under controlled conditions, may be utilized to determine biological processes and regulatory pathways linked to the contrasting *ACD6* phenotypes found in different environments. In particular, changes in gene expression could be studied as a function of sampling time, genotype, and environmental factors like temperature, humidity, and light intensity. Genotype $\times$ time interactions could be investigated to see if the transcriptional response to *ACD6* is different throughout the growing

season. Since different plants were sampled at each time point, statistical models could control for plant-to-plant variation and test for the effects of genotype, sampling time, and environmental variables. Genes and biological processes associated with specific stages of the seasonal response or environmental conditions could be further identified by differential expression and pathway-level analyses. Further, detailed environmental measurements and increased sampling density and biological replication across multiple growing seasons could help identify environmental factors associated with variation in *ACD6*-dependent immune responses.

In summary, the use of stable PR1:RUBY reporter lines in controlled-environment experiments, together with transcriptomic analysis of field-grown plants, would provide complementary approaches for studying how environmental conditions regulate *ACD6*-dependent immunity. The optimized RNA extraction and in-house library preparation workflows could also enable larger transcriptomic studies, as they allow for higher sample throughput at comparatively lower reagent costs. This could make experiments involving greater biological replication and more frequent sampling more feasible. These strategies could be combined to help identify intrinsic responses to the *ACD6* genotype and to gain insight into the plant's ability to modulate immune responses in response to environmental conditions to optimize growth and fitness.

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Popular science summary

Insects, pathogens, and changing weather conditions cannot be avoided by plants. Rather, they have a complex immune system that enables them to identify threats and trigger protective responses. However, plant immunity is not controlled by genes alone. Plants can respond strongly to their environment, including temperature, humidity, light, and interactions with microorganisms.

This thesis examined the influence of the environment on plant immunity in the small plant *Arabidopsis thaliana*, which is widely used in biological research. The research was centered on a gene called *ACD6*, which is crucial for controlling plant immune responses. A particular *Arabidopsis* accession, Est-1, carries a naturally occurring version of this gene that causes unusually strong immune activation when the plants are grown under controlled laboratory conditions. Interestingly, this strong immune phenotype is greatly reduced when the same plants grow under natural field conditions. This difference brings up an important question: why do the same genetic change result in different immune responses in different environments?

To address this question, a visual reporter system, PR1:RUBY, was created. This system links the activity of a plant defence-related gene, *PR1*, to the production of a red pigment. The immune system is activated, causing the pigment to build up in the plant tissues and making the response more visible and measurable. The reporter was introduced into three genetic backgrounds: the standard Col-0 accession, Est-1, and an Est-1 plant in which the gene *ACD6* was knocked out. The plants were treated with heat-killed bacteria to stimulate immune responses, and the immune response was assessed by examining the activity of genes and the appearance of the red pigment.

The experiments demonstrated that the activity of the *PR1* and the *RUBY* reporter was significantly enhanced by the bacterial treatment. Differences between the plant genotypes also supported the role of *ACD6* in determining the strength of the immune response. The *RUBY* signal was visible and measurable by image analysis, thus offering a practical tool to study changes in plant immunity.

Plants were also grown in the field and sampled multiple times during the growing season to investigate what happens under natural conditions. Because these samples were collected over time and under changing environmental conditions, they provided an opportunity to investigate how the plant's immune state changes in a more natural environment. To analyse these samples at the molecular level, an RNA extraction method and an RNA sequencing library preparation workflow were successfully optimized. These methods allow the study of the activity of numerous genes simultaneously.

Overall, this study highlights that plant immune responses cannot always be understood from genetics alone. The differential response of the Est-1 plants to controlled and field conditions is consistent with the idea that the environment is a critical factor in determining genetically determined immune activation. The PR1:RUBY reporter and optimized transcriptomic workflow developed in this study offer valuable tools for future studies of how plants coordinate genetic information with environmental cues to control their immune responses.

Acknowledgements and Dedication

I would like to express my sincere gratitude to my supervisors, **Dr. Derek Lundberg and Dr. Shailja**, for their guidance, support, and valuable feedback throughout my thesis work. Their encouragement and scientific insights greatly contributed to my learning and to the development of this project.

I would also like to thank the members of the laboratory and department for their help, support, and for creating a friendly and stimulating research environment. I am grateful for the many discussions, assistance, and encouragement I received during my time at SLU.

I am deeply grateful to my **husband and my children** for their constant love, patience, encouragement, and support throughout my studies. Their belief in me helped me through the challenges and made this journey possible.

This thesis is dedicated to the loving memory of my **late parents, whom I miss dearly**. Their love for plants and their joy in planting and caring for them inspired my own connection with the plant world. I carry their love, memories, and inspiration with me in everything I do.

8. Appendix

8.1 Complete nucleotide sequence of the PR1:RUBYconstruct

***PR1* promoter sequence**

PR1 promoter a bit shorter than 1293 bp functional zone from the SnaBI site onwards

Tacgtaataatcctatggtgtcattttataagtttagcacaaagctgttttaactataaaatgattctccctccatataaaa
aagtttgattttatagaatgtttataccgattaaaaaataataatgcttagttataaattactattttatcatgctaaactatttc
tcgtaactattaaccaatagtaattcatcaaattttaaaattctcaattaattgattcttgaaattcataaccttttaattgatt
gataaaaatatacataaactcaatcttttaatacaaaaaaactttaaaaaatcaatftttctgattcggaggaggatataatgt
attgcttagaatcacagattcatatcaggattggaaaattttaagccagtgcatatcagtagtcaaaattggtaaagat
atacgaaggcgggtacaaaattaggtatactgaagatagaagaacacaaaagtagatcggtcacctagagttttcaatt
taaactgcgtattagtgttggaaaaaaaaacaaagtgtatacaatgtcaatcggtgatcttttttttttttttttttttttc
ttttggataaatctcaatgggtgatctattgactgtttctctacgtcactattttacttacgtcatagatgtggcggcatatat
tcttcaggacttttcagccataggcaagagtgtatagatactcatatgcatgaaacactaagaacaaataattcttga
ctttttcttttattgaaaattgactgtatataaactttttttttctgactgtaaatataatcttaattgccaaactgtccga
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RUBY CDS

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cagcctcgatcccgacctccgcgattacatctccaggcatttcagcccagccaggccaccactctgcgtgatccatga
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gagcgcaacgaggggtccaaaggctgggagatgaagaatcgcgccgtggaaatgggcaaaaagctcaaggacg
ccgtgaacgagggaaaagagctgaagggtcctcctgaaggcgatcgacgatttctcgacgccgtcatgcagg
ccaaactgagccaagcctccagtga

HSP18.2 terminator

Gcttatatgaagatgaagatgaaatatttggtgtgtcaataaaaagggtgtgtgcttaagttgtgttttttcttggt
tgtgtgttatgaattgtggctttttctaataataatgaatgtaacatctcattataatgaataaacaatgtttctataatcc
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aag

8.2 Appendix Figures

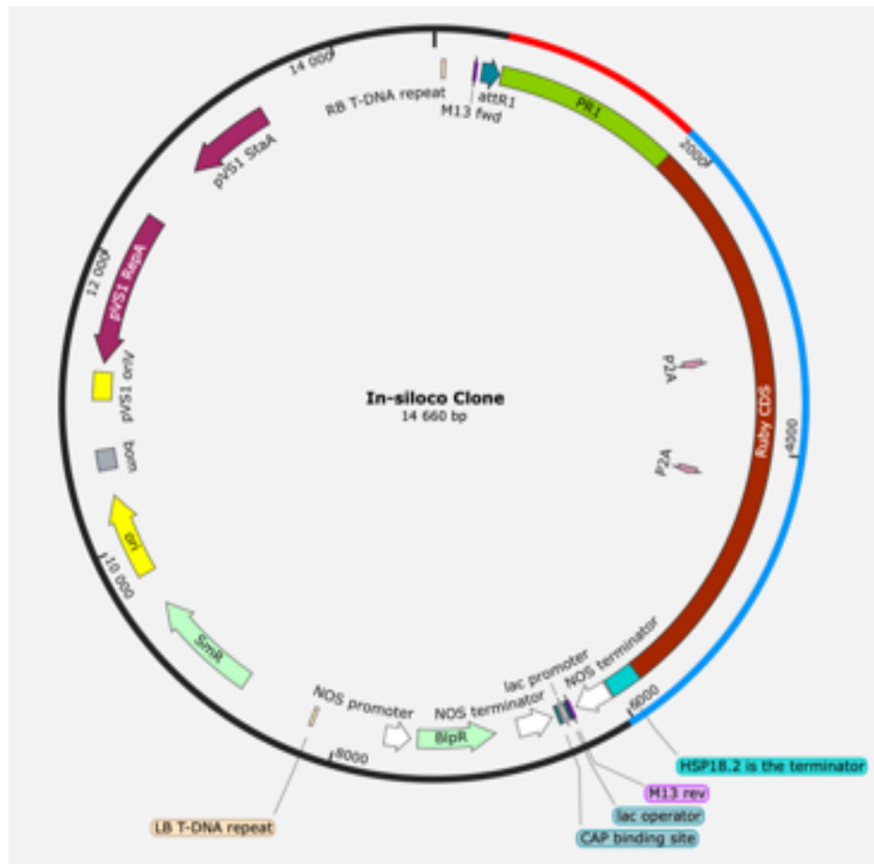


Figure A1: In silico map of the recombinant pGWB601 plasmid containing the PR1:RUBY construct.

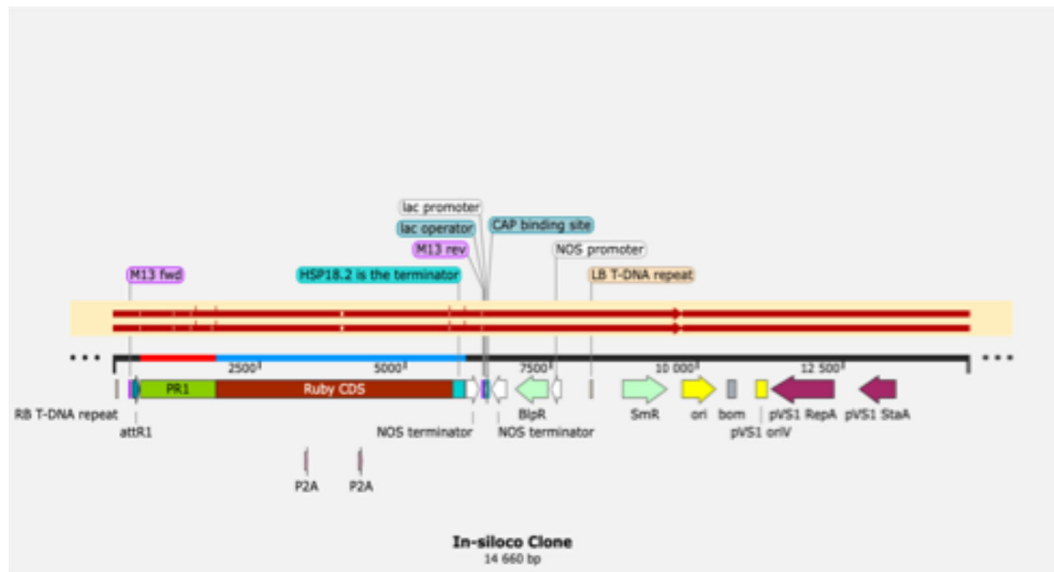


Figure A2: SnapGene alignments of two independently sequenced recombinant plasmids against the in silico-designed PR1:RUBY reference construct. The upper panel shows the alignment for plasmid 1, and the lower panel shows the alignment for plasmid 5.

8.3 Appendix Tables

Table A1: Primer used for PCR and RT-qPCR analysis in this study.

Primer ID	Target Gene	Direction	Sequence (5'-3')	Application
L0368	<i>PR1</i> promoter	Forward	CTTTGTACAAAAAGCAGGCTggtgtcattttataa gtagcacaagc	<i>PR1</i> promoter amplification (Gibson assembly)
L0390	<i>PR1</i> promoter	Forward	CACCgtgtcattttataagtagcacaagct	Alternative <i>PR1</i> promoter amplification
L0369	<i>PR1</i> promoter	Reverse	GCGAGGGTCGCATGATCCATTTTTCTAAGT TGATAATGGTTATTGTTG	<i>PR1</i> promoter amplification (Gibson assembly)
L0370	<i>RUBY</i> fragment 1	Forward	accattatcaactagaaaaatggatcatcgaccctcgccat	<i>RUBY</i> fragment 1 amplification
L0371	<i>RUBY</i> fragment 1	Reverse	GTAGTGCGGTGTCTCATCGAGTGGATGTG TGGCGCTGCCGG	<i>RUBY</i> fragment overlap assembly
L0372	<i>RUBY</i> fragment 2	Forward	ccggcagcgccacacatccactcgatgagacaccgcactac	<i>RUBY</i> fragment overlap assembly
L0373	<i>RUBY</i> fragment 2	Reverse	CTGGGTCGGCGCGCCACCCCTTATCTTTA ATCATATTCCATAGTCCATAC	<i>RUBY</i> fragment 2 amplification
L0380	<i>RUBY</i> fragment 2	Reverse	TTGTACAAGAAAGCTGGGTCGGCTTATCT TTAATCATATTCCATAGTCCATACC	Alternative <i>RUBY</i> reverse primer
L0374	pENTR backbone	Forward	atggactatggaatatgattaaagataagGGGTGGGCGCG CCGACCCAG	Backbone amplification
L0379	pENTR backbone	Forward	atggactatggaatatgattaaagataagCCGACCCAGCTT TCTTGACAA	Alternative backbone primer
L0384	pENTR backbone	Reverse	GCTCTGCCAGTGTTACAACCAATTAACCA ATTCTG	Backbone amplification
L0385	pENTR backbone	Forward	CAGAATTGGTTAATTGGTTGTAACACTGG CAGAGC	Backbone amplification
L0375	pENTR backbone	Reverse	AATGACACCATAGGATATTATTACGTAAG CCTGCTTTTTTGACAAAGTTG	Backbone connection to <i>PR1</i>
L0389	pENTR backbone	Reverse	AGCTTGTGCTAACTATAAAATGACACGG TG*GGCGGCCGCGGAGCCTGCT	Alternative backbone primer
L0439	<i>PR1</i> promoter	Forward	atcgatCTCGAGgtgtcattttataagtagcacaagct	<i>PR1</i> amplification with XhoI site
L0440	<i>PR1</i> promoter	Reverse	TAAGTTGGTACCGGTTATTGTTGTGTTATG ATTTTGG	<i>PR1</i> amplification with KpnI site
L0441	<i>RUBY</i>	Forward	ATTATCGGTACCACTTAGAAAAatggatcatgc gaccctcgccat	<i>RUBY</i> amplification with KpnI site
L0442	<i>RUBY</i>	Reverse	TTCGATGAGCTCCTTATCTTTAATCATATT CCATAGTCCATACC	<i>RUBY</i> amplification with SacI site
L0465	<i>PR1</i>	Reverse	ATGAGTGGGCCGTGAATCTTG	Colony PCR screening
L0466	<i>RUBY</i>	Reverse	TAACATATACTCCCTCCGAATCAG	Colony PCR screening
L0326	<i>Actin2</i>	Forward	CTTGACCAAGCAGCATGAA	qPCR Housekeeping gene

L0327	<i>Actin2</i>	Reverse	CCGATCCAGACACTGTACTTCCTT	qPCR Housekeeping gene
L0330	<i>PR1</i>	Forward	AGGCACGAGGAGCGGTAGG	qPCR <i>PR1</i> expression analysis
L0331	<i>PR1</i>	Reverse	CATGTTACGGCGGAGACG	qPCR <i>PR1</i> expression analysis
L0491	<i>RUBY</i>	Forward	CGCAACTACACCAAGCTGCCGA	qPCR <i>RUBY</i> expression analysis
L0492	<i>RUBY</i>	Reverse	CTGAGCCACTGCACGAACTCGG	qPCR <i>RUBY</i> expression analysis
L0493	<i>RUBY</i>	Forward	TCATGCTCGCCACACTCCTCCA	qPCR <i>RUBY</i> expression analysis
L0494	<i>RUBY</i>	Reverse	GTAGCGCGGAATCGGGATGAGC	qPCR <i>RUBY</i> expression analysis
L0512	TDNA intergrate	Forward	ACACTCTTTCCCTACACGACGCTTCCGA TCT*TCACGACGTTGTAAAACGACGGC	TagIM primer to check where TDNA intergrate
N701	Nextera_index		CAAGCAGAAGACGGCATACGAGATTCGCC T TAGTCTCGTGGGCTCGG	Sequencing library preparation
N702	Nextera_index		CAAGCAGAAGACGGCATACGAGATCTAGT ACGGTCTCGTGGGCTCGG	Sequencing library preparation
L0429	Barcode		AATGATACGGCGACCACCGAGATCTACAC GGCTCTGA ACACTCTTTCCCTACACGACGC	Sequencing library preparation
L0430	Barcode		AATGATACGGCGACCACCGAGATCTACAC CCTATCCT ACACTCTTTCCCTACACGACGC	Sequencing library preparation
L0431	Barcode		AATGATACGGCGACCACCGAGATCTACAC ATAGAGGC ACACTCTTTCCCTACACGACGC	Sequencing library preparation
L0432	Barcode		AATGATACGGCGACCACCGAGATCTACAC TATAGCCT ACACTCTTTCCCTACACGACGC	Tegmentation based sequence library preparation
L0361	Tn5ME_A	reverse	TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAG	Tegmentation based sequence library preparation
L0360	Tn5ME_B	reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAG	Tegmentation based sequence library preparation
L0026	Tn5ME	reverse	5'-phosphorylated CTGTCTCTTATACACATCT	Tegmentation based sequence library preparation

Appendix Table A2. Stock solutions used in this study.

Stock solution	Concentration	Preparation	Storage
EDTA	0.5 M	18.61 g disodium EDTA dihydrate dissolved in autoclaved Milli-Q water; pH adjusted to 8.0 with NaOH; volume made up to 100 mL; filter-sterilized (0.2 μ m)	Room temperature
MES	1 M	19.52 g MES dissolved in autoclaved Milli-Q water; volume made up to 100 mL; filter-sterilized (0.2 μ m)	Room temperature
Glucose	1 M	9.0 g glucose dissolved in sterile water; volume made up to 50 mL; filter-sterilized	4°C
Tris-Cl	1 M	6.057 g Tris dissolved in sterile water; pH adjusted with concentrated HCl; volume made up to 50 mL	Room temperature
NaOH	10 N	20 g NaOH dissolved in sterile water; volume made up to 50 mL	Room temperature
Potassium acetate	5 M	49.071 g potassium acetate dissolved in sterile water; volume made up to 100 mL	4°C
Acetic acid	3 M	Prepared from glacial acetic acid	Room temperature
PIPES	0.5 M, pH 6.7	Prepared in sterile water and adjusted to pH 6.7	Room temperature
Ampicillin	100 mg/mL	Prepared as antibiotic stock solution	Not specified
Spectinomycin	100 mg/mL	5 g dissolved in 50 mL sterile Milli-Q water; filter-sterilized (0.22 μ m); aliquoted	-20°C
Kanamycin	100 mg/mL	5 g dissolved in sterile Milli-Q water; filter-sterilized (0.22 μ m); aliquoted	-20°C
Gentamicin	50 mg/mL	0.5 g dissolved in 10 mL sterile Milli-Q water; filter-sterilized (0.22 μ m); aliquoted	-20°C
Rifampicin	25 mg/mL	0.25 g dissolved in 10 mL DMSO; vortexed; filter-sterilized (0.22 μ m); aliquoted in light-protected tubes	-20°C

Table A3: Buffers and solutions used in this study.

Buffer / solution	Composition	Preparation	Storage
DNA/RNA lysis buffer	100 mM Tris-HCl, pH 8.0; 100 mM NaCl; 10 mM EDTA; 1.5% SDS; 2% β -mercaptoethanol	Components dissolved in Milli-Q water; Proteinase K (100 μ g/mL) added immediately before use	Room temperature
Guanidine HCl RNA extraction buffer	8 M Guanidine HCl; 20 mM MES; 20 mM EDTA	76.4 g Guanidine HCl, 2 mL 1 M MES, and 4 mL 0.5 M EDTA; volume made up to 100 mL with DEPC-treated water	Room temperature
Inoue transformation buffer	55 mM MnCl ₂ ·4H ₂ O; 15 mM CaCl ₂ ·2H ₂ O; 250 mM KCl; 10 mM PIPES, pH 6.7	Components dissolved in water; volume made up to 1 L; filter-sterilized (0.45 μ m); aliquoted	-20°C
Alkaline lysis Solution I	50 mM glucose; 25 mM Tris-Cl, pH adjusted; 5 mM EDTA	5 mL 1 M glucose, 2.5 mL 1 M Tris-Cl, and 1 mL 0.5 M EDTA; volume made up to 100 mL with water; autoclaved	Room temperature
Alkaline lysis Solution II	0.2 N NaOH; 0.1% SDS	200 μ L 10 N NaOH and 1 mL 1% SDS; volume made up to 10 mL with water; prepared immediately before use	Freshly prepared
Alkaline lysis Solution III	3 M potassium acetate with acetic acid	60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid, and 28.5 mL water; volume 100 mL	Room temperature
TE buffer	10 mM Tris-HCl; 1 mM EDTA	Prepared using Tris-HCl and EDTA stocks; RNase A added when required	Room temperature

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