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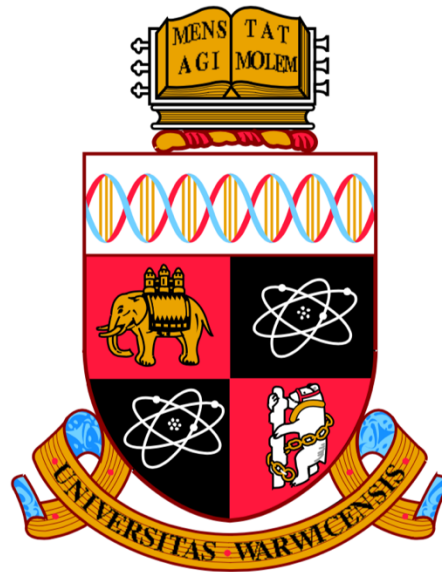
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**Understanding the roles of beneficial microbe
effectors in plant growth and stress resistance**

by

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Thesis

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for the degree of

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Abbreviations

| | |
|-----------------------|--|
| aa | Amino acid |
| ABA | Abscisic acid |
| <i>A. thaliana</i> | <i>Arabidopsis thaliana</i> |
| cDNA | Complementary Deoxyribo- Nucleic Acid. |
| CIM | Callus-inducing media |
| CT | Cycle threshold |
| dai | Days after inoculation |
| DNA | Deoxyribonucleic acid |
| <i>DREB2A</i> | <i>Dehydration-responsive element-binding protein 2A</i> |
| <i>ERF1</i> | <i>Ethylene response factor 1</i> |
| ETI | Effector-triggered immunity |
| <i>F. graminearum</i> | <i>Fusarium graminearum</i> |
| FHB | Fusarium head blight |
| <i>FLS2</i> | <i>FLAGELLIN SENSITIVE2</i> |
| <i>FRK1</i> | <i>FLG22-INDUCED RECEPTOR-LIKE KINASE 1</i> |
| GFP | <i>GREEN FLUORESCENT PROTEIN</i> |
| GUS | β GLUCURONIDASE |
| H | Hygromycin |
| HIGS | Host-induced gene silencing |
| LUC | Luciferase |
| M | Maltose |
| MAMPs | Microbe-associated molecular patterns |
| n | Number of scutella |
| NHL10 | <i>NDR1/HIN1-like 10</i> |
| <i>PHI-1</i> | <i>PHOSPHATE-INDUCIBLE1</i> |
| PRR | Pattern-recognition receptors |
| PTI | Pattern-triggered immunity |
| <i>PvUbi1</i> | <i>Polyubiquitin 1 promoter</i> |
| <i>PvUbi2</i> | <i>Polyubiquitin 2 promoter</i> |
| qRT-PCR | Quantitative reverse transcription-polymerase chain reaction |
| RFP | Red fluorescent protein |
| RIM | Root inducing media |
| RNA | Ribonucleic acid |
| SIE | <i>Serendipita indica</i> effector |
| <i>S. indica</i> | <i>Serendipita indica</i> |
| SMART | Simple Architecture Research Tool |
| STARTS | Stable root transformation system |
| VIGS | Virus induced gene silencing |
| WGA | Wheat germ agglutinin |
| Y2H | Yeast 2 hybrid |
| <i>ZmUbi1</i> | <i>Maize ubiquitin 1 promoter</i> |

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Declarations

I, Emeka Chibuzor Okechukwu hereby confirm that this thesis was written in accordance with the regulations of the university for the degree of Doctor of Philosophy. This thesis was composed by me and has not been submitted in parts or full in any previous application for any degree. The work in this thesis was undertaken by me except the screening of *S. indica* effectors in protoplasts for abscisic and flg22 stress resistance, and transformation and generation of T2 *Arabidopsis thaliana* lines expressing the effectors that Dr Silke Lehmann performed.

Abstract

Beneficial microbes have a mostly untapped potential to serve as bio-fertilizers and soil remediators in enhancing crop growth, yield and stress resistance. Understanding the molecular mechanisms of how microbes activate benefits in plants will be crucial for its application in larger-scale crop production systems. It is postulated that beneficial microbes could employ small secreted proteins (termed effectors) to reprogram and facilitate the transmission of those positive benefits in host plants. Recent studies have attempted to investigate the functions of ‘beneficial’ effectors in crop improvement. *Serendipita indica*, which is the beneficial fungus studied in the project, was used as a model to study the function of its effectors and reveal their beneficial activity in improving plant growth, abiotic and biotic stress resistance. The aim of this project was to investigate the roles of a selected set of *S. indica* effectors in single cell, whole plant and crop-based systems. *In-planta* analyses of *S. indica* effectors showed that some effectors such as *SIE44*, *SIE76*, *SIE106* and *SIE120* could enhance plant growth. In addition, *SIE67* could be a candidate in improving plant stress resistance. In support of this, a conserved protein domain known to be important in stress resistance was identified in *SIE67*. In turn, *SIE10* was identified as a potential effector that facilitates *S. indica* host colonization. To further investigate the roles of the effectors in a crop-based system, a barley transformation system was optimised to allow rapid functional analyses. This system was used to scrutinise effector functions in crucial crop improvement parameters such as abiotic and biotic stress resistance. While further confirmatory studies are needed, this research showed a first holistic functional view of effectors from beneficial microbes that could be important in sustaining crop productivity.

1 Introduction

1.1 Biotic/abiotic stress, a global concern for sustaining food security

1.1.1 Why global food security is increasingly under threat?

A food source is one of the essential needs of every living thing. All the food we eat comes directly or indirectly from plants. Food insecurity is the major challenge that humankind faces at present, which will further worsen with the climate change (Myers et al., 2017; Chakraborty and Newton, 2011). Food security can be defined as a state whereby all people at all times, have unrestricted physical and economic access to sufficient, safe, and nutritious food to meet their dietary needs and food preferences for an active and healthy life (FAO, 1996). From the inception, agricultural crop production has been the main contributor to global food security. We need agriculture to provide us with all necessary nutrients, economically and socially accepted foods, fibres and other needed food products (SDSN, 2013). Improvement of agricultural techniques was very important in the survival and rise of the human population in the early period of human civilization reviewed in Diamond (2005). Significant progress in increasing food production over the past 100 years and making food relatively affordable for the vast majority of the world was achieved by agricultural communities (SDSN, 2013). In addition, the green revolution era pioneered the period of massive technological and innovative investments in agriculture that resulted in unparalleled agricultural yield increases that preserved about 18-27 million hectares of natural ecosystems from being used for agriculture (Stevenson et al. 2013). For instance, world production of key cereal crops such as rice, wheat and maize has approximately increased by 300 % since 1960, resulting in lowering of food prices (Godfray et al., 2010). It was also reported that some progress has been made in tackling global hunger by decreasing it from almost 1 billion people in 1990-1992 (18.6% of world population) to about 870 million in 2010-2012 (12.5%) (FAO-WFP-IFAD, 2012).

Despite the progress made in ensuring food security, positive impacts of reducing hunger differed significantly by region and the global efforts of tackling hunger had slowed since 2007 (SDSN, 2013). Consequently, more than 8,000 children are dying every day directly and indirectly from malnutrition (people from South and Southeast Asia, and Sub-Saharan Africa where about 75 % of the poor live in rural areas are the most affected) despite the massive

global advancement in wealth, innovation and technology (SDSN, 2013). **Figure 1.1** shows that about 868 million are seriously malnourished because of hunger between 2010-2012, with the highest proportion from Southern Asia (304 million) due to their explosive increase in population. In Sub-Saharan Africa about 234 million people suffer from malnourishment mainly because of extremity of abiotic stresses peculiar to the region, while other countries (mostly developed regions) have relatively low number of persons exposed to hunger. SDSN (2013) further emphasized that the sharp increase in the world population from 800 million at the beginning of the industrial revolution (1790) to over 7 billion at present, and with the estimation of approximately 9.3 billion in 2050, has triggered concerns on how to provide sufficient food for the world population in a sustainable fashion. As shown in **Figure 1.2**, the estimated increase in the world population to over 9 billion people by 2050 (34 % increase) will mostly come from developing countries.

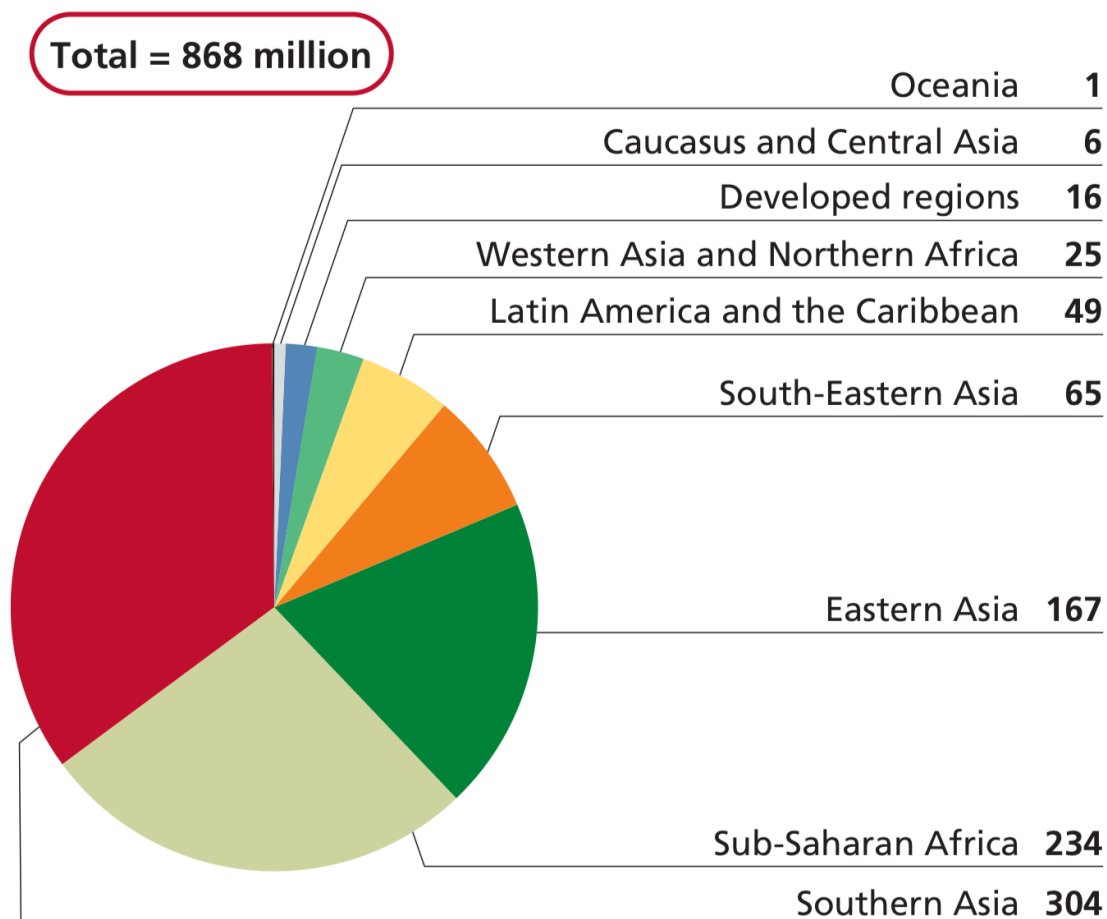


Figure 1.1: Global distribution of malnourished people in 2010-2012.

Source: FAO (2012). Numbers = millions of people.

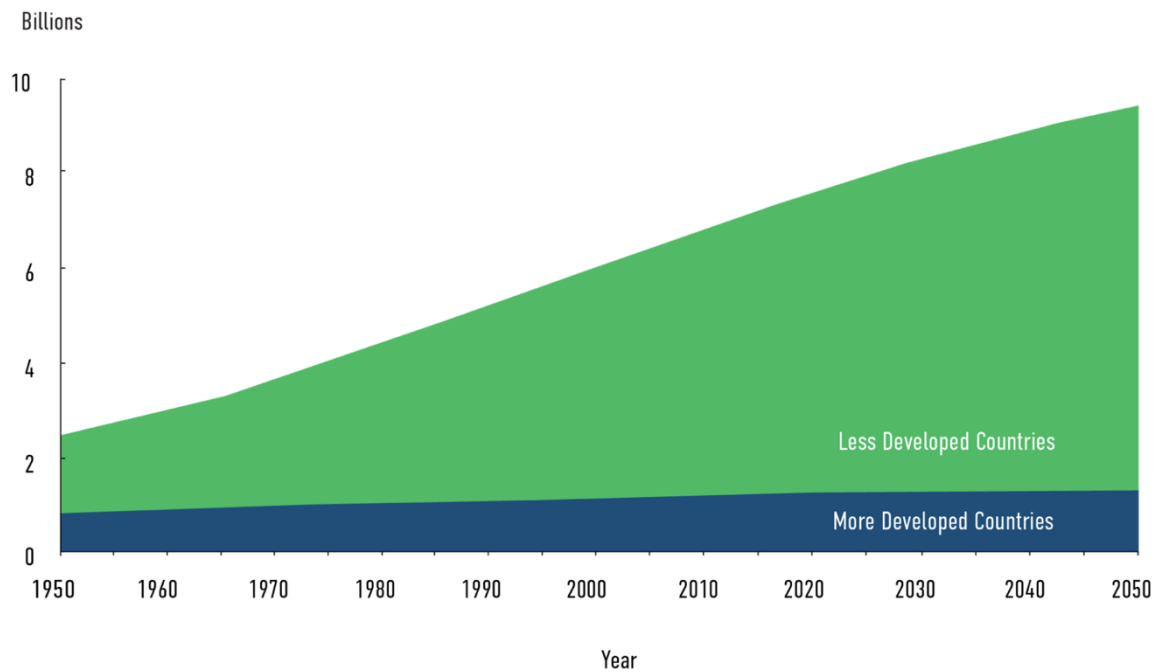


Figure 1.2: World population growth and predicted increases.

Source: United Nations Population Division (2012).

In order to sustain the world population in 2050, total food production will have to rise by 60–110 % (Pretty 2008; IAASTD 2008; Tilman et al. 2011; Ray et al. 2013; Pardey et al. 2014). There are many factors that challenge global efforts in achieving sustainable food security such as an increase in urbanization and industrialization which involves the conversion of cultivated lands and also rise in demand of crops especially cereals for animal nutrition and generation of biofuel (Murchie et al., 2009; Zhu et al., 2010). These conscious and other unconscious human activities have resulted in climate change. Climate change is evident in the forms of rising of global temperature, sea level, desertification, erosion, land degradation, storms as well as changing the amount and patterns of precipitation (incessant flooding and drought).

The impact of climate change can differ by regions, countries, season and time. It has significantly accelerated different kinds of abiotic stresses (e.g. salinity, drought, heat, flooding) and biotic stresses (pathogen and pest attack) resulting in global crop yield losses. This situation is likely to worsen with the implication that hunger, malnutrition, human diseases, death and poverty will continue to increase especially in developing countries that do not have sufficient technologies to manage the precarious incidents to a permissible level. Human activities are rapidly altering the viable environmental conditions, thereby making it harder to achieve global food security (Whitmee et al., 2015.). In addition, climate change poses a major long-term threat to food security and could render additional 600 million people

to undernourishment by 2080 (United Nations, 2015) if effective mitigating approaches are not employed globally.

1.1.2 General overview of impact of biotic and abiotic stresses on global crop productivity

As immobile organisms, plants are constantly challenged with different kinds and levels of biotic and abiotic stresses that severely reduce their growth, productivity and fitness (Walley et al., 2007; Rejeb et al., 2014, Shao et al., 2008). Biotic stresses are caused by living organisms mainly pathogens (viruses, bacteria, fungi, nematodes) and pests (such as insects, rodents, birds, and other animals) whereas abiotic stresses are caused by environmental threats such as soil salinity, low pH, drought, heat/cold stress, poor soil fertility and excess accumulation of heavy metals and other toxic compounds in the soil. Biotic and abiotic stresses can induce a mild, moderate or severe breakdown in plant metabolism (Heil and Bostock, 2002; Swarbrick et al., 2006; Bolton, 2009; Massad et al., 2012) that can result in up to 70% yield loss (Datta, 2004). FAO (2005) revealed that out of a US\$1.3 trillion global annual food production capacity, biotic stresses alone cause 31 to 42% loss (US\$500 billion) with an addition of 6 to 20% (US\$120 billion) due to pathogens and insects associated with post-harvest loss. Abiotic stresses can reduce economic yields by > 50% for most major plants with an estimated loss of billions of dollars annually (Wang et al. 2003; Pereira, 2016; Pareek et al., 2009).

Climate change has been predicted to continue to rise, consequently plants could be exposed to more severe stresses. The typical example of the global yield loss due to biotic and abiotic stresses are presented in **Figure 1.3**. It shows that proportion of yield loss due to abiotic stress was much higher than the proportions of average yield production (in 2002), production increment from 2002-2013 and the yield loss due to biotic stress combined together. This might also explain why more research attention has been given to confronting the impact of abiotic stresses on crops than biotic stress (**Figure 1.4**). It further shows that more research attentions should be channelled towards the identification of effective ways to recover global yield loss due to stresses and sustain food security.

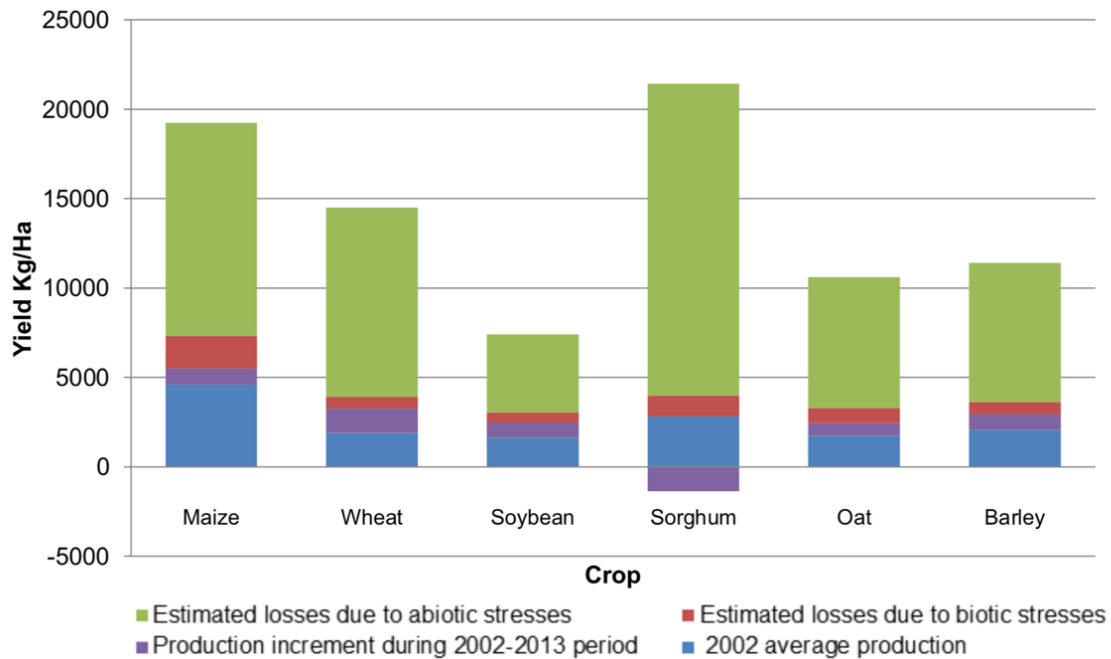


Figure 1.3: Increase in yield (kg/ha) and losses due to biotic and abiotic stresses of globally important crops from 2002-2013.

It was a global estimate. Biotic stresses include diseases, insects and weeds. Abiotic stresses include, but are not limited to drought, salinity, flooding and extreme temperatures. Source: Bray et al. (2002) and Jiménez-Arias et al. (2017).

In natural habitats, plants can experience single but more often combinations of stresses at the same time. Although the combined impact on plants depend mainly on the kinds of stresses involved, most of the time the outcome of the interaction is usually devastating for the plants. Plants try to overcome stress by activating stress adaptive responses to stop or circumvent the threat. They also employ their innate resilience to cope with stress to certain extent by specifically altering their morphology, physiology, biochemistry and molecular integrity, but often at the expense of their growth, development and yield (Herms and Mattson, 1992). Crop yield depends on outcome of growth/immunity trade-off (Coley et al., 1985; Simms and Rausher, 1987; Herms and Mattson, 1992). Understanding how plants respond to different stresses will be very crucial in modern plant improvement programmes (Dresselhaus and Hüchelhoven, 2018) for breeding for enhanced biotic and abiotic stress resistance in major global crops. In order to gain more insight into the impact of a single biotic and abiotic stress inducers, impact of *Fusarium graminearum* (biotic), a devastating fungus that attack cereals (mostly wheat and barley) and salt stress (abiotic) on global crop productivity will be specifically discussed here.

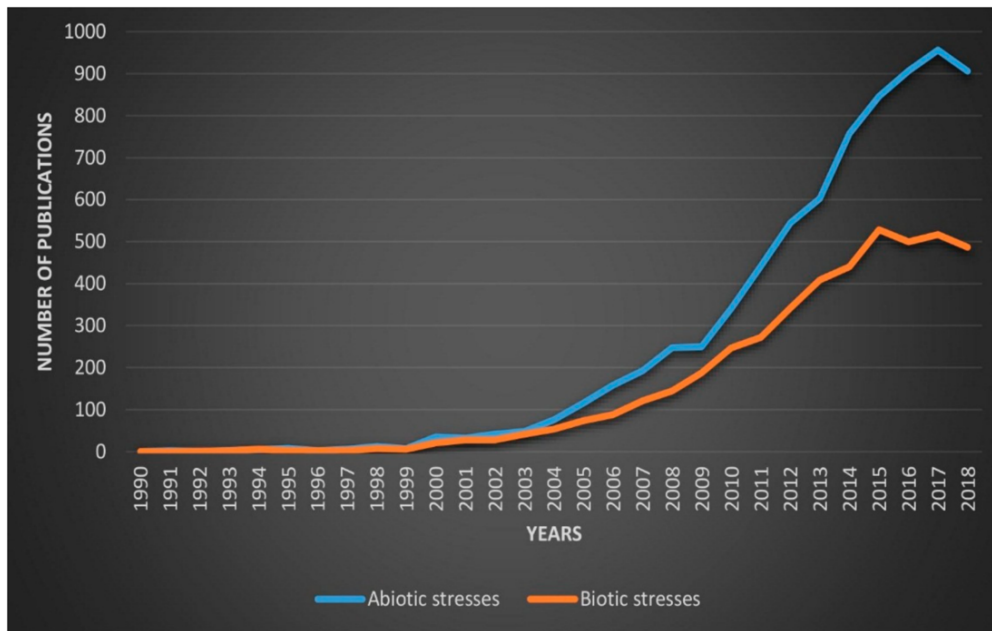


Figure 1.4: The number of publications per year related to abiotic and biotic stresses from Jan/1990–Nov/2018.

No overlapping publications. PubMed (Keywords: abiotic stresses, drought, cold, heat, salinity and water-logging or biotic stresses, bacteria, virus, fungi, insects, parasites, and weeds) was used to determine the number of publications in 1990-2018. Source: Raza et al. (2019).

1.1.3. Impact of *Fusarium graminearum* and salt stresses on global crop productivity

One of the most devastating fungal pathogens that causes biotic stress in cereals is *Fusarium graminearum*. It attacks mostly barley and wheat causing economically important diseases such as root, crown, floral and seed rot, and Fusarium head blight (FHB) (Machado et al., 2018; Taheri, 2018). **Figure 1.5** further showed Fusarium head blight (FHB) disease symptoms on wheat crop. The fungal disease was first identified and described in 1884 in England, but was recognized a main problem to wheat and barley productivity during the early years of the twentieth century (Muriuki, 2001; Stack, 1999, 2003). Following host plant infection, *F. graminearum* also destroys and contaminates the seeds with different kinds of mycotoxins (such as deoxynivalenol (DON) and zearalenone). These toxins can harm human and animal immune, gastrointestinal and reproductive systems if those contaminated seeds are consumed (Goswami and Kistler, 2004; Desjardins and Proctor, 2007). In addition, accumulation of the mycotoxins poses serious problems in malt production and brewing quality (Chehri and Godini, 2017). The incidences of *F. graminearum* diseases have increased recently due to global climate change and modifications in farming practices (Feng et al., 2018).

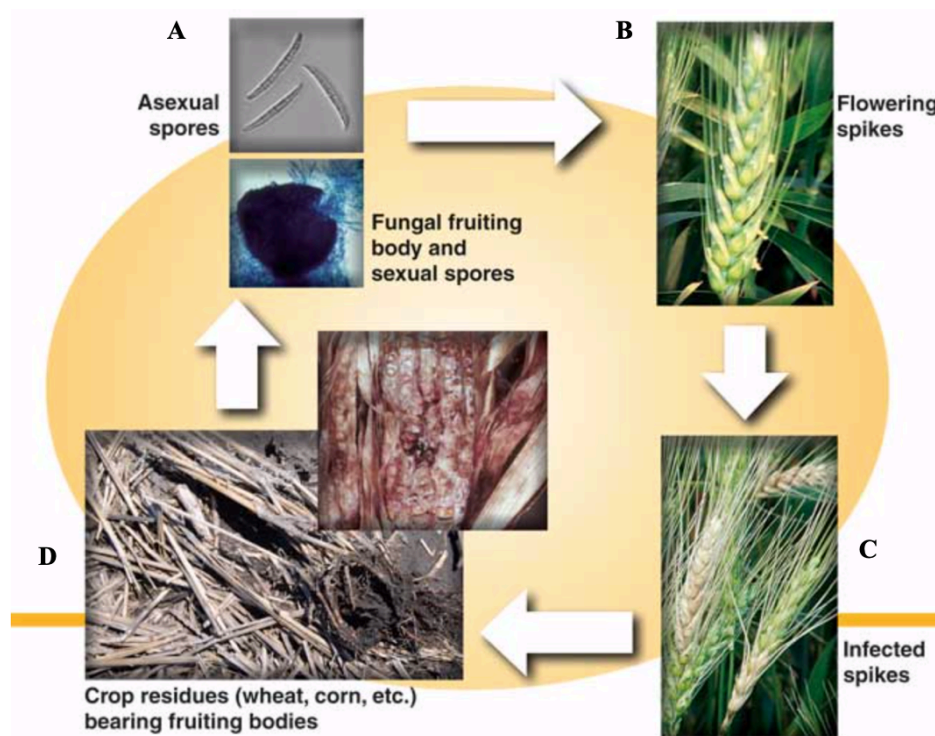


Figure 1.5: Life cycle of *Fusarium graminearum* pathogen on wheat crop.

A: Conidia (spores) from *Fusarium graminearum*. **B:** Spores in contact with the heads of the cereal crop. **C:** The head shows Fusarium head blight (FHB) disease symptoms. **D:** The crop residues harbouring the fruiting spores.

Source: Pessl Instruments (<http://docs.metos.at/Fusarium+Head+Blight+Biology>)

Continuous outbreaks of this fungal disease in major cereal-producing parts of the world have led to significant reduction in yield, seed quality and value of cereal crops with economic losses in the range of billions of US dollars (Dubin et al., 2002; Nganje et al., 2004). Nganje et al. (2002) determined that losses in the Northern Great Plains and Central United States equal approximately \$2.7 billion from 1998 to 2000.

The fungus exhibits a necrotrophic mode of action (Quarantin et al., 2019); it kills the living cells of its host and then feeds on them. Disease infection begins when the fungal spores (either asexual conidia or sexually developed ascospores) come in contact with the surface of host plant tissues (for instance florets or seedlings) (Zhang et al., 2012). The spores germinate and form germ tubes, which further spread and develop into hyphae (Zhang et al., 2012). Hyphae have the ability to directly penetrate the florets (Boenisch and Schäfer, 2011) or other plant tissues through natural openings (Bushnell et al., 2003) or wounds created by pests or winds

(Reid et al., 1994). As soon the fungus is inside the host, it grows within and between host cells prior to the formation of spores (Jansen et al., 2005; Rittenour and Harris, 2010). Disease symptoms gradually develop as the hyphal growth increases in the host plants (Zhang et al., 2012). There are several methods to control the fungal disease such as use of effective cultural methods (e.g. crop rotation, weeding, tilling) and use of fungicides (Parry et al., 1995). However, use of resistant varieties that limits the fungal development is the most effective method (Steiner et al., 2009; Son et al., 2013).

With respect to abiotic stress, salinity (salt stress) is one of the most harmful environmental stresses that severely limits crop growth and productivity in many parts of the world (Shrivastava and Kumar, 2015; Flowers, 2004). Soil salinity is the result of a high accumulation of soluble salts (such as NaCl) in the soil (Jouyban, 2012) and causes more than 50 % yield loss in all major crops (Bray et al., 2000). It negatively alters normal morphological, physiological, biochemical, and molecular processes thereby affecting seed germination, plant growth, as well as water and nutrient absorption (Akbarimoghaddam et al., 2011; Singh and Chatrath, 2001). The intake of high amounts of Na⁺ and Cl⁻ by roots also reduces metabolic processes and photosynthetic efficiency in plants (Flowers and Yeo, 1995; Tester and Davenport, 2003) primarily by a severe decrease in leaf area/size, chlorophyll content and stomatal conductance, and to a lesser extent through a reduction in photosystem II efficiency (Netondo et al., 2004). In addition, salt stress interferes with reproductive development (such as flowers and seeds/fruits formation) by hindering microsporogenesis and stamen filament elongation, and through accelerating programmed cell death in some reproductive related tissues, leading to ovule abortion and abnormal senescence of fertilized embryos (Shrivastava and Kumar, 2015). High build-up of sodium ion in the cell wall can instantly trigger osmotic stress and cell death (Munns, 2002).

It is estimated that more than 20% of arable land is currently impoverished by salt stress (Shrivastava and Kumar, 2015), and this continues to increase on a daily basis (Gupta and Huang, 2014). Apart from natural causes of salinity such as high build-up of salts over extended periods of time especially in arid and semiarid areas (Rengasamy, 2002) and deposition of oceanic salts by wind and rain, human activities such as land clearing (Munns and Tester, 2008), and irrigation water that contains some amounts of sodium chloride (NaCl) contribute significantly to salinization of cultivated lands (Flowers and Yeo, 1995; Tester and Davenport, 2003). Annually, 27.3 billion US dollars are spent to ameliorate irrigation-triggered salinity

(Qadir et al., 2014). The impact of salt stress will continue to worsen with the progress of climate changes (Dahal et al., 2019; Rahmstorf, 2007; Dasgupta et al., 2009). It has been projected that by 2050, above 50% of the arable land will be salinized (Jamil et al., 2011). The continuous increase in salinization of agricultural land is a major threat to global food security (Oyiga et al., 2016). Witzel et al. (2014) stressed that with the challenge of sustaining food security for the growing world population, improving salt stress tolerance in major crops is of increasing global priority.

1.2 How do plants respond to biotic and abiotic stresses?

1.2.1 Plant-pathogen interactions

Plants are resistant to the majority of potential pathogens (Bigeard et al., 2015), and only some pathogens have the ability to infect plants, cause diseases or even kill plants as part of their strategy to access nutrients (e.g. carbohydrates) from plants (Kemen and Jones, 2012). Plants have pre-formed physical barriers (e.g. cuticle and cell wall), as well as the ability to produce antimicrobial compounds (such as phenols, alkaloids, tannins) for protection against invading pathogens (Bigeard et al., 2015). The cuticle is found on the outer surface of the leaf epidermis of all terrestrial plants and mostly consists of cutin and waxes (Yeats and Rose, 2013). Apart from its importance in regulating evapo-transpiration to protect plants against drought and water loss, the cuticle forms a physical barricade against plant pathogens and pests (Bigeard et al., 2015). However, most fungal pathogens have the ability to enter the cuticle by physical means and/or the secretion of cutinases that can hydrolyse cutin (Mendgen et al., 1996; Longhi and Cambillau, 1999). Plant cell walls are composed of cellulose microfibrils, pectin, hemicelluloses, proteins, and other polymers such as lignin, suberin, and cutin in some cases (Somerville et al., 2004). These components give cell walls a tough, flexible and rigid identity and provides both structural strength and protection against pathogens and abiotic stresses (Hamann, 2012). Although some fungal pathogens can penetrate both plant cuticle and cell walls, bacteria have developed other strategies to enter host tissue (Bigeard et al., 2015). Some bacteria can enter through natural openings such as stomata, lenticels, hydathodes, and nectarhodes (Melotto et al., 2008) or through wounds in plant tissues created by pests, winds, rain storms, among others. Apart from the presence of those intricate physical barriers that can prevent pathogen invasion, plants produce antimicrobial compounds (such as phytoanticipins and phytoalexins) that hinder growth and proliferation of plant pathogens (Osbourn, 1996).

Also, several plant proteins, secondary metabolites and their derivatives can exhibit some level of antimicrobial activities against some pathogens (Tierens et al., 2001).

After physical barriers (cuticle and cell wall) and antimicrobial and secondary metabolites that plants produce for self-protection, host-adapted pathogens that can penetrate plant cells are challenged by a well-coordinated and sophisticated network of plant immunity signalling components (Bigeard et al., 2015). Entering plant cells is however essential for pathogens to access host nutrients, which they require for their growth and proliferation. Different from mammals, plants do not have an adaptive immune system or specialized immune cells to detect and eliminate pathogens (Hou et al., 2019). Nevertheless, plants have evolved a complex innate immune system capable of identifying all classes of pathogens (Chiang and Coaker, 2015). Plants have evolved pattern-recognition receptors (PRR) embedded in plasma membranes to recognize distinctive molecular signatures/patterns, so called microbe-associated molecular patterns (MAMPs) (Buscaill et al., 2019; Ausubel, 2005), that can be conserved among plant pathogens. PRRs are typically plasma membrane-associated receptor-like kinases (RLKs) or receptor-like proteins with extracellular domains allowing recognition of MAMPs (Böhm et al., 2014). Microbes usually depend on these molecular patterns for their adaptability and survival (Macho and Zipfel, 2014; Zipfel, 2014). Apart from MAMPs, plants can also detect damage-associated molecular patterns (DAMPs) that are of plant origin and released, for instance, by pathogen-induced cell degradation or are produced by plants in response to pathogen attacks such as endogenous peptides (e.g. AtPep1) (Kutschera et al., 2019; Boller and Felix, 2009). Detection of DAMPs also activates plant immunity (Yamaguchi and Huffaker, 2011). MAMPs include proteins (such as bacterial flagellin and elongation factor Tu), carbohydrates (such as fungal chitin) lipopolysaccharides and peptidoglycans (Boller and Felix, 2009; Felix et al., 1999; Kunze et al., 2004; Albert, 2013).

To date, a few PRR/MAMP interactive partners have been identified. For instance, *Arabidopsis* FLAGELLIN SENSITIVE2 (FLS2), a protein kinase receptor, recognizes the bacterial epitope flg22 (a stretch of 22 amino acid) embedded in the N terminus of flagellin of gram-negative bacteria (Buscaill et al., 2019; Zipfel et al., 2004). The *Arabidopsis* EF-Tu receptor (EFR) has the ability to detect an 18 amino acid epitope of elongation factor Tu (elf18) from *Escherichia coli* (Zipfel et al., 2006). The MAMP chitin, which is an important component of fungal cell walls (Silipo et al., 2010), can be detected by CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) and LYSM-CONTAINING RECEPTOR-LIKE KINASE 5 (LYK5) (Miya et al.,

2007; Wan et al., 2008; Cao et al., 2014). Plasma membrane-bound receptor-like kinases (RLK) PEPR1 and PEPR2 have been identified as the receptor of DAMP AtPEPs including Pep1 (Yamaguchi et al., 2006).

The recognition of MAMPs triggers the first line of immunity in plants called pattern-triggered immunity (PTI) (Kutschera et al., 2019). This happens when PRRs perceive MAMPs/DAMPs, which results in the downstream activation of immunity pathways that include a rapid cytosolic increase in Ca^{2+} and reactive oxygen species (ROS), induction of Ca^{2+} -related protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs), generation of phytohormones, transcriptional induction of defence related genes and reprogramming of metabolic processes (Boller and Felix, 2009; Macho and Zipfel, 2014). Induction of PTI inhibits pathogen colonisation of host cells. Once this first layer of immunity is active, pathogens cannot invade host plants. Therefore, adapted pathogens secrete effectors to suppress or reduce PTI response to enable pathogen establishment and invasion (Jones & Dangl, 2006, Lindeberg, 2012). Plants that are not capable of detecting effectors or counteract their activities become susceptible to the pathogen; a phenomenon called effector-triggered susceptibility (ETS). Those plants that can recognize effectors through cognate resistance (R) proteins will switch on the second tier of immunity called effector-triggered immunity (ETI). For instance, *AvrRpt2* and *AvrRpm1* from the bacterial pathogen *Pseudomonas syringae* are detected by Arabidopsis R proteins, RPS2 and RPM1, respectively (Bent et al., 1994; Mindrinos et al., 1994; Grant et al, 1995). Generally, ETI is associated with stronger and faster immune responses, typically including programmed cell death of plant cells at the infection site, often referred to as hypersensitive response (HR) (Mur et al., 2008; Coll et al., 2011). With this HR established, plants stop translocation of nutrients to the infection site to starve and limit pathogens further proliferation. In summary, the two tiers of plant immunity are presented in **Figure 1.6**.

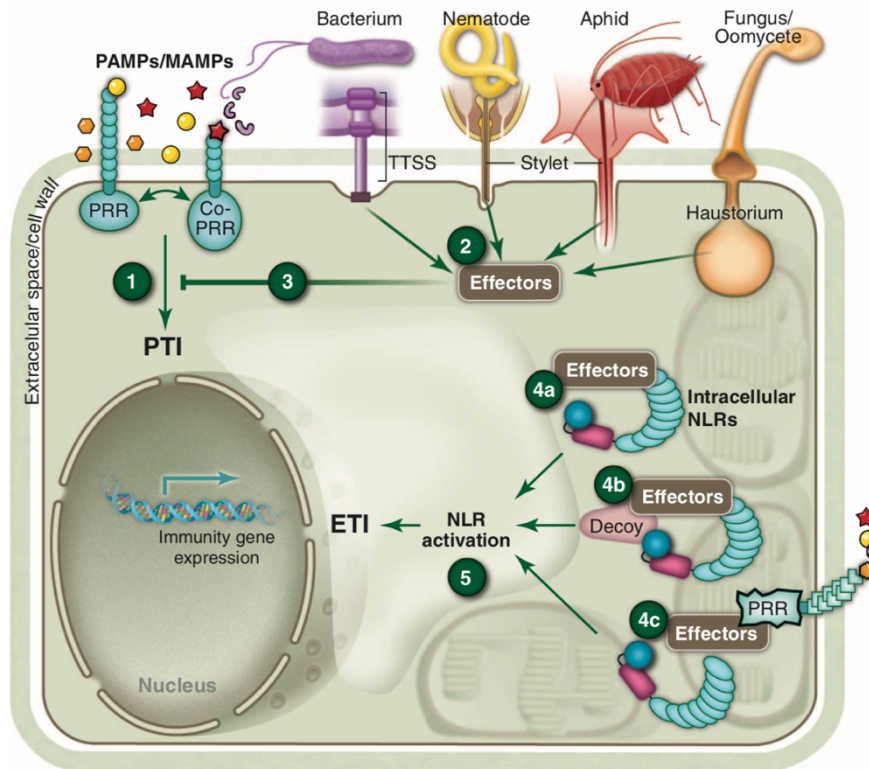


Figure 1.6: Schematic diagram of the plant immune system.

The pathogens (bacterium, nematode, aphids, and fungus) and their respective PAMPs/MAMPs (pathogen/microbe associated molecular patterns) are colour-coded accordingly. Plants recognize these MAMPs through extracellular PRRs (Pattern-Recognition Receptors), this recognition triggers the first tier of immunity, PTI (Pattern Triggered Immunity) (1). Host adapted pathogens deliver the effectors (2). These effectors are delivered to suppress the host PTI and aid pathogen invasion (3). Host cognate resistance (R) proteins that has intracellular NLR (nucleotide binding leucine-rich repeat) domain can perceive effectors in three main ways: through R-protein interacting directly with the virulence effector (4a), by recognizing effector-induced modulation of host decoy proteins (4b); and third, perception of effector-induced modulations of the host target protein, e.g the cytosolic domain of PRR (4c) (Dangl et al., 2013).

1.2.2 How do plants cope with salt stress?

For salt stress, osmotic stress is the first indication for plants, which is accompanied by inhibition of the growth of young leaves (Horie et al., 2012; Munns and Tester, 2008). Ion toxicity occurs later when the plant cannot sustain ion homeostasis (Munns and Tester, 2008), which is manifested in abnormal and rapid senescence of mature leaves (Munns and Tester, 2008). Occurrence of osmotic stress and ion toxicity can stimulate oxidative and other secondary stresses. Plants differ in their ability to tolerate salt stress. For instance, rice is the most sensitive among cereals, bread wheat is moderate while barley is the most tolerant (Munns

and Tester, 2008). Plants try to cope with salt stress in three distinctive ways: osmotic stress tolerance, Na⁺ or Cl⁻ exclusion, and by activating the innate ability (salt tolerant genes) to tolerate higher Na⁺ or Cl⁻ levels (Munns and Tester, 2008). Muchate et al. (2016) summarized that plants respond to salt stress by the activation of salt stress responsive genes, the induction of plant hormones (such as abscisic acid, jasmonic acid, ethylene) as well as an increase in antioxidant activity, Ca²⁺ signalling, reactive oxygen species scavenging, osmolytes accumulation and ion homeostasis. These programmed alterations enable plants to cope with salt stress; however, if the salt concentration is more than the tolerable level, plants will be overpowered.

Currently, there are two main cropping methods in use to reduce the impact of salt stress: ameliorating the soil by chemical or physical methods, and cultivation of salt-tolerant crop varieties (Glenn et al., 1999). The chemical methods include leaching the soil with low-salt water, addition of gypsum, acid-containing fertilizers (ammonium sulphate, anhydrous ammonia, and liquid ammonia), while the physical methods comprise proper drainage construction, proper soil tillage, reducing evaporation using mulches, use of organic manures such as farm yard manures, green manure, and mixed cropping. Use of salt tolerant crops that can withstand the negative impacts of salt stress are in use. A combination of these methods is important in overcoming salt stress.

1.3 Exploiting *Serendipita indica* (a beneficial microbe) for elevating yield and stress resistance

Many studies have shown that plant-microbe interactions in native ecosystems affect plant overall performance (Ganley et al., 2004; Van der Heijden et al., 2006). Beneficial microbes provide a wide range of vital functions to support plant development by improving nutrient cycling, bioremediation, mineralization of soil organic matter in addition to enhancing biotic and abiotic stress resistance (Singh et al., 2011; Zolla et al., 2013). Significant attention has been given to beneficial microbes in boosting crop productivity because of their unique ability to confer beneficial functions in host plants (Bhattacharyya et al., 2016; Singh et al., 2011; Khalid et al., 2009). *Serendipita indica* (formerly known as *Piriformospora indica*) is a filamentous root endophytic fungus that belongs to the phylum Hymenomycetes and the order Sebaciales (Basidiomycota) (Waller et al., 2005; Nizam et al., 2019). It was first discovered in Indian Thar desert in northwest Rajasthan (Verma et al., 1998). The fungus colonizes root

epidermal and cortex cells without entering the central cylinder (stele) and shows two colonisation phases (Deshmukh et al., 2006; Lahrman et al., 2013; Zuccaro et al., 2011; Qiang et al., 2012). In the first, biotrophic phase the fungus colonises living root cells (Schäfer and Kogel, 2009; Jacobs et al., 2011). Finally, it switches to a programmed cell death phase without causing tissue necrosis (Schäfer and Kogel, 2009; Deshmukh et al., 2006; Lahrman et al., 2013; Qiang et al., 2012) as illustrated in **Figure 1.7**.

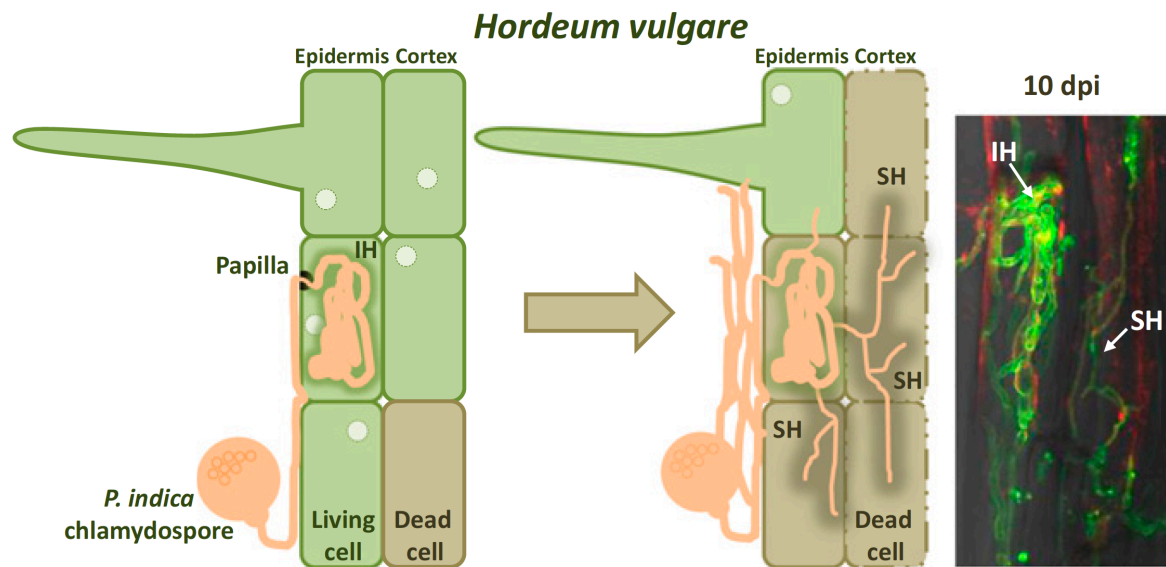


Figure 1.7: Schematic diagram of *S. indica* colonization strategies in root of barley crop (*Hordeum vulgare*).

Invasive hyphae (IH) and secondary thin hyphae (SH) of *S. indica* in dead cells of barley roots 10 days post-inoculation (dpi). The fungal structures were stained with WGA-AF488 (green) while the plant cell membranes were stained with FM4-64 (red). The fungal structures were detected also in dead host cortical cells. Source: Lahrman et al. (2013).

The fungus can colonize roots of virtually all plants. Thus, it exhibits no sign of host specificity, which makes the fungus ideal for a wide range of applications in biotechnology and crop production (Franken, 2012; Qiang et al., 2012). *S. indica* can be cultured on a variety of synthetic media such as Hill and Kaefer, potato dextrose, yeast Malt Pepton agar (YMP) and Murashige and Skoog (MS) (Attri and Varma, 2018). This amenability makes it very suitable to be used as biofertilizers in commercial production (Varma et al., 1999). *S. indica* confers versatile benefits to host plants such as increased growth, yield and stress resistance (Schäfer et al. 2009; Unnikumar et al., 2013; Deshmukh et al., 2006, Akum et al., 2015), which indicates the fungus as a potential bio-tool for biotechnology and bio-engineering (Oelmüller et al.,

2009). Understanding the molecular mechanisms on how this beneficial microbe activates these benefits in plants will be crucial for its application in crops beside using it as biofertilizer and bio-remediator in large-scale crop production systems. Knowing how plant pathogens overcome host immunity has accelerated development of possible ways (such as host induced gene silencing, virus induced gene) to control plant diseases (Qi et al., 2019; Koch and Kogel, 2014; Kant and Dasgupta, 2019). In turn, we still have often a limited understanding on how beneficial microbes activate benefits in their host plants which hinders their exploitation in terms of elevating crop productivity and stress resistance.

Recently, it has been shown that effector proteins are not solely secreted by pathogens, but by beneficial microbes as well (Rovenich et al., 2014). A few effectors from other beneficial microbes (e.g. mycorrhizal fungi), rhizobia (legume-nodulating bacteria) have been identified to play vital roles in plant colonization (Plett et al., 2014; Klopffholz et al. 2011; Tsuzuki et al., 2016; Miwa and Okazaki, 2017; Kambara et al., 2009). In the same vein, *Serendipita indica* has also been confirmed to secrete effectors used for reprogramming host plant cells (Nizam et al., 2019; Akum et al., 2015). It is postulated that *S. indica* (including other beneficial microbes) could use effectors to re-wire host cell signalling to facilitate the transmission of benefits to colonized plants. Attempts have been made to understand the functions of *S. indica* effectors in host plants and Akum et al. (2015) reported the function of *S. indica* effector PIIN_08944 in the colonization of Arabidopsis roots. However, the roles of *S. indica* effectors in activating beneficial effects in host plants remain unknown. This project aimed to fill this gap of knowledge. Understanding the function of *S. indica* effectors could help in identifying new plant components and pathways involved in growth regulation as well as plant resilience against abiotic and biotic stresses. This dearth of information motivated this PhD research to investigate the potentials *S. indica* effectors can offer in crop improvement.

1.4 Aims of the research

Investigating the function of *S. indica* effectors could open up new opportunities to engineer crops with improved yield and stress resilience. This requires adequate analyses of *S. indica* effector functions *in-planta*. Therefore, the broad aim of this study was to analyse the function of 150 *S. indica* effectors and reveal to what extent these effectors mediate beneficial activities reported for *S. indica*-colonised plants. To do this, the roles of *S. indica* effectors in plant growth and stress (biotic and abiotic) resistance were investigated in a single cell and in a whole

plant approach. The effectors were transiently expressed in leaf protoplasts from *Arabidopsis thaliana* for the single cell studies, while for the whole plant studies, the candidate effectors (selected from the single cell studies) were stably transformed into *A. thaliana* to generate stably transgenic lines. Chapter 1 presents findings of *S. indica* effector function in *Arabidopsis*. To further test effectors in a crop-based system, a barley scutella transformation system for rapid functional studies of effectors was optimized. This work and the outcome are described in Chapter 2. The roles of the effectors in biotic (*Fusarium graminearum*) and abiotic (salt stress) stress resilience were further investigated in barley and results are presented in Chapter 3.

2 Materials and Methods

2.1 Functional studies of the *S. indica* effectors in protoplasts

2.1.1 Plant and growth conditions

Arabidopsis wild-type line (accession Col-0) was used for the experiment. The seeds sown on damp compost/sand/vermiculite mix (6:1:1) and stratified in the dark at 4°C for 2-3 days. The seedlings were transplanted and grown in pots filled with compost/sand/vermiculite mix (6:1:1) for 4-5 weeks in growth compartments at 20-22°C under short-day light duration (10-12 h) and intensity ($(100 \mu\text{mol m}^{-2} \text{s}^{-1})$) with 40–65% relative humidity. The *A. thaliana* was also used to generate lines stably expressing the effectors.

2.1.2 Gene constructs used for protoplast transformation

The gene constructs used for protoplast transformation were:

1. The *luciferase* gene fused to the promoter of the PTI marker *PHI-1* (*pPHI1::LUC*) or to the promoter of ABA responsive marker *RD29A* (*pRD29A::LUC*).
2. *GUS* (β -*GLUCURONIDASE*) marker encoding glucuronidase fused to a plant constitutive promoter; *pUBQ10* (*pUBI10:: β -Gluc*).
3. *S. indica* effectors (SIEs) were fused to the *CaMV35S* promoter (*p35S::SIEs*) for overexpression of all effectors.

2.1.3 Protoplast isolation

The protoplast extraction was carried out as described by Yoo *et al.* (2007). Broad and healthy leaves were harvested (from 4-5 weeks old plants) and sliced into 1 mm strips using a scissor and blade. The sliced leaves were immediately transferred to 6 ml of enzyme solution (20 mM MES, pH 5.7, 0.4 M mannitol, 20 mM KCl, 1.5% (w/v) cellulose R10 (Duchefa Biochemie), and 0.4% (w/v) macerozyme R10 (Duchefa Biochemie) in a 5.5 cm petri dish. Vacuum infiltration (-0.8 bar) for 5 min was applied twice. The mix was incubated at 25°C for 3 h with occasional shaking. The protoplasts were filtered through a 70 μm nylon cell mesh into a 50 ml falcon tube and rinsed with the same volume of cold W5 buffer (2 mM MES, pH 5.7, 154

mM NaCl, 125 mM CaCl₂ and 5 mM KCl, autoclaved). The filtrate containing the protoplasts was centrifuged at 100 g for 2 min at 4°C and the supernatant was discarded. The protoplasts were re-suspended in the same cold W5 buffer. The number of protoplasts yielded was estimated using a Fuchs-Rosenthal haemocytometer and adjusted to a concentration of 300,000 protoplasts/ml using W5. Protoplasts were then centrifuged again for 1-2 min at 100 g and the supernatant was discarded. Protoplasts were diluted again to the required volume in MMG (4 mM MES, pH 5.7, 0.4 M mannitol and 15 mM MgCl₂).

2.1.4 Protoplast transformation

The protoplasts were transiently transformed as described by Yoo *et al.* (2007). For each well of a multiple plate (96 wells), 1 µg each of *pPHI1::LUC* or *pRD29A::LUC*, *pUBI10::β-Gluc* and *p35S::SIEs* were added in duplicate. For the control, empty vector (*p35S*) was used. 30 µl of protoplast solution (about 10000 cells) was added to each well. Thereafter, 33 µl of freshly prepared PEG solution (40% w/v PEG 4000, 0.2 M mannitol and 100 mM CaCl₂) was added to initiate protoplast transformation. The mix was shaken at 1000 rpm on an orbital shaker for 1 min and incubated for 15 min at room temperature. Thereafter, 170 µl of W5 was added and mixed by shaking for 1 min at 1000 rpm to stop the transformation processes. The transformed cells were centrifuged again at 100 g for 2 min and the supernatant was removed. 140 µl of W1 solution (4 mM MES, pH 5.7, 0.5 M mannitol and 20 mM KCl, autoclaved) was added under shaking at 1000 rpm for 1 min. The transformed protoplasts were incubated in a growth cabinet (22°C, 12 h light period) overnight.

2.1.5 Application of flg22/ABA treatment to the transformed protoplast

The overnight transformed protoplasts were spun down at 100 g for 2 min and 100 µl of the supernatant was removed. The protoplasts treated with flg22 / ABA at a final concentration of 100 nM and 10 µM, respectively. Water was used as a mock. The treated protoplasts were shaken on a shaker for 1 min and were incubated again in the same growth chamber for 5 h.

2.1.6 LUC and GUS detection

For LUC assays, white 96-well 0.5 ml polypropylene plates (Nunc™ #267350) were used. 10 µl of treated protoplasts were pipetted into each well. 20 µl of beetle luciferin luciferase substrate (Promega, #E1602) was then added quickly. Luminescence was captured after 4.5 h and 2h for flg22 (*pPHI-1::LUC*) and ABA (*pRD29A::LUC*) assays, respectively with a Photek

camera and analysed with Image32 software. To measure the GUS intensities. 100 µl of 1x lysis buffer (Luciferase Assay System, Promega # E1500) was added to the protoplasts and then the protoplasts shaken at 1000 rpm at room temperature for 30 sec and spun down for 2 min at 1000 g at room temperature. 10 µl of each lysate was pipetted into separate 96-well plates (transparent flat bottom). 100 µl of GUS substrate (1 mM 4-methylumbelliferyl-beta-d-glucuronide (MUG), 10 mM Tris-HCl pH 8, 2 mM MgCl₂) was added to each lysate. The mix was incubated at 37°C for 1 h after which GUS emission for each sample was quantified using a TECAN GENios microplate reader (excitation: 360 nm, emission: 465 nm)

2.1.7 Normalization of LUC data

Prior to the analyses of the luciferase emission, GUS data were used for LUC normalization to consider protoplast transformation efficiency. Firstly, the highest 10 GUS values were averaged to calculate the mean value of GUS for the normalization. Any sample (well) in each plate with GUS value less than the half of the mean of the 10 highest GUS values as a threshold were excluded from the analysis. The samples that met the threshold were normalized. Normalization was carried out using the following formula: average of the 10 highest GUS values divided by the GUS value of the individual well and then multiplied by the well corresponding luciferase value. The obtained values were used for the comparisons.

$$\text{Normalized LUC value for each well} = \frac{\text{Average of the 10 highest GUS x LUC values}}{\text{Individual actual GUS value}}$$

In order to identify the effectors that altered the protoplast response to stress, each SIE normalized LUC value was compared to that of the empty vector in mock, and stressed conditions (flg22 or ABA). The comparisons were carried out as follows; Ratio mock (SIEs luminescence/empty vector luminescence under mock- treated condition), ratio flg22/ABA (SIEs luminescence/empty vector luminescence under flg22/ABA- treated condition). Finally, multiplication of the two ratios were calculated as ratio mock x ratio flg22/ABA.

2.2 Functional analysis of *S. indica* effectors in a whole plant

2.2.1 Cloning of *S. indica* effectors into the vector

Selected candidate effectors from the protoplast were cloned into a Gateway[®]-compatible binary plasmid, pEarleyGate201, following Gateway[®] Technology methods (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/lr_clonaseii_man.pdf).

The plasmid (destination vector) used has selective markers for kanamycin resistance for selection of transformed bacteria and Basta resistance for selection of transformed plants. The effectors were sub-cloned into entry vector pDONR221 by GeneArt[™] (Thermo Fisher Scientific). The effectors that were cloned into pEarleyGate201 were driven by the cauliflower mosaic virus (CaMV) 35S promoter.

2.2.2 Transformation of effector constructs into *Agrobacterium tumefaciens*

The effector constructs generated above were transformed into the GV3101 strain of *Agrobacterium tumefaciens* with kanamycin for effector selection, and rifamycin and gentamycin for GV3101 selection. Colony PCR was used to identify effector-harboring colonies. These colonies identified were streaked on fresh agar containing the selective antibiotics and incubated in incubator (no shaking) at 28°C for two days. Single colonies of the transformed GV3101 were inoculated in 5 ml overnight culture in LB with selective antibiotics (kanamycin, rifamycin and gentamycin) in 50 ml falcon tubes in incubator (28°C, 220 rpm). The cells were harvested at 3000 g for 10 min at 4°C. The supernatant was discarded and the pellets were re-suspended in 7 ml of infiltration medium (5%, w/v of sucrose and 0.02%, v/v of Silwet-L-77). The culture was used for the transformation of *A. thaliana*.

2.2.3 Transformation of *Arabidopsis thaliana* plants

Arabidopsis wild-type (accession Col-0) seeds were germinated on agar media. 7-day old seedlings were transferred to soil in individual pots. All the plants in the pots were transferred to trays and grown under long day conditions until flowering. The first bolts that emerged (about 4-5 weeks old) were cut off with scissors. The plants were usually ready for transformation within 3-6 days of bolt cutting, when the secondary bolts are 1-5 cm. Sterile paint brushes were used to apply the bacterial suspension carrying the effectors onto the flowers and buds of the plants. The transformed plants were covered with film for two days to increase humidity. The cover was removed and the plants were left to grow and set seeds. The transformed (T1) plants were selected using Basta antibiotics. Three independent T1 plants

were selected for each effector to separately produce T2 seeds. T2 seeds expressing green fluorescent protein (GFP) as a control were also generated.

2.2.4 Screening of *Arabidopsis thaliana* lines expressing effectors for flg22 sensitivity

Pooled T2 seeds comprising three independent lines for each effector were initially screened. The individual lines of some identified effectors were thereafter tested. Sterilization of the seeds was carried out with the wet method. Seeds in 2 ml tubes were first washed with 1 ml of 0.02% tween H₂O by shaking and flipping by hand. The seeds were spun down in a centrifuge, then Tween H₂O was replaced with 1 ml of 70% ethanol, flipping by hand and spun down in centrifuge. The ethanol was replaced with 1 ml NaOCl (mixture of 15 ml of Domestos bleach + 10 ml sterile H₂O; ~ 3% NaOCl) and incubated for 5 mins with occasional flipping. It was then spun down again. The bleach was removed by washing with sterile H₂O and spinning down for 8 times. After removing the bleach, 0.2% gelrite was added to the tube until the seeds were entirely submerged. The seeds sown were stratified for 2 days at 4°C.

The lines expressing the effectors and GFP were sown on ATS medium in a single line (one seed per spot) with an average of 25 seeds per plate. The components of ATS for 1 litre media were 4.5 g gelrite (Wako Pure Chemical), 5 ml of 1 M KNO₃, 3 ml of 1 M MgSO₄, 3 ml of 1M Ca (NO₃)₂, 2.5 ml of 1 M KPO₄, 2.5 ml of 20 mM Fe-EDTA and 1 ml of micronutrients solution (70 mM H₃BO₃, 14 mM MnCl₂, 0.5 mM CuSO₄, 1 mM ZnSO₄, 0.2 mM Na₂ MoO₄, 10 mM NaCl and 0.01 mM CoCl₂). 6 plates per effector were sown; 3 plates were treated with either flg22 or H₂O (control). They were later transferred to growth cabinet at 22 °C under 24 h light and grown for 9 days. The plates containing the seedlings were treated with 1 ml of 1 µM flg22 or 1 ml of sterile water as a control. The application was carried out on the roots and then was evenly distributed on the plates by shaking uniformly in all directions. The plates were allowed to dry for 15 mins in laminar flow hood before they were moved back to the growth cabinet. The plates were scanned after 4 days and the root growth was measured with ImageJ 1.5 1F version. Root growth of the lines expressing the effectors was individually calculated relative to 100% of the control line (*35S::GFP/Col-O*) under mock and flg22-treated conditions. Also, root growth sensitivity to flg22 of the transgenic lines was calculated as the percentage reduction in root growth due to flg22 treatment. The percentage reduction in root length obtained for the control line was set to 0 % and the percentage reduction in root length for the lines expressing the effectors were calculated relative to the control line.

2.2.5 RNA extraction

11-day old *Arabidopsis* seedlings expressing effectors (e.g. identified in the flg22 assay) were treated with either 1 μ M flg22 or sterile H₂O as the control. Whole seedlings were harvested after 2 hr into 2 mL RNase free reaction tubes, frozen in liquid nitrogen and stored at -80°C until needed for RNA extraction. The plant tissues were kept in liquid nitrogen or -80°C at every stage to avoid RNA degradation. The frozen plant materials were quickly ground into fine form using a Tissue Lyser for 2 min. For each tube containing the frozen plant materials, 1 ml Trizol was ground material and vortexed thoroughly. 200 μ l chloroform was also added, vortexed for 15 sec and the samples were incubated on ice for 2-3 min. They were centrifuged for 20-30 min at 13,500 rpm (or full-speed) at 4°C and the supernatant (the genomic DNA-containing interphase was avoided) was carefully transferred into 1.5 ml RNase-free tubes. 500 μ l isopropanol was added to each tube and incubated over night at -20°C to increase precipitation of RNA. The precipitated RNA was harvested by centrifugation for 50 min at 13,500 rpm at 4°C. The supernatant was discarded and the RNA pellets were washed with 1 ml 75% ethanol (cold and prepared with nuclease free water). The ethanol was removed and the pellet dried for 5 mins on ice in the laminar flow hood. The pellets were resuspended in 10-30 μ l nuclease free water, vortexed and incubated at 35°C for 10 mins. RNA concentration of each sample was measured using NanoDrop.

2.2.6 cDNA synthesis

Firstly, genomic DNA was removed. For each sample, a reaction mix was made: 2 μ g RNA sample, 2 μ l 10x buffer supplemented with MgCl₂, 2 μ l DNase 1 (1 U/ μ l, Thermo Scientific), 0.5 μ l Ribolock RNase inhibitor (40 U/ μ l, Thermo Scientific) and nuclease-free water was added to make up the final volume 20 μ l. The reaction mix was incubated at 37°C for 30 mins in a thermocycler. 2 μ l of 50 mM EDTA (Thermo Scientific) was added and incubated further at 65°C for 10 min in a thermocycler to inactivate DNase enzyme. The concentration of the RNA was re-measured. The cDNA synthesis was carried out using Quanta Biosciences qScript™ cDNA Synthesis Kit (#95047-100). For each sample, a reaction mix was prepared: 1 μ g DNase- treated RNA, 4 μ l 5x qScript Reaction Mix, 1 μ l qScript reverse transcriptase and nuclease free water was added to make up the total volume to 20 μ l. For the cDNA synthesis, the reaction mix was run in a thermocycler for 5 mins at 25°C, 30 mins at 24°C, 4 mins at 85°C, then cooled down at 8°C. The cDNA synthesized was diluted to 5 ng/ μ l by adding 180

μl sterile H_2O . The integrity of the cDNA of each sample obtained was checked by PCR. A reaction mix for each sample was made: 2 μl of cDNA template, 17.9 μl , 2.5 μl 10x buffer A, 0.5 μl 10 mM dNTPs, 1 μl 10 μM *AtUBQ5* (forward primer), 1 μl 10 μM *AtUBQ5* (reverse primer), and 0.1 μl KAPA Taq DNA polymerase (KAPA Biosystems). The PCR was carried out as follows: 95°C for 3 min, 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, 72°C for 5 min, cooling down at 8°C and 24 cycles. 7 μl of the PCR product was loaded onto a 1.2% agarose gel and presence of expected *AtUBQ5* band size was checked for each sample.

2.2.7 qPCR analyses of immunity markers

qPCR was performed on three immunity induced markers, *FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)* with gene ID of AT5G52310, *PHOSPHATE-INDUCIBLE1 (PHI-1)* with gene ID of AT1G35140 and *NDRI/HINI-like 10 (NHL10)* with gene ID of AT2G35980 in the Arabidopsis lines expressing SIE and the control line (GFP) treated with flg22 or H_2O . The reaction mix per sample was: 8 μl SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich), 0.4 μl 10 μM forward primer, 0.4 μl 10 μM reverse primer, 2 μl DNA template, 9.2 μl MilliQ H_2O , was used. There were three technical replicates for each biological replicate and two biological replicates analysed. The PCR reaction was run in a thermocycler Agilent Technologies Stratagene Mx3005P. The PCR programme was : 5 minutes at 95°C, 40 cycles of (15 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C), followed by 1 min at 95°C, 10 sec at 55°C, and 10 sec at 95°C. Gene expression level of the immunity markers in each sample was normalised against the expression of the housekeeping gene (*AtUBQ5*) by calculating the average $2^{-\Delta\text{CT}}$ of the three technical replicates. ΔCT is the difference between cycle threshold (CT) value of the housekeeping gene and the corresponding CT value of the immunity marker (Schmittgen and Livak, 2008). The value obtained was used for comparing expression levels of the immunity markers in the lines expressing the effectors with the control line in mock conditions. For fold induction due to flg22 treatment relative to the respective mock of each sample, average $2^{-\Delta\Delta\text{CT}}$ was also calculated (Schmittgen and Livak, 2008). The primers used are presented in **Table 1**.

Table 1: List of primers used for the qPCR analysis of immunity response genes

| Name of primers | Sequence (5' to 3') |
|--------------------------|------------------------|
| <i>AtUBQ5</i> (forward) | CCAAGCCGAAGAAGATCAAG |
| <i>AtUBQ5</i> (reverse) | ATGACTCGCCATGAAAGTCC |
| <i>AtFRK1</i> (forward) | CGGTCAGATTTCAACAGTTGTC |
| <i>AtFRK1</i> (reverse) | AATAGCAGGTTGGCCTGTAATC |
| <i>AtPHI-1</i> (forward) | TTGGTTTAGACGGGATGGTG |
| <i>AtPHI-1</i> (reverse) | ACTCCAGTACAAGCCGATCC |
| <i>AtNHL10</i> (forward) | CTCATCTTCTGGCTCATCGTC |
| <i>AtNHL10</i> (reverse) | GTTACGGACAGGAACAGTGAG |

2.2.8 Screening of *Arabidopsis thaliana* lines expressing effectors for ABA sensitivity

Pooled T2 seeds were tested initially and the individual lines of the candidate effectors were further screened. Sterilization of the seeds was done using dry method. Seeds placed in 2 ml open tubes were put in a rack. A solution of 50 ml NaOCl (14% aqueous solution, 27900.365 VWR) and 3 ml concentrated HCl (32%) were prepared in a beaker. The seeds and mixed solution of NaOCl and HCl were placed in a desiccator in a fume hood. The desiccator lid was sealed with tape to ensure airtight. The seeds were incubated for 3 hours. After the sterilization, the tubes containing the seeds were removed from the desiccator. The tubes remained open to allow the chlorine gas to diffuse out in a sterile hood. Phyto agar (0.2%) was added to the seeds. The seeds were stratified for 2 days at 4 °C. The seeds were sown on ½ MS medium (2.151g/L MS basal salts, 0.5 g/L MES hydrate and 7 g/L phyto agar; pH adjusted to 5.7 – 5.8 with KOH). The plates were moved to growth cabinet at 22 °C under 24-h light for 7 days. Fourteen uniform growth seedlings were transferred to 0 µM (sterile H₂O) and 10 µM ABA containing ½ MS media. 6 plates per effectors were sown; 3 plates were treated with either ABA or H₂O (control). The plates were returned to the growth cabinet for another 7 days. The plates were then scanned and the primary root was measured with ImageJ 1.5 1F. Root growth of the lines expressing the effectors was individually calculated relative to 100% of the control line (*35S::GFP*) under mock and abscisic acid (ABA)-treated conditions. Root growth sensitivity to ABA of the transgenic lines was calculated as the percentage reduction in root growth due to ABA treatment. The percentage reduction in root length obtained for the control line was set

to 0 % and the percentage reduction in root length for the lines expressing the effectors were calculated relative to the control line.

2.2.9 Identification of putative protein domains in effector protein sequences

Simple Architecture Research Tool (SMART) web-based tool (<http://SMART.embl-heidelberg.de>) was used to search for known protein domains in the protein sequence of the effectors. The amino acid sequences of the effectors were used for the protein domain search.

2.2.10 qPCR analyses of ABA markers

11-day old Arabidopsis seedlings expressing effectors (some candidates identified in the ABA assay) were moved to either 50 or 0 μ M ABA containing media. Whole seedlings were harvested after 5 hr of stress. The qPCR analyses are the same as described for immunity markers in flg22 assay; the primers used are presented in Table 2.

Table 2: List of primers used for the qPCR analysis of ABA markers

| Name of primers | Sequence (5' to 3') |
|--------------------------|------------------------|
| <i>AtUBQ5</i> (forward) | CCAAGCCGAAGAAGATCAAG |
| <i>AtUBQ5</i> (reverse) | ATGACTCGCCATGAAAGTCC |
| <i>AtRAB18</i> (forward) | GGAACCGGGACCGGGACTGA |
| <i>AtRAB18</i> (reverse) | CCAGATCCGGAGCGGTGAAGC |
| <i>AtRD29A</i> (forward) | ACCAAAGAAGAACTGGAGGAG |
| <i>AtRD29A</i> (reverse) | TCTCTTTCAAATTGTCCTGGCT |
| <i>AtRD29B</i> (forward) | GCAAGCAGAAGAACCAATCA |
| <i>AtRD29B</i> (reverse) | CTTTGGATGCTCCCTTCTCA |

2.3 Optimization of barley scutella transformation system

2.3.1 Barley seed germination and growth

Spring barley seeds (Golden Promise) were germinated on a petri dish for 3 days before they were transplanted on growth media as follows: Firstly, the seeds were washed with 0.02% tween water, incubated in 70% ethanol for 5 min with occasional shaking and then incubated further in 60% sodium hypochlorite (12% active ingredient) for 1hr 30 min on an orbital shaker.

The seeds were washed 3 times with distilled water and incubated again in distilled water for 10 min on an orbital shaker. After sterilization, the seeds were carefully extracted from their husk/hull with sterilized tweezers. The seeds were then placed on a petri dish containing moistened filter paper. The seeds were incubated in the dark for 3 days to germinate. Watering was carried out when needed to avoid water stress. 6 seedlings were transplanted in each plastic pot (3 litre capacity) containing modular compost with Osmocote Exact fertilizer (N (16)- P (9)- K (12) + MgO (2)) and the plants were grown in a growth compartment at 18°C with 65% relative humidity, with a 16 h photoperiod. The plants were irrigated to avoid water stress. Amblyline CU (*Amblyseius cucumeris*) was used as a bio-pesticide to control thrips. Staking of the tillers was carried out 3-4 weeks after transplanting.

2.3.2 Gene cloning and transformation into *Agrobacterium tumefaciens*

A Gateway®-compatible plasmid; *pPS2015::GFP* (size: 18,527 bp) was used. The vector constitutively expresses green fluorescent protein (GFP) under the control of *UBI* promoter. It also has two hygromycin genes as selective markers under the control of either *CaMV35S* or *ZmUBI* promoter for selection of transformed scutella. It also has both spectinomycin and streptomycin for selection of transformed bacterial cells. *S. indica* effectors (SIEs) sub-cloned into entry vector pDONR221 by GeneArt™ M (Thermo Fisher Scientific) were cloned into the destination vector (*pPS2015::GFP*) using the Gateway® technology procedure presented in this link:

(https://assets.thermofisher.com/TFS-Assets/LSG/manuals/lr_clonaseii_man.pdf). Before the effector constructs were transformed into AGL-1 *Agrobacterium tumefaciens*, AGL-1 chemically competent cells were produced as follows: 100 ml YEB-medium (5 g/l peptone, 1 g/l yeast extract, 5 g/l beef extract, 5 g/l sucrose, 0.49 g/l MgSO₄·7H₂O), supplemented with respective antibiotic (for AGL-1: 100 mg/l Rif and 50 mg/l Carb), was inoculated with 1-2 colonies of *A. tumefaciens* and cultivated for 2 days at 28°C and 220 rpm until an OD₆₀₀ of above 1 was obtained. From this culture, a preparatory culture was prepared in 50 ml flask of OD₆₀₀ of 0.3-0.5 (YEB-medium plus antibiotic was used for the dilution) and incubated at 28°C and 220 rpm until the OD₆₀₀ of 0.7-0.8 is reached. The culture was portioned into 25 ml aliquots, cooled down on ice and centrifuged for 5 minutes at 3200 g. Pellets were resuspended in 1 ml sterile ice-cold CaCl₂ (20 mM) each and subsequently combined. The competent cells in CaCl₂ solution were aliquoted (50 µl) into autoclaved and pre-cold tubes, frozen in liquid nitrogen and stored at -80°C until when needed.

The effector constructs (*pPS2015::GFP-SIE*) were transformed into the AGL-1 chemo-competent cells produced. 2 µg plasmid DNA was added to 25 µl thawed (on ice) cells. After 5 minutes incubation on ice, then 5 minutes in liquid nitrogen and then 5 minutes at 37°C, 1 ml YEB medium was added and cells were grown for 3 hours at 28°C and 220 rpm. The culture was centrifuged and most of the supernatant was discarded. After resuspension in 50 µl of YEB medium, 30 µl of the culture were plated on solid agar plates with low salt content containing adequate antibiotics (100 mg/l spectinomycin, 100 mg/l Rif and 50 mg/l Carb). Plates were sealed with parafilm and incubated for 3 days at 28 °C. Positive colonies were confirmed by colony-PCR. The procedure is summarised as follows; a mix of the following PCR components were used per sample (2 µl of 1 mM dNTP, 0.2 µl Taq polymerase enzyme, 1 µl of 10 µM forward primer (5'-CGCGCGCGTATAAGAGCTCTATTTTAC-3') 1 µl of 10 µM reverse (5'-ACTAGTCCCGGGTCTTAATTA ACTCTC-3') primer and 13.8 µl H₂O). A small amount of each colony was added in separate PCR tube containing the mix. PCR run was carried out using the programme: (initial denaturation = 94°C, 3 min; final denaturation = 94°C, 30 sec; annealing = 55°C, 30 sec; initial extension/ elongation = 72°C, 1 min/kb; final extension/ elongation = 72°C, 5 min, cooling down = °C, ∞; 32 cycles). Gel electrophoresis was run to confirm that the size of the band obtained was the same with the expected size of the insert (effector). This was further confirmed by DNA sequencing. Glycerol stocks were made from the confirmed transformed colonies and stored at -80°C until needed.

2.3.3 Surface-sterilization of seeds for scutella transformation

Barley spikes were harvested at around 2-week post- anthesis when the seeds were 1.5–2 mm in diameter. Awns were removed and kernels were put in an autoclaved bottle placed on ice. About 100–200 kernels were surface-sterilized in 70% ethanol for 5 min under ice and afterward incubated in 6% sodium hypochlorite for a further 10-15 min. Thereafter, the kernels were incubated in autoclaved water of pH 3, pH 7 and tap water in that order for 10-15 min each. In all the incubations, a stirrer placed in the bottle was used to stir the seeds on a rotary shaker. The bottle containing the seeds was placed in fridge until it was used the same day. Overnight soaked seeds were not used.

2.3.4 Media preparation

Two different kinds of media were used: Callus Inducing Medium (CIM) and Root inducing medium (RIM). For 1 litre of CIM, 4.3 g MS stock (M0221, Duchefa), 30 g maltose, 0.25 g myo-inositol, 1 g casein hydrolysate, 0.69 g L-proline, 48 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 40 mg thiamine HCl, 50 mg Dicamba, 500 ml dH_2O and adjusted to pH 5.8-5.9 were filtered (using a vacuum filtration system (500 ml, PES membrane, 0.22 μm pore size) into autoclaved 6 g phyto agar in 500 ml dH_2O . 150 mg/l ticarcillin and required concentration of hygromycin were added for media used for selection of transformed plant tissues. The antibiotics were added when the media had cooled down. The media were poured out in petri dishes then the lids closed. They were stored at 4°C and used within 2 weeks; plates without antibiotics were used within a month.

For RIM, a stock solution was made: 10 mg nicotinamide, 10 mg pyridoxine HCl, 100 mg thiamine HCl, 0.25 mg cobalt (II) chloride hexahydrate, 0.25 mg copper (II) sulphate pentahydrate, 30 mg boric acid, 50 mg manganese (II) sulphate monohydrate, 2.5 mg sodium molybdate dihydrate, 50 mg zinc sulphate heptahydrate, 250 mg arginine, 500 mg asparagine monohydrate, 300 mg aspartic acid, 1.2 g glutamine, 500 mg proline, 250 mg threonine, 2.95 g calcium chloride dihydrate, 1.7 g potassium phosphate monobasic, 22 g potassium nitrate, 3.1 g magnesium sulphate heptahydrate, 750 mg sodium phosphate monobasic monohydrate, 670 mg ammonium sulphate, 6 g ammonium nitrate, 200 mg ferrous citrate, 280 Mg FeEDTA and 1 l dH_2O . In order to make 1 litre of RIM, 100 ml from the stock solution was added into a bottle containing 500 ml dH_2O , 20 g sucrose, 7 g glucose, 125 mg casein hydrolysate and 100 mg myo-inositol, pH adjusted to 5.3 with KOH. After adjusting the pH, 25 ml sterile coconut H_2O was added. The entire solution was filtered into autoclaved 6 g phyto agar + 1 g charcoal in 475 ml of dH_2O . The other processes were the same as described in CIM (Section 2.34)

2.3.5 Transformation of barley scutella

Barley scutella transformation was carried out using *Agrobacterium tumefaciens* strain AGL-1 as described by Imani et al. (2011) with some optimizations. Scutella (embryos) from the sterilized seeds were isolated in sterile conditions with sharp edged-pointed tweezers under a microscope. The embryonic axis was carefully removed from each scutellum. The extracted scutella were dipped into full strength overnight culture of AGL-1 containing effector

constructs (*pPS2015::GFP-SIE*) and were placed (on the callus inducing medium (CID) without selective antibiotics. The part where embryonic axis was removed facing upwards.

2.3.6 Callus induction and root generation

The transformed scutella were incubated on CIM with no selective antibiotics for 30 minutes. The scutella were turned upside down and the plate was tilted for more than 30 mins to remove excess *A. tumefaciens*. The scutella were later moved to a new plate with the scutella facing upwards (i.e. the part where embryonic axis was removed facing upwards). The transformed scutella were left at 24°C in the dark for 2 days. Thereafter, the scutella were transferred to CIM medium supplemented with 25 mg/l hygromycin and 150 mg/l of ticarcillin for selection of transformed scutella and to remove the persisting *Agrobacterium*, respectively. The scutella were cultivated on this medium for 4 weeks to calli formation. The scutella were not kept on a medium older than 2 weeks from date of preparation. In course of optimization of the transformation system, it was observed that the calli produced did not have a uniform transformation pattern. In other words, the transformation intensity (the area fully transformed relative to the entire plant area intended to be transformed) was low. Although the scutella transformed showed high transformation intensity, calli generated were consistently composed of transformed and untransformed fragments that were randomly interspersed. In order to solve this, the calli produced were sub-cultured on the same medium for further 2 weeks to facilitate fragmentations of the calli. Fully transformed and healthy calli fragments/parts were harvested from the ‘mother’ calli and either used for salt stress tests or regenerated to form mature calli. The fully mature regenerated calli were cultivated on RIM for 3 weeks to generate roots. Each root was carefully separated from the calli but still retained part of the calli as a crown. These roots were grown on RIM for an additional 1 week to recover from the shock of separation. These roots were used for *Fusarium graminearum* assays.

2.3.7 Confocal microscopy

MZFL-stereo fluorescence microscope was used in selection of transformed scutella/calli/roots. A GFP filter with excitation of 488 nm and emission peak of 509 nm setting was used to visualise the transformed scutella/calli/roots expressing GFP. To check for auto-fluorescence, a red filter setting with excitation of 561 nm and emission peak of 575 nm was used. Only scutella expressing GFP were sub-cultured to produce calli and roots.

2.3.8 Culturing of *Fusarium graminearum* spores

Fusarium graminearum strains CC19 was used. It was cultured by pipetting 10 µl glycerol stock of the spores on the centre of potato dextrose agar plates (39 g/l potato dextrose agar (PDA), 100 mg/l chloramphenicol) and was incubated at 24°C in the dark. The spores were harvested (scratching off the spores using a Drigalski spatula) after 11 days of incubation using autoclaved 0.02% tween-20- containing tap H₂O. The spore suspension was sieved into an autoclaved beaker via a sieve placed in a funnel. The number of spores in the filtrate was counted using haemocytometer under the microscope. Dilution to the required concentration or amount was done.

2.3.9 *Fusarium graminearum* inoculation of barley

For barley seedlings, the roots of 2-day old seedlings were immersed and swirled in the required spore concentration. The seedlings were transferred on CIM medium without antibiotics. The plates were monitored for development of disease symptoms up to 4 days after inoculation. However, for the roots from calli, the same method as above was used and spores evenly distributed using either spreaders or glass beads on the media before the roots were placed there. Images of the disease symptoms were taken with a MZFL-stereo fluorescence microscope and Panasonic DMC-FX33 Lumix.

2.3.10 Visualization of *F. graminearum* colonization of roots from barley calli.

The roots were fixed in 2 ml of distaining solution (80 ml chloroform, 20 ml ethanol and 1.5% trichloroacetic acid) for 2 days before the solution was decanted. The samples were then washed in tap H₂O and vacuum-infiltrated with 1 X PBS (pH 7.4). Thereafter, the roots were incubated for 20 minutes in 1 X PBS. They were moved into fresh 1 X PBS solution and 10 µg/ml of wheat germ agglutinin (WGA) Alexa Fluor 488 (Thermo Fisher Scientific) containing 0.02% Silwet L-77 (Sigma) was added. The roots were then vacuum-infiltrated three times, wrapped with foil and incubated for 1-2 days at 4°C. Roots were visualized under bright field and GFP settings (excitation: 488 nm, emission: 509 nm).

2.3.11 Cloning of effectors for salt stress assay

A Gateway®-compatible vector (*pANIC 10A::pporRFP*) was used as the destination vector for cloning of the effectors using the cloning procedure as described above. The vector contained the switchgrass polyubiquitin 2 promoter (*PvUbi2*) to drive the hygromycin gene, while the

switchgrass polyubiquitin 1 promoter (*PvUbi1*) controlled *pporRFP* (red fluorescent protein). SIEs were driven by the maize ubiquitin 1 promoter (*ZmUbi1*).

2.3.12 Screening for salt stress

The transformed calli parts expressing effectors or *GFP* (control) were subjected to salt stress by cultivating them on CIM supplemented with either 300 mM or 0 mM (mock) NaCl for 14 days. The initial and final weight of each callus was taken at day 0 and 14, respectively. In addition, average green fluorescence intensities of the calli on mock and salt stressed conditions were measured using ImageJ 1.5 1F. The mean gray value (the sum of the gray values of all the pixels in the selection divided by the number of pixels) was selected in the software for the measurement of the green fluorescence intensities.

2.3.13 Statistical testing for bioassays.

T-test (a two-tailed, unpaired, heteroscedastic) was used to determine if there was a significant difference between the line/calli expressing the effectors and the control using Microsoft Excel.

3 Chapter 3

S. indica effectors modulate *Arabidopsis* responses to biotic and abiotic stress elicitors

3.1 Introduction

Plants can be potentially infected by different types of pathogens which include viruses, bacteria, fungi, oomycetes and nematodes (Toruño et al., 2016). Over millions of years, plants have developed a complex immune system capable of detecting potential plant pathogens (Toruño et al., 2016). There is constant pressure on plants to continue to improve their innate ability to cope with the attacks from the persistently evolving pathogens. Consequently, plants to continue to build and accumulate large arsenals of immune receptors in their genomes. Specifically, plants have two modes of plant immunity to be able to resist pathogens attack: Pattern-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI) (Kutschera et al., 2019; Jones and Dangl, 2006; Dodds and Rathjen, 2010). PTI is triggered by recognition of molecular cues that are conserved among related microbes by cognate pattern-recognition receptors (PRR) on the plant cell membranes. FLAGELLIN SENSITIVE2 (FLS2) FLS2, a protein receptor kinase in *Arabidopsis thaliana* recognizes a bacterial flg22 peptide (a stretch of 22 amino acids) conserved in the N terminus of bacterial flagellin (Buscaill et al., 2019; Zipfel et al., 2004). This perception induces PTI following downstream activation of plant immunity genes. In addition, plants can activate a second line of immunity (ETI) when the invading pathogen suppresses or overcomes the first line of defence (PTI). For pathogens to colonise host plants and use them; the pathogens must subdue these two lines of host immunity (Toruño et al., 2016).

In order to overcome plant immunity, plant pathogens secrete small protein molecules, known as effectors to suppress the host immunity in order to facilitate invasion and infection (Cook et al., 2015; Rovenich et al., 2014). For instance, *Ustilago maydis*, a fungus that is responsible for smut disease in maize, secretes Pep1 effector required for successful invasion of the host tissues (Doehlemann et al., 2009). Ahmed et al. (2015) reported that *CSEP0105* and *CSEP0162* effectors produced by powdery mildew fungi contributed to immunity suppression in barley. Effector HopM1 from bacterial pathogen *Pseudomonas syringae* have been identified to subdue stomatal defence by targeting a 14-3-3 protein, GRF8/AtMIN10, in *Arabidopsis thaliana* (Lozano-Durán et al., 2014). Nematodes also use hundreds of effectors to modulate significant molecular and physiological changes associated with feeding site stimulation and

maintenance (Lilley et al., 2018). Most of the effectors are produced from three pharyngeal gland cells through a needle-like stylet and are secreted into the host plant (Lilley et al., 2018). The green peach aphid *Myzus persicae* feeds on host plants by thrusting its stylet between layers of cells in order to get to the phloem. Aphids produce saliva that contains a diversity of effectors that have been recognized to reduce plant immune responses and, hence, to improve host feeding (Rodriguez et al., 2017). Suppression of plant immunity often leads to diseases, which can ultimately result in substantial economic yield losses.

Effectors have become a class of protein molecules of interest in plant–microbe interaction studies. Understanding the functions of plant pathogenic effectors has significantly contributed to increase our knowledge of important molecular components involved in plant–pathogen interactions such as plant hormones, plant receptors, and signal transduction proteins (Win et al., 2012). In addition, information about the structure and functions of key pathogenic effectors has the potential to provide new strategies of controlling plant disease. For instance, engineering small RNAs with silencing activities against conserved effectors when in host plants or by partially/completely modifying the host effector target proteins through gene editing can disrupt the dogmatic interaction that mostly favours the pathogens. Genetically engineering crops through transgene-based Host-Induced Gene Silencing (HIGS) or Virus Induced Gene Silencing (VIGS) could be a new effective and environmentally-friendly approach to controlling crop diseases caused by different pathogens and pests (Qi et al., 2019; Kant and Dasgupta, 2019). It has been shown to modulate the growth and development of pathogens such as fungi, bacteria, nematodes, bacteria, viruses and pests on their plant hosts (Koch and Kogel, 2014).

Recent studies have shown that effectors may have other functions apart from suppressing host immunity. These include pathogen self-defence against antimicrobial compounds and hydrolytic enzymes produced by plants and other competing microbes (Rovenich et al., 2014). They are also used for mineralization of nutrients from host tissues (Fatima and Senthil-Kumar, 2015). In addition, it has been shown that effectors are not exclusively secreted by pathogens. Other symbiotic organisms such as endophytes, mutualists and even saprophytes do also secrete effectors (Rovenich et al., 2014). Rovenich et al. (2014) therefore argued that instead of defining effectors as proteins used by pathogens to manipulate host plant immune responses, effectors should be described as proteins that microbes use to facilitate niche colonization in host plants. Thus, improvement of plant growth and resistance/tolerance to biotic and abiotic

stresses following *S. indica* colonisation have been extensively documented and considered as a potential agent to sustainably improve crop production (Singh et al., 2011).

Although hundreds of genes coding for *S. indica* effectors have been recently identified, which provides opportunity to study them in plants (Zuccaro et al., 2011; Akum et al., 2015), little attempts have been made to investigate their functions in plant growth and stress resistance. Hassing et al. (2019) reviewed that only a small number of effectors from plant beneficial microbes have been known and functionally described. This gap of knowledge hinders the exploitation of those effectors in promoting plant growth, yield and stress resistance as an alternative to the application of beneficial microbes as bio-fertilizers. Plant molecular breeding has immensely contributed to sustaining global food security, but the major challenge is finding new key genes that can boost plant yield and stress resistance in the face of steadily increasing global food crisis. There is possibility that *S. indica* uses its effectors in conferring benefits to host plants, unlike pathogens that engage their effectors to cause disease in host plants. Therefore, the activities of ‘beneficial’ effectors can help us to identify plant mechanisms involved in the establishment of effector-driven benefits. Potentially, effectors from *S. indica* could be a novel genetic source for engineering economic crops for enhanced yield and stress resistance attributes.

3.2 Aims and objectives

This research chapter tries to investigate the roles of *S. indica* effectors in plant growth under stress and in biotic and abiotic stress resistance. This work was done in collaboration with and based on work done by Dr. Silke Lehmann in the same research group (Patrick Schäfer’s lab), who transiently transformed 150 *S. indica* effectors into protoplasts from the model plant, *Arabidopsis thaliana*. These transformed protoplasts were treated with flg22 and abscisic acid (ABA) to trigger early immunity (biotic) and abiotic stress responses, respectively. The top 10 suppressors and 10 top inducers identified in the protoplast assay were used by Dr. Silke Lehmann to generate Arabidopsis stable lines expressing those candidate effectors. These transgenic lines were screened for altered flg22 and ABA responses to further understand the functions of *S. indica* in plant growth and stress resistance.

3.3 Results

3.3.1 *S. indica* effectors modulate *Arabidopsis thaliana* response to flg22 treatment

Arabidopsis thaliana leaf protoplasts transformed with a library of 150 *S. indica* effectors were challenged with 1 μ M flg22 to induce PTI. The luciferase marker fused to the promoter of the PTI marker PHI-1 (*pPHI1-LUC*) means the intensities of luminescence produced as influenced by different effectors (*35S::SIEs*) in the protoplasts under mock and flg22-treated conditions can be measured. Based on this assay, the top 10 PTI-inducing and repressing effectors were selected. The 10 top suppressors consistently reduced protoplast response under mock and flg22-treated conditions, while the top 10 inducers increased the LUC activity of protoplast under both conditions (**Figure 3.1**). SIE 10 exhibited the highest impact and increased PTI-indicative LUC activity in protoplasts in response to mock and flg22. *SIE67* showed the strongest repressive effect on the PTI reporter under mock and flg22-treated conditions.

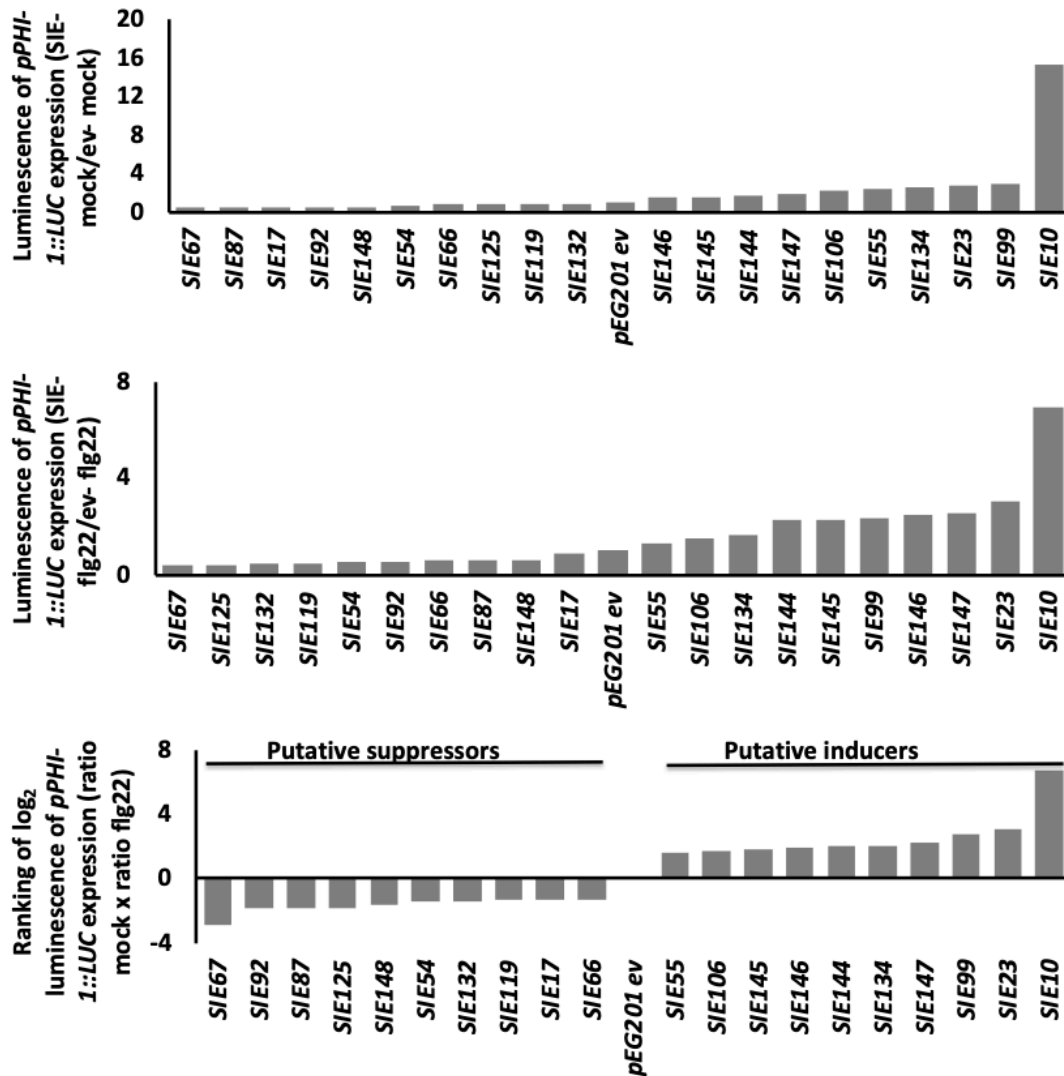


Figure 3.1: *S. indica* effectors (SIE) with most significant effects on the expression of PTI marker *PHI-1* in Arabidopsis protoplasts upon mock or flg22 treatment.

Protoplasts were co-transformed with *35S::SIE*, PTI marker construct *pPHI-1::LUC*, and *pUBI10::β-glucuronidase* (for internal control). **A:** *S. indica* effectors (SIE) that change *PHI-1*-regulated luminescence in Arabidopsis protoplasts under mock conditions. Bars show ratios of luminescence produced by SIEs and the empty vector (pEG201 ev) under mock conditions. **B:** *S. indica* effectors (SIEs) that change *PHI-1*-regulated luminescence in Arabidopsis protoplasts upon flg22 treatment. Bars show ratios of luminescence produced by SIEs and the empty vector (pEG201 ev) under flg22 conditions. **C:** *S. indica* effectors (SIE) that change Arabidopsis protoplasts response under both mock and flg22 treated conditions. Ratio mock (SIEs luminescence/pEG201 luminescence under mock- treated condition) x ratio flg22 (SIEs luminescence/pEG201 luminescence under flg22- treated condition) adjusted relative to 0 % of the control (pEG201).

For further studies on the functions of *S indica* effectors in plant, *Arabidopsis thaliana* lines expressing each of the top 10 repressing/inducing candidate effectors were screened for flg22 responses. Primary root growth was used as basis to screen for the flg22 response. For each effector a pool consisting of three independent lines was used for the screening. Apart from those top 20 candidates, other SIE candidates identified in same research group (Patrick Schafer) to influence other hormone or abiotic stress pathways (e.g. *SIE126* and *SIE69* in ABA, *SIE88* and *SIE64* in auxin, *SIE109* in cytokinin, *SIE76* in salicylic acid, and *SIE91* in salt stress assays) were included. In total, 32 effectors were investigated and ten effectors significantly promoted primary root growth as compared to the control line (*35S::GFP/Col-0*) in *Arabidopsis thaliana* in mock conditions (**Figure 3.2A**). In comparison with the control line (*35S::GFP/Col-0*), the highest increase in root length was observed in lines expressing *SIE99* (38%) followed by *SIE23* (27%). 14 effectors had significantly longer primary roots than the control line under flg22-treated conditions (**Figure 3.2B**). Interestingly, 8 effectors significantly and consistently exhibited growth promotion under mock and flg22-treated conditions. These effectors were *SIE120*, *SIE43*, *SIE126*, *SIE133*, *SIE66*, *SIE106*, *SIE99* and *SIE23*. Only 4 effectors significantly reduced root length under mock or flg22-treated conditions as compared to the control treatment. Among them, *SIE10* reduced primary root length by 10% and 19% upon mock and flg22 treatment, respectively. Finally, root flg22 sensitivity to show the extent of reduction in root growth of each SIE-expressing line treated with flg22 relative to its mock treated was quantified (**Fig. 3. 2C**). 9 effectors significantly modified root sensitivity to flg22 compared to the control line. The lowest sensitivity was observed in lines expressing *SIE76* (19%), while the highest sensitivity was obtained in lines expressing *SIE146* (22%).

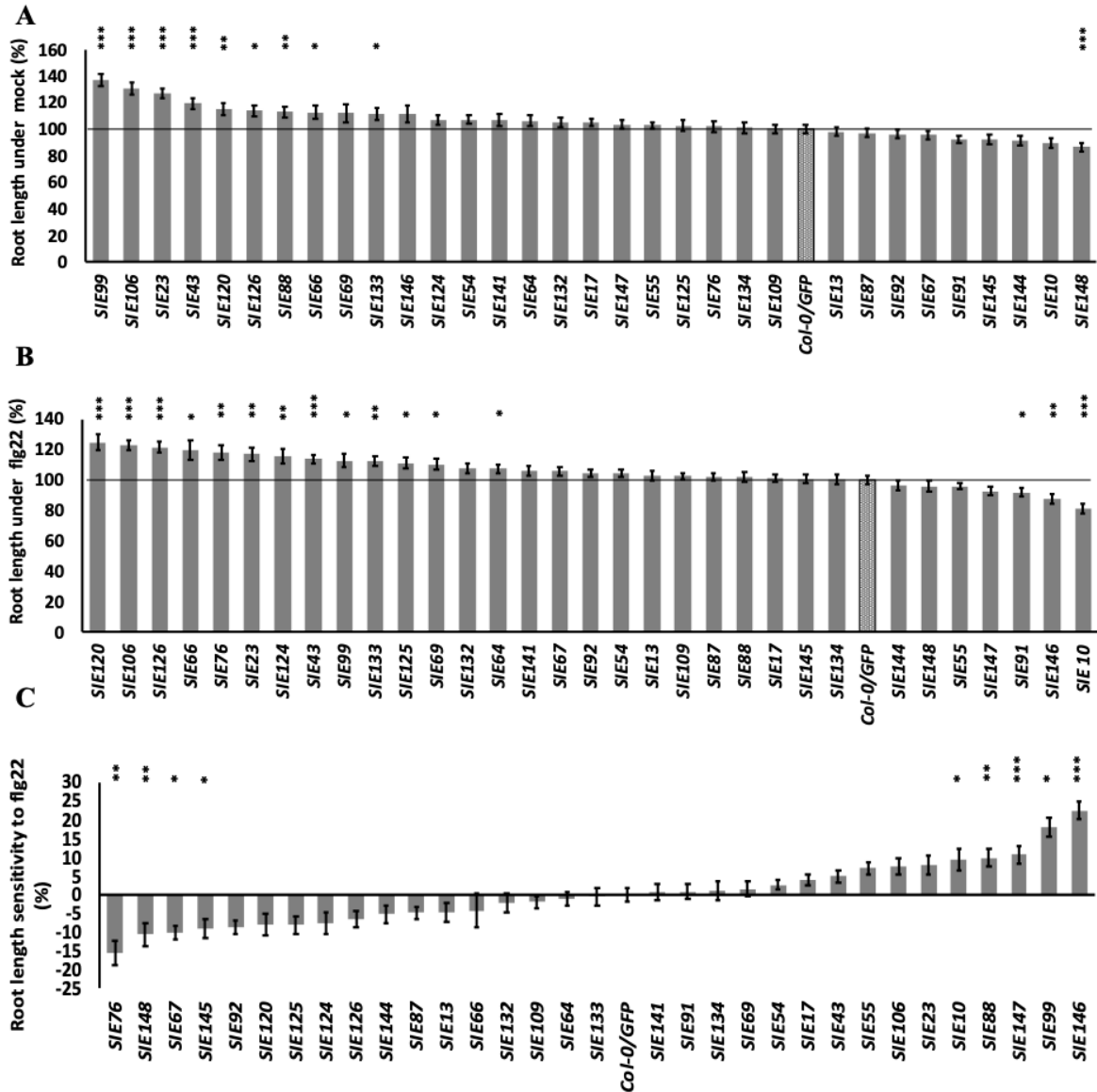


Figure 3.2: Root length of *Arabidopsis thaliana* lines expressing *S. indica* (SIE) effectors.

Each SIE line represents a pool consisting of three independent lines. 9-day old seedlings were treated with sterile H₂O (mock) and 1 μM flg22. After 4 days of treatment, the primary root length of each line was measured. Data from at least 42 plants per treatment is shown with standard errors. **A:** Root length of SIE-expressing lines relative to 100 % of control lines (35S::GFP/Col-0) under mock condition. **B:** Root length of SIE-expressing lines relative to 100 % of control lines upon flg22 treatment. **C:** Root growth sensitivity of SIE-expressing lines to flg22 treatment relative to the control line. Differences (root length of SIE lines + flg22 / root length + mock) are shown as percentages (adjusted relative to 0% of control lines). Number of plants per line =75. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

To confirm the results obtained with SIE pool lines, each line per pool was analysed individually. The selection was based on effectors that altered root sensitivity to flg22 and those that promoted growth. Independent lines for six effectors (*SIE23*, *SIE67*, *SIE76*, *SIE99*, *SIE106* and *SIE146*) were selected and tested (**Figures 3.3- 3.8**). Growth promotion observed for *SIE76*, *SIE99* and *SIE106* was confirmed in independent lines treated with mock or flg22. Only *SIE23*-expressing lines only changed in one independent line. Significantly, all independent lines expressing *SIE76* consistently showed substantial increase in root growth under mock and flg22 treated conditions. It is important to mention that the pool of *SIE76* showed significant growth promotion only under flg22 conditions. The highest increase in root length compared to the control line was observed in one of the independent lines of *SIE106* that ranged from 33% in mock to 38% under flg22 conditions. In terms of root sensitivity to flg22, *SIE67* and *SIE76* (except in one independent line) showed nearly similar results as observed with the line pools. All independent lines (except in one independent line of *SIE76*) showed reduced root sensitivity to flg22 as compared to the control line.

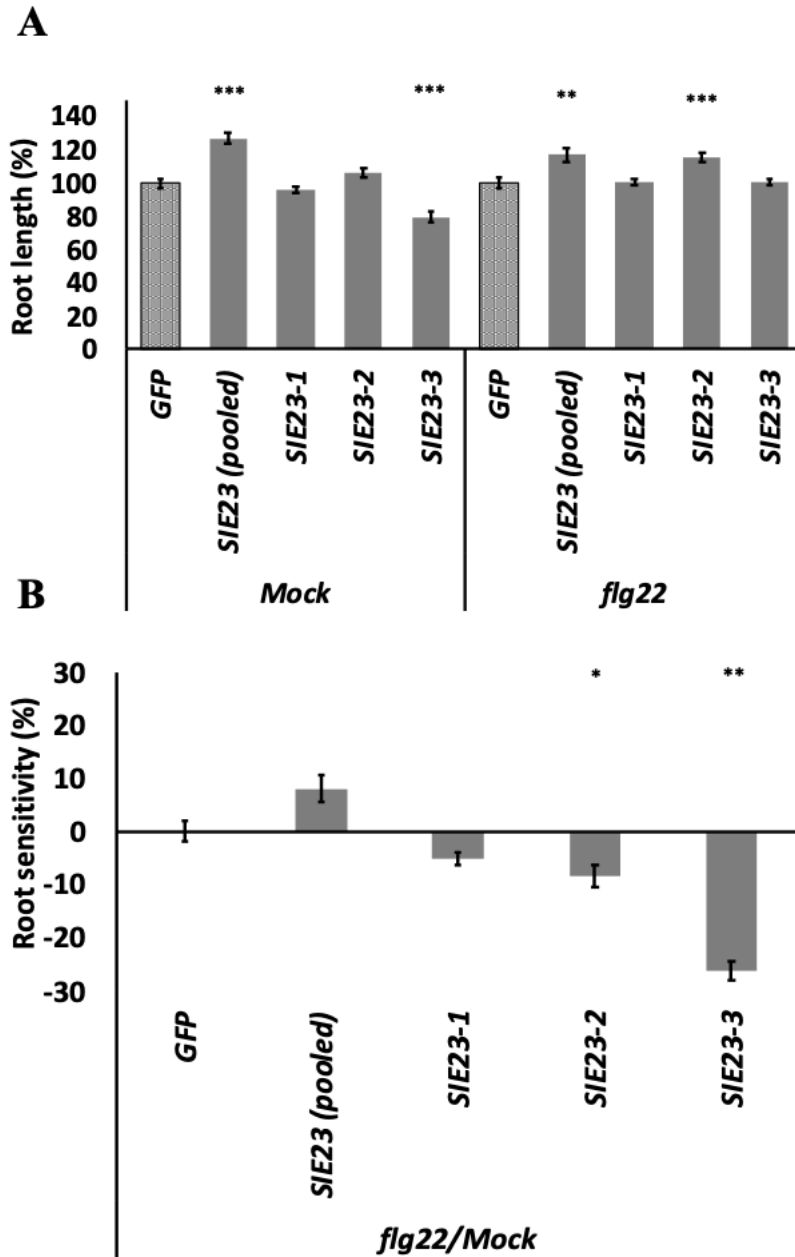


Figure 3.3: Phenotypic analyses of *A. thaliana* lines expressing *SIE23*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE23* relative to 100% of the control line (*35S::GFP/Col-0*) under mock and flg22-treated conditions. 9-days old seedlings were treated with sterile H₂O (mock) or 1 μ M flg22. 4 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to flg22 treatment of *SIE23* lines relative to the control line. Number of plants per line =75. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

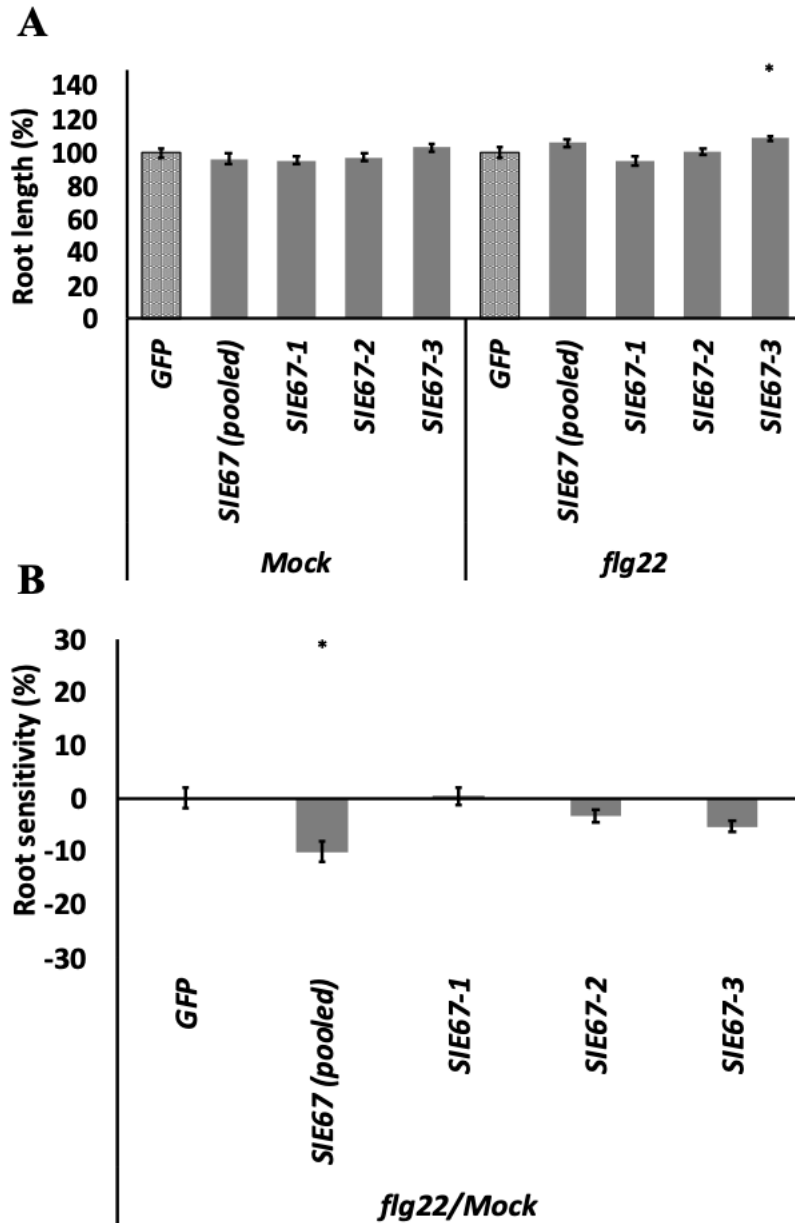


Figure 3.4: Phenotypic analyses of *A. thaliana* lines expressing *SIE67*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE67* relative to 100% of the control line (*35S::GFP/Col-0*) under mock and flg22-treated conditions. 9-days old seedlings were treated with sterile H₂O (mock) or 1 μM flg22. 4 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to flg22 treatment of *SIE67* lines relative to the control line. Obtained differences in root length were adjusted relative to 0% of the control line. Number of plants per line =75. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05.

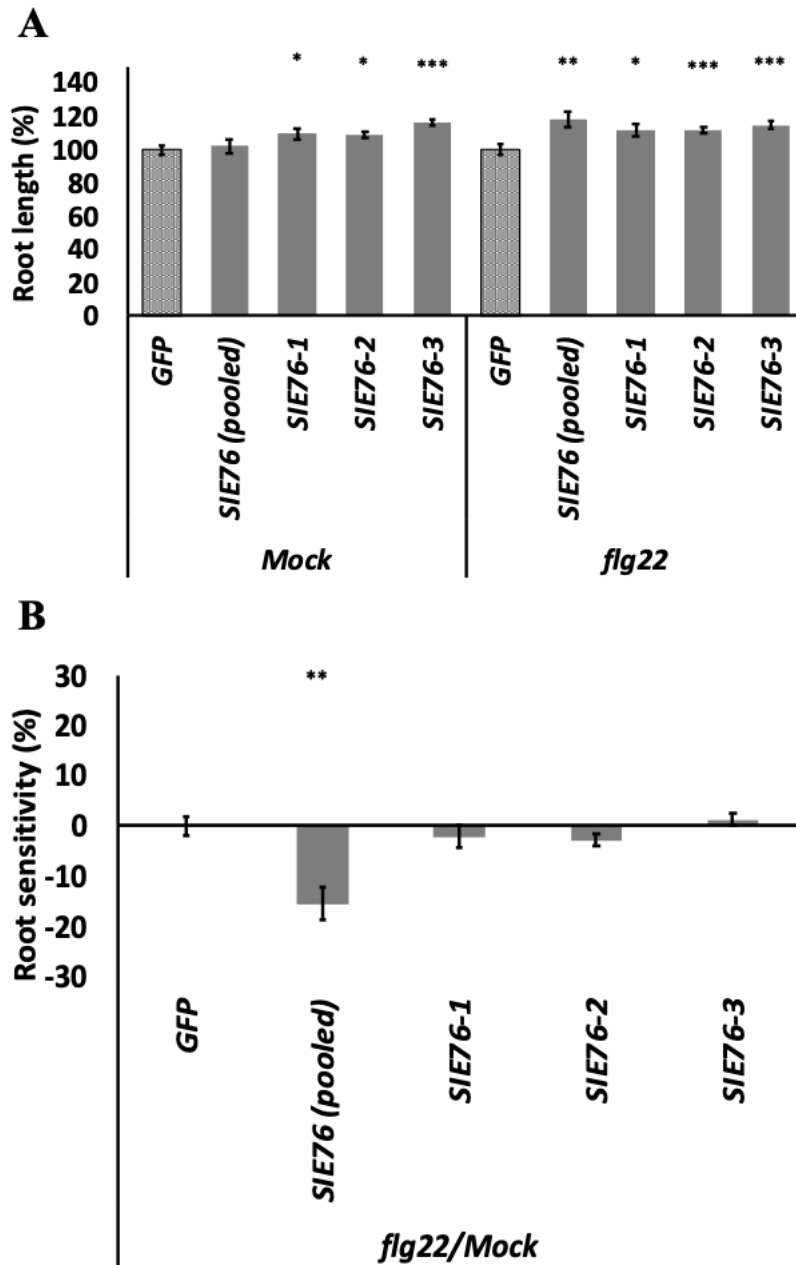


Figure 3.5: Phenotypic analyses of *A. thaliana* lines expressing *SIE76*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE76* relative to 100% of the control line (*35S::GFP/Col-0*) under mock and flg22-treated conditions. 9-days old seedlings were treated with sterile H₂O (mock) or 1 μM flg22. 4 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to flg22 treatment of *SIE76* lines relative to the control line. Obtained differences in root length were adjusted relative to 0% of the control line. Number of plants per line =75. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

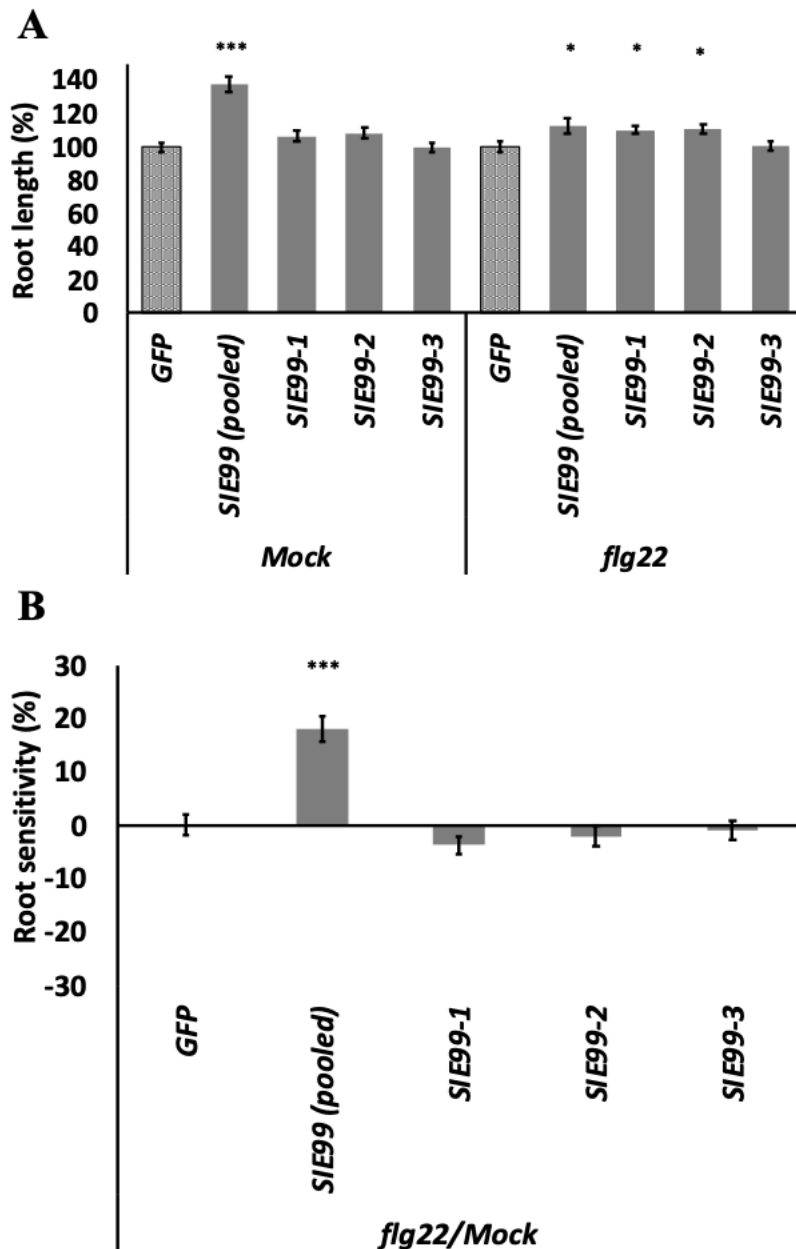


Figure 3.6: Phenotypic analyses of *A. thaliana* lines expressing *SIE99*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE99* relative to 100% of the control line (*35S::GFP/Col-0*) under mock and flg22-treated conditions. 9-days old seedlings were treated with sterile H₂O (mock) or 1 μM flg22. 4 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to flg22 treatment of *SIE99* lines relative to the control line. Obtained differences in root length were adjusted relative to 0% of the control line. Number of plants per line =75. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. ***=significant at p-value of <0.001.

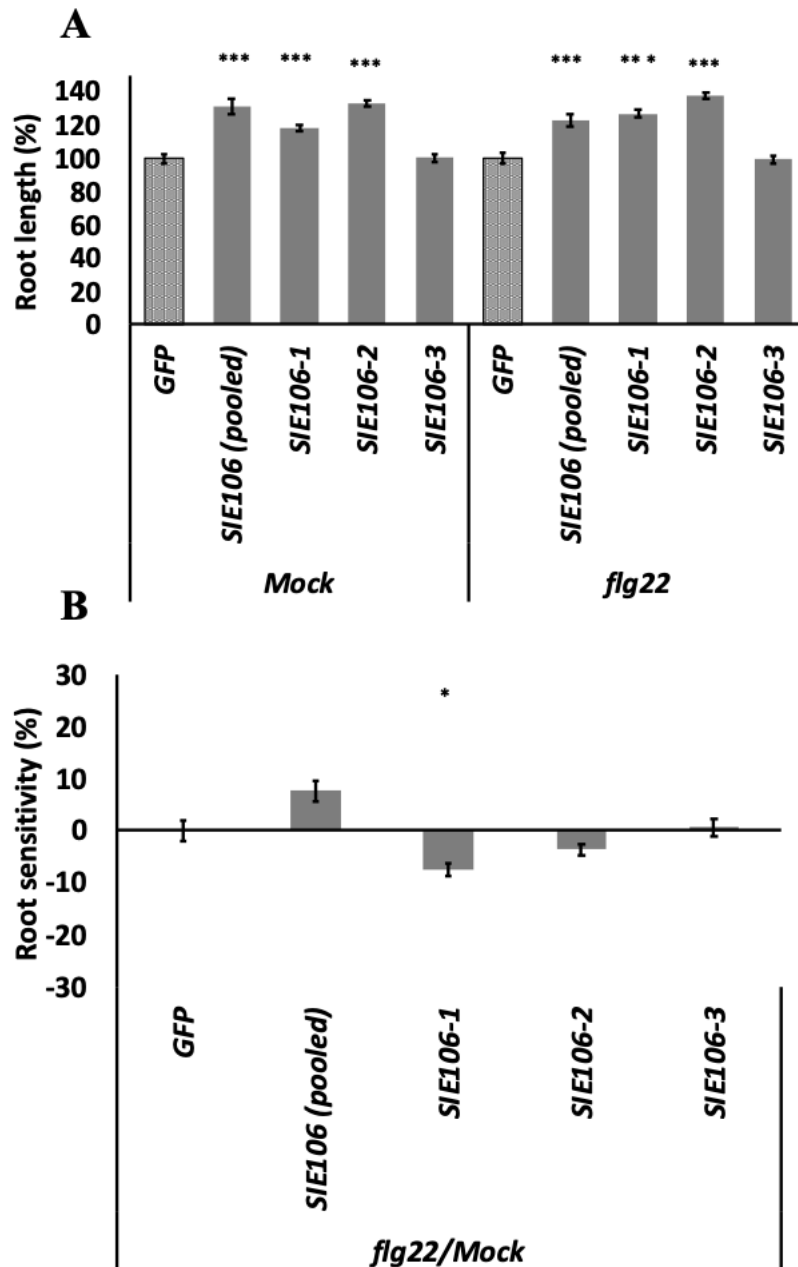


Figure 3.7: Phenotypic analyses of *A. thaliana* lines expressing *SIE106*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE106* relative to 100% of the control line (*35S::GFP/Col-0*) under mock and flg22-treated conditions. 9-days old seedlings were treated with sterile H₂O (mock) or 1 μM flg22. 4 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to flg22 treatment of *SIE106* lines relative to the control line. Number of plants per line =75. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. ***=significant at p-value of <0.001.

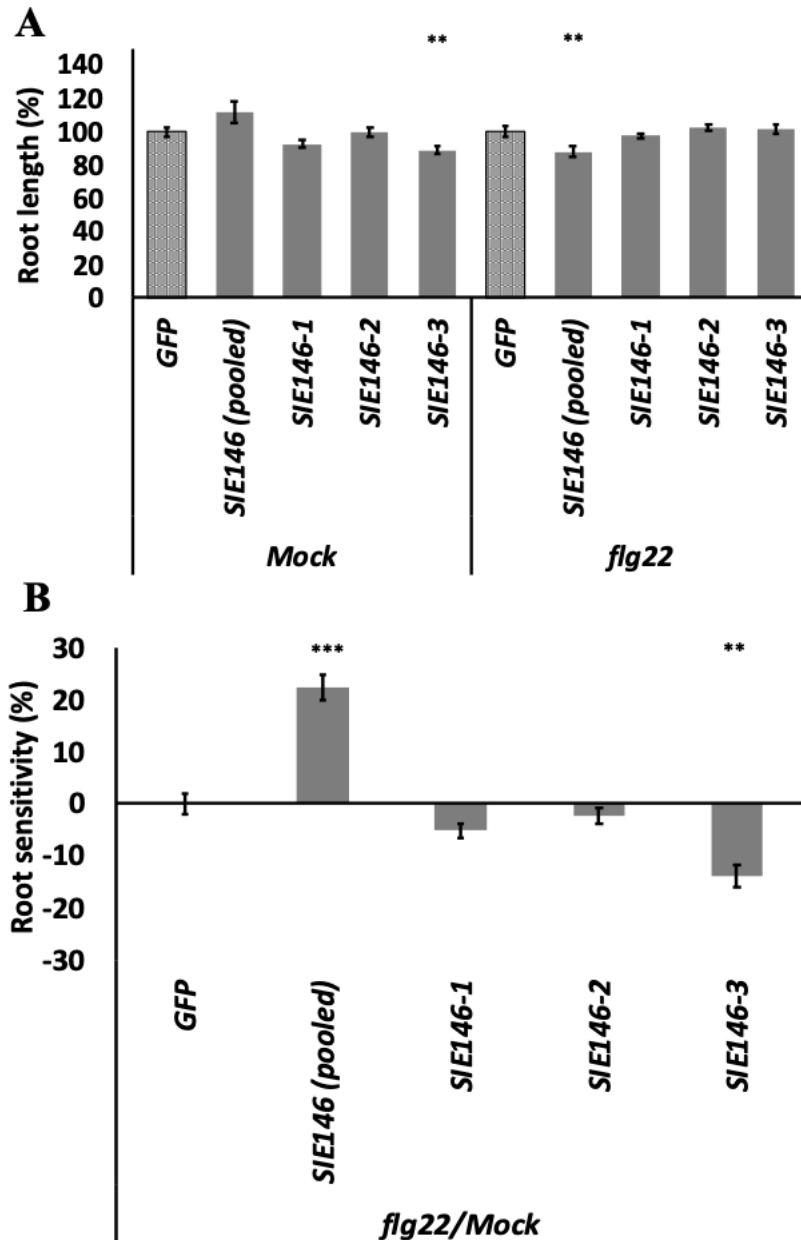


Figure 3.8: Phenotypic analyses of *A. thaliana* lines expressing *SIE146*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE146* relative to 100% of the control line (*35S::GFP/Col-0*) under mock and flg22-treated conditions. 9-days old seedlings were treated with sterile H₂O (mock) or 1 μM flg22. 4 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to flg22 treatment of *SIE146* lines relative to the control line. Obtained differences in root length were adjusted relative to 0% of the control line. Number of plants per line =75. T-test was used to compare the lines expressing effectors with the control lines. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

In order to understand the molecular basis of *S. indica* effector activities, known protein domains in the protein sequences of these six selected effectors (*SIE23*, *SIE67*, *SIE76*, *SIE99*, *SIE106* and *SIE146*) were analysed using SMART (Simple Modular Architecture Research Tool). For *SIE67*, DJ-1_PfpI protein domain was identified (**Figure 3.9 A**) while there was no known protein domain found in any of the other five effector sequences. To gain an insight into the molecular mode of action of these effectors, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to study the expression of three immunity marker genes; *FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)*, *PHOSPHATE-INDUCIBLE1 (PHI-1)* and *NDR1/HIN1-like 10 (NHL10)* in *A. thaliana* lines stably expressing *SIE67*, *SIE76*, *SIE99* and *SIE146* upon flg22 or mock treatment (**Figures 3.9 – 3.12**). As expected, all markers were induced by flg22 in the control line (**Figures 3.9 – 3.12**). Under mock condition, lines expressing *SIE67* did not show similar pattern of expression of the three immunity markers in both replicates (**Figure 3.9**). Lines expressing *SIE76* showed significant increase expression of *FRK1* and *PHI-1* in both replicates (**Figure 3.10**). The lines expressing *SIE99* and *SIE146* did not show significant and consistent pattern of the PTI markers expression in the two replicates (**Figures 3.11 & 3.12**). Upon flg22 treatment, *FRK1* marker had significant reduced expression in lines expressing *SIE76* (**Figure 3.10**). Similarly, there was reduced expression of *FRK1* marker in line expressing *SIE99* but significant difference was observed in one replicate (**Figure 3.11**).

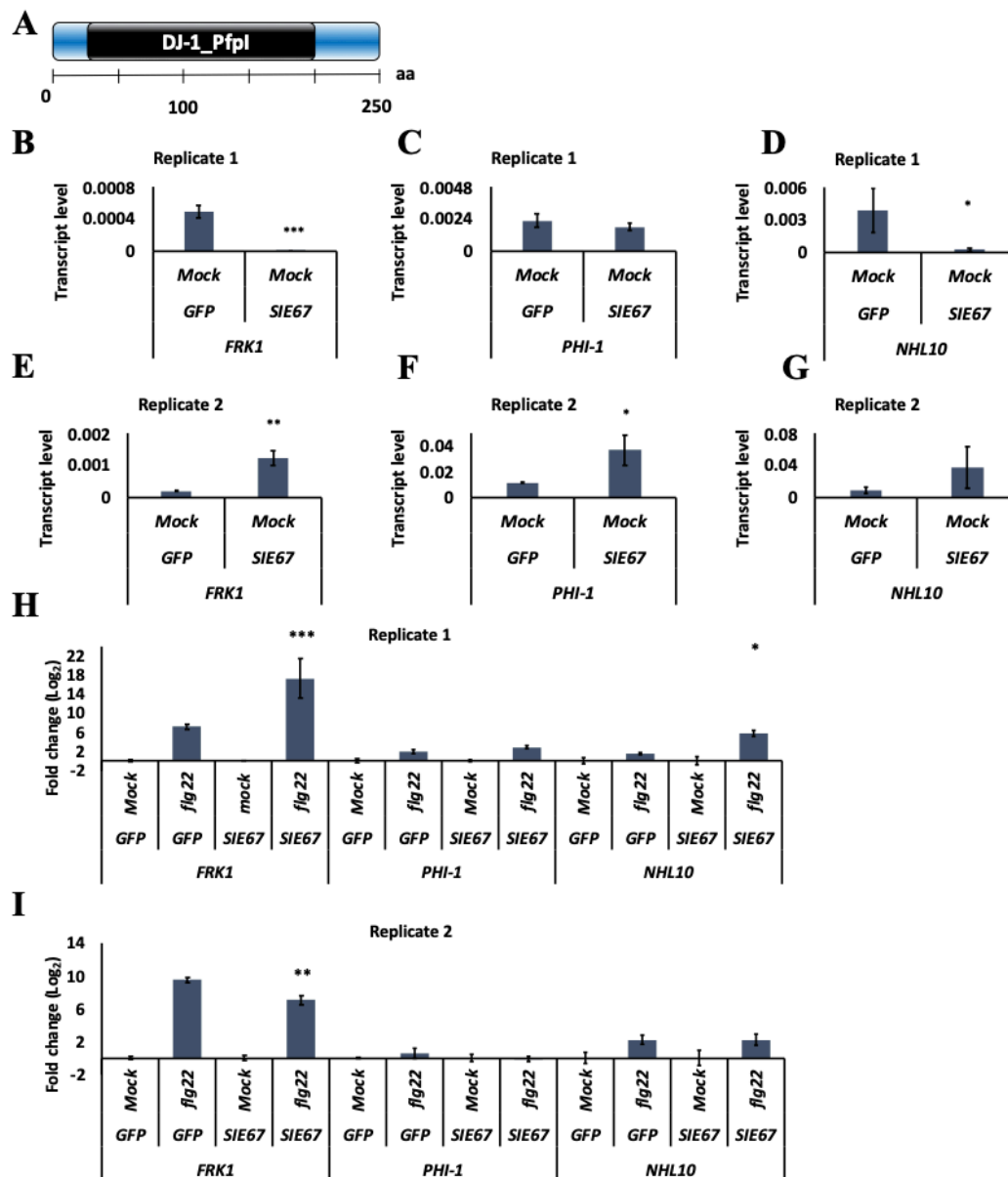


Figure 3.9: Functional analyses of *A. thaliana* lines expressing *SIE67*.

A: Schematic representation of DJ-1_PfpI protein domain (e-value = 1.5×10^{-18}) identified in protein sequence of *SIE67* using Simple Architecture Research Tool (SMART). The domain (amino acid (aa) position 31-203) is represented in black, while blue indicates the total length of *SIE67*. **B-G:** Transcriptional expression of immunity markers *FLG22-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*), *PHOSPHATE-INDUCIBLE1* (*PHI-1*) and *NDR1/HIN1-like 10* (*NHL10*) in *A. thaliana* lines expressing *SIE67* in mock conditions. 11-day old seedlings comprising pools of three independent lines treated with sterile H₂O (mock) or 1 μ M flg22 and the whole seedlings harvested after 2 hours for qRT-PCR. *UBQ5* was used as a housekeeping gene. **H, I:** Fold change of immunity marker expression in control and *SIE67*-expressing lines upon flg22 relative to mock treatment. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

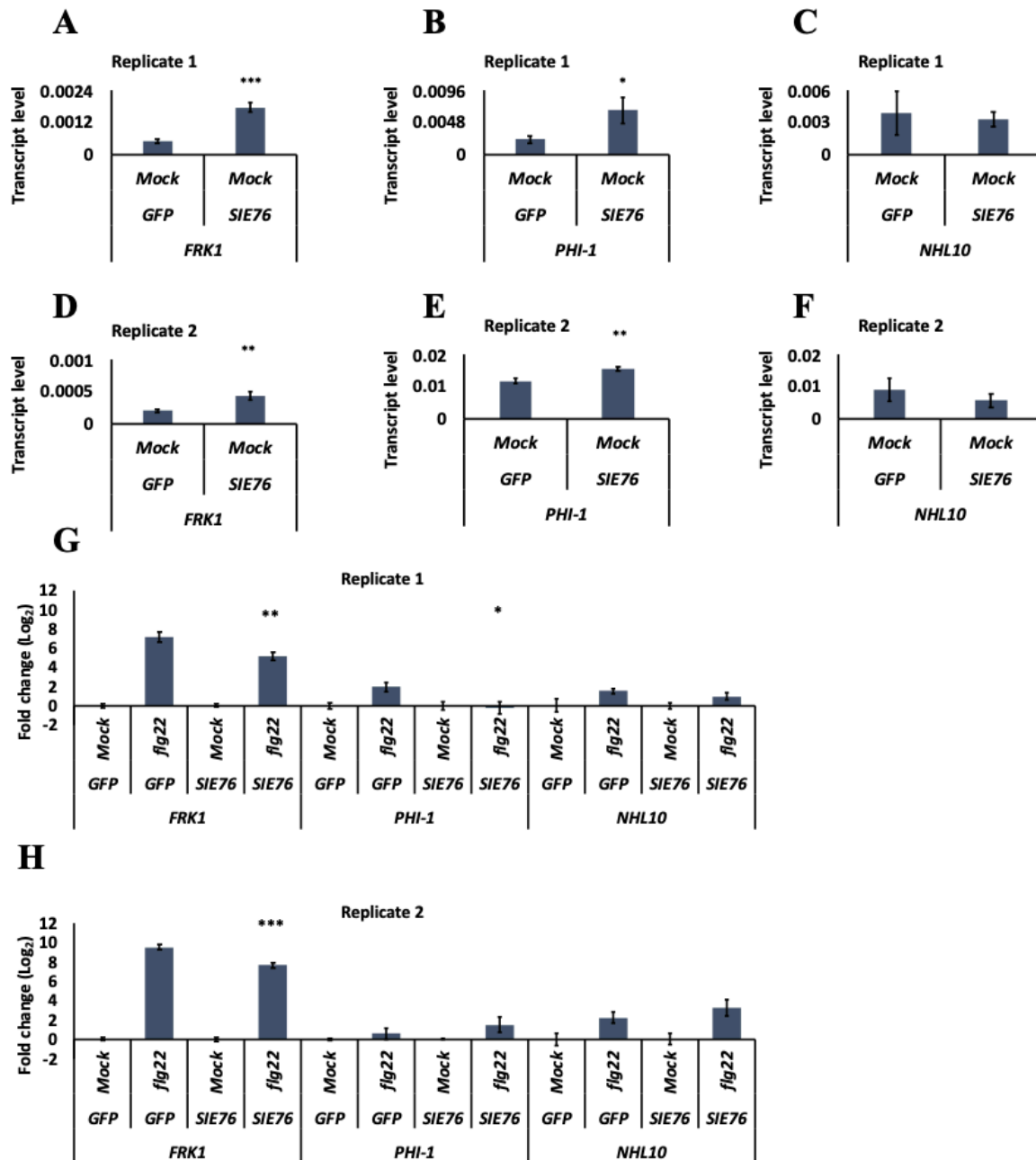


Figure 3.10: Functional analyses of *A. thaliana* lines expressing *SIE76*.

A- F: Transcriptional expression of immunity markers *FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)*, *PHOSPHATE-INDUCIBLE1 (PHI-1)* and *NDR1/HIN1-like 10 (NHL10)* in *A. thaliana* lines expressing *SIE76* on mock condition. 11-day old seedlings comprising pool of three independent lines were treated with sterile H₂O (mock) or 1 μM flg22 and the whole seedlings were harvested after 2 hours for qRT-PCR. *UBQ5* was used as a housekeeping gene. **G, H:** Fold change of immunity marker expression in control and *SIE76*-expressing lines upon flg22 relative to mock. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

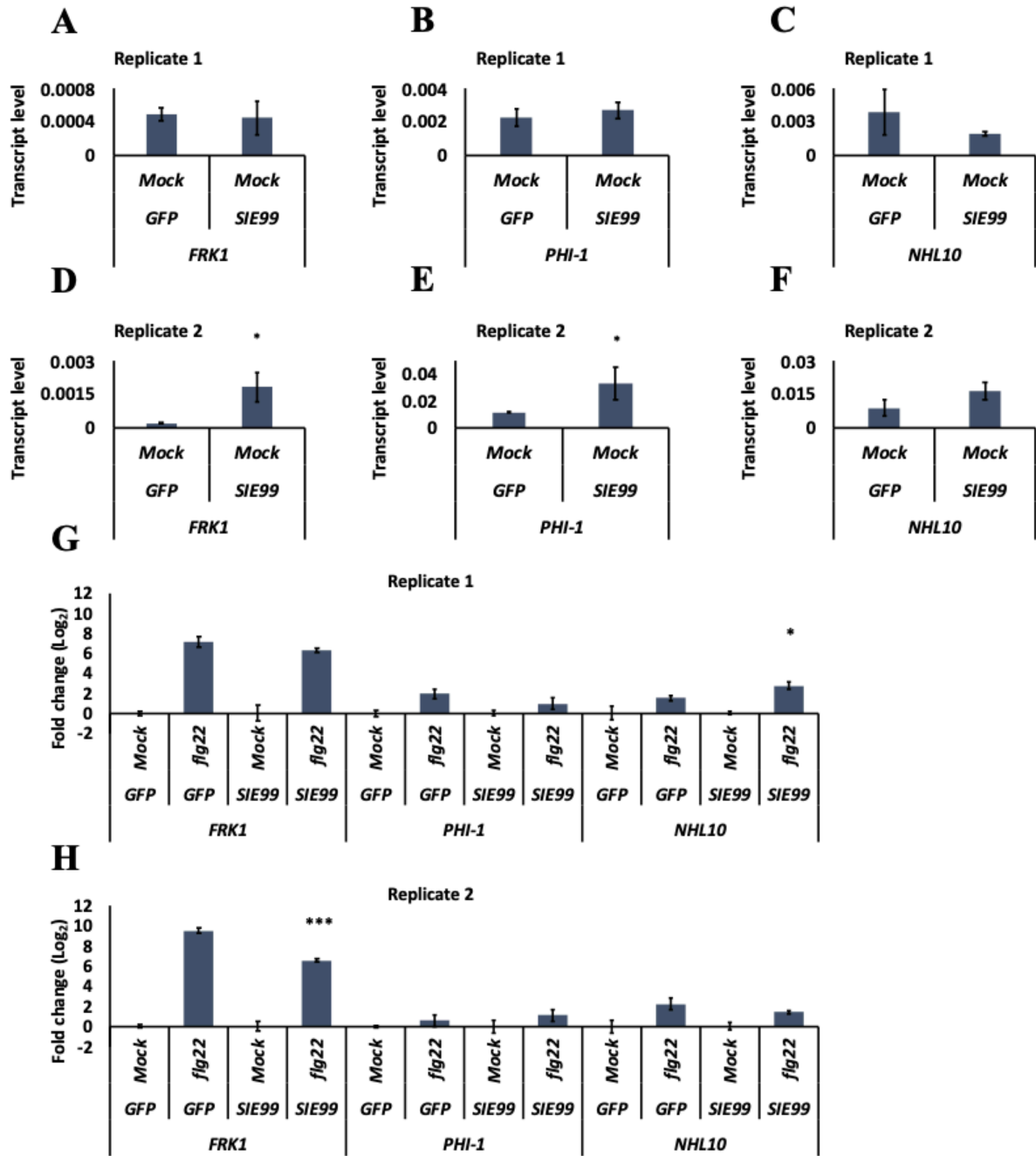


Figure 3.11: Functional analyses of *A. thaliana* lines expressing *SIE99*.

A-F: Transcriptional expression of immunity markers *FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)*, *PHOSPHATE-INDUCIBLE1 (PHI-1)* and *NDR1/HIN1-like 10 (NHL10)* in *A. thaliana* lines expressing *SIE99* on mock condition. 11-day old seedlings comprising pool of three independent lines were treated with sterile H₂O (mock) or 1 μM flg22 and the whole seedlings were harvested after 2 hours for qRT-PCR. *UBQ5* was used as a housekeeping gene. **G, H:** Fold change of immunity marker expression in control and *SIE99*-expressing lines upon flg22 relative to mock. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. ***=significant at p-value of <0.001.

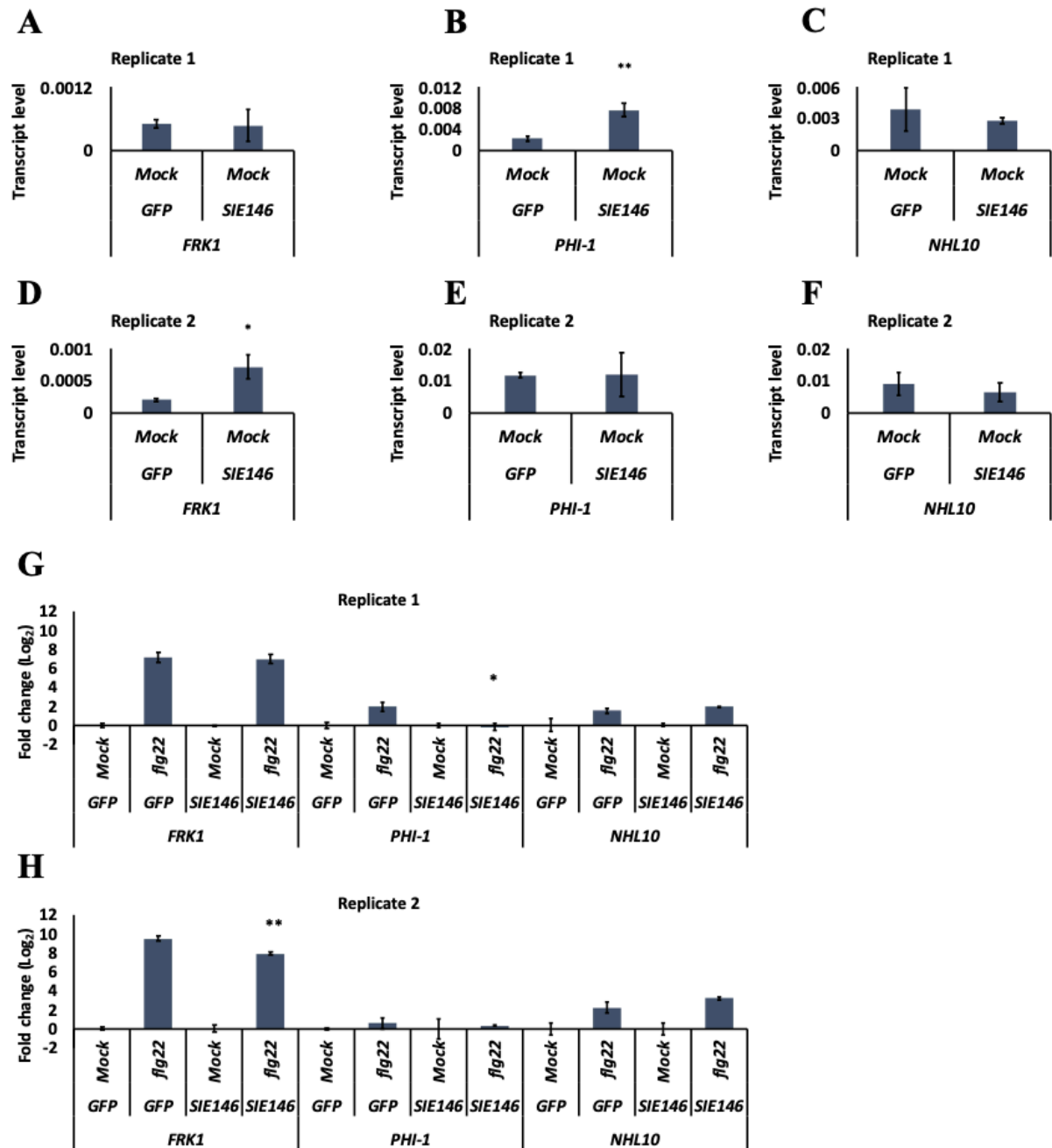


Figure 3.12: Functional analyses of *A. thaliana* lines expressing *SIE146*.

A- F: Transcriptional expression of immunity markers *FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)*, *PHOSPHATE-INDUCIBLE1 (PHI-1)* and *NDR1/HIN1-like 10 (NHL10)* in *A. thaliana* lines expressing *SIE146* on mock condition. 11-day old seedlings comprising pool of three independent lines were treated with sterile H₂O (mock) or 1 μ M flg22 and the whole seedlings were harvested after 2 hours for qRT-PCR. *UBQ5* was used as a housekeeping gene. **G, H:** Fold change of immunity marker expression in control and *SIE146*-expressing lines upon flg22 relative to mock. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01.

3. 3. 2 *S. indica* effectors modulate *Arabidopsis* response to abscisic acid treatment

To understand the functions of the 150 effectors in abiotic stress signalling, protoplasts were co-transformed with *pRD29A::LUC*, *35S::SIEs* and *pUBI10:: β -Gluc* and subjected to abscisic acid (ABA) as abiotic stress-mitigating hormone. Based on those analyses 20 effectors (10 suppressors and 10 inducers) with the strongest effect on ABA signalling were selected for further analyses (**Figure 3.13**). As in the *flg22* assay, *SIE10* exhibited the strongest effects in protoplasts exposed to mock or ABA treatment. These 20 effectors and three SIEs identified in other hormone assays (e.g. *SIE88* in auxin). In total, 23 effectors) were used to generate stable *SIE*-expressing *Arabidopsis* lines. These lines were challenged with ABA to confirm if *SIE*-associated alterations in protoplast assays were transferable to whole plants. ABA-induced root growth inhibition was selected as representative ABA response. Prior to the screening, different concentrations of ABA were tested to identify the most suitable concentration for the assay (**Figure 3.14**). When challenged with 5, 10, and 20 μ M ABA for 7 days, the primary root length was reduced by 19, 26 and 33 %, respectively. Thus, 10 μ M ABA that reduced the root length moderately was chosen for screening *SIE* expressing *Arabidopsis* lines.

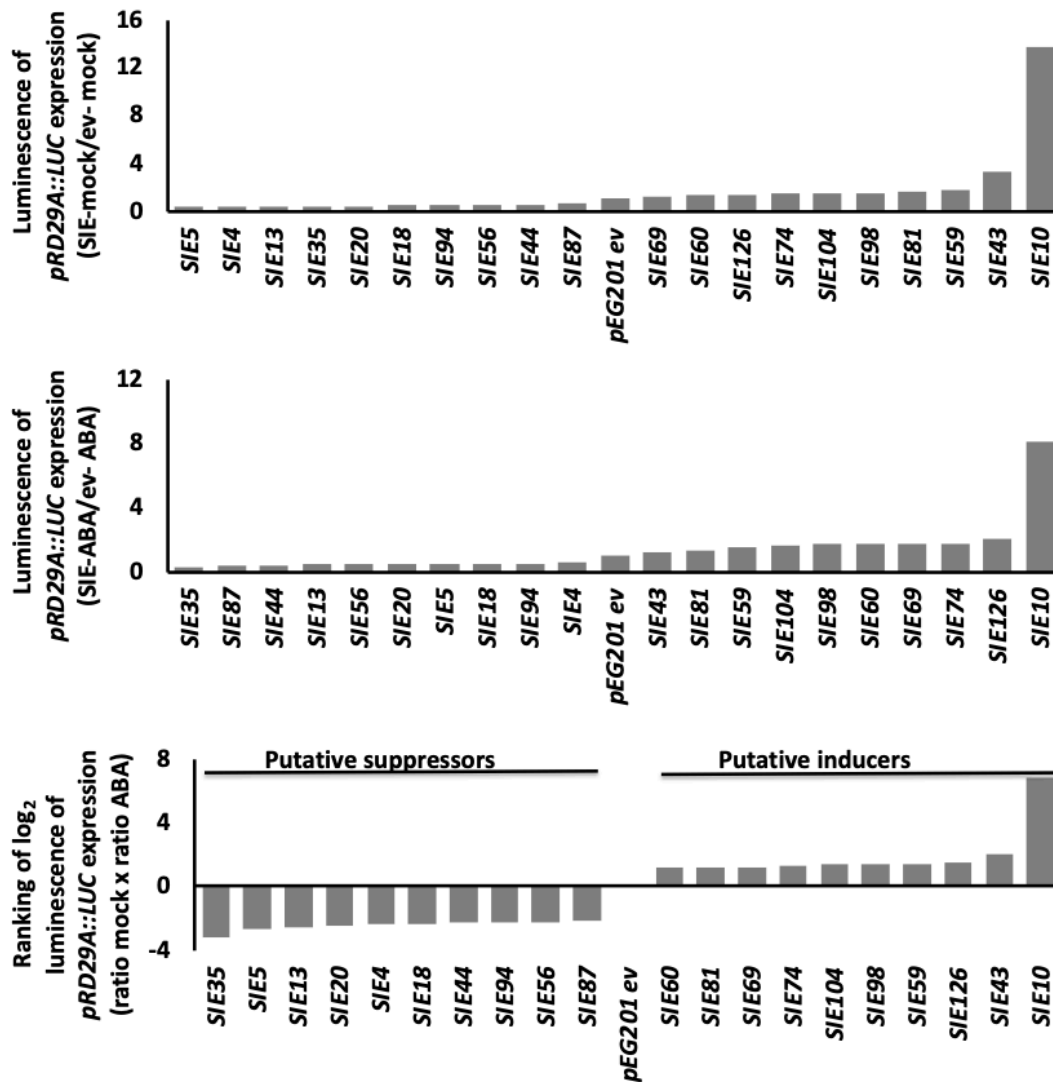


Figure 3.13: *S. indica* effectors (SIE) with most significant effects on the expression of abscisic acid (ABA) marker *RD29A* in *Arabidopsis* protoplasts upon mock or ABA treatment.

Protoplasts were co-transformed with *35S::SIE*, ABA response marker construct *pRD29A::LUC*, and *pUBI10::β-glucuronidase* (for internal control). **A:** *S. indica* effectors (SIE) that change *RD29A*-regulated luminescence in *Arabidopsis* protoplasts under mock conditions. Bars show ratios of luminescence produced by SIEs and the empty vector (pEG201 ev) under mock conditions. **B:** *S. indica* effectors (SIEs) that change *RD29A*-regulated luminescence in *Arabidopsis* protoplasts upon flg22 treatment. Bars show ratios of luminescence produced by SIEs and the empty vector (pEG201 ev) under ABA conditions. **C:** *S. indica* effectors (SIE) that change *Arabidopsis* protoplasts response under both mock and ABA treated conditions. Ratio mock (SIEs luminescence/pEG201 luminescence under mock-treated condition) x ratio flg22 (SIEs luminescence/pEG201 luminescence under ABA-treated condition) adjusted relative to 0 % of the control (pEG201).

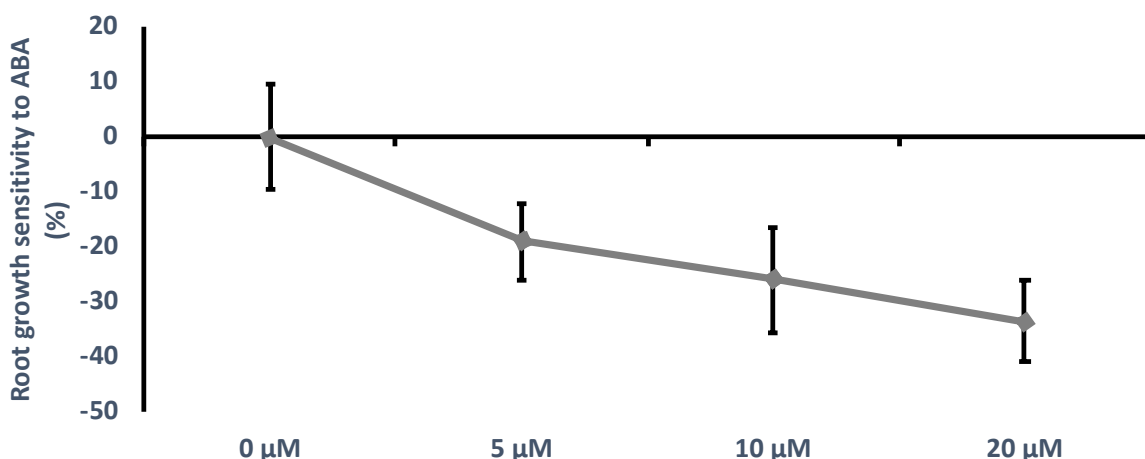


Figure 3.14: Root sensitivity of *Arabidopsis thaliana* to different concentrations of abscisic acid.

7- day old seedlings were transferred to mock and abscisic acid (ABA) containing media. After 7 days of treatment, the primary root length was measured and the percentage reduction relative to 0 % of 0 μM ABA (mock) was plotted as root growth sensitivity to ABA. Standard deviation was used for estimation of the errors.

For each effector seedlings of a pool consisting of three independent lines was used and primary root growth was examined under both mock and ABA conditions. 4 effectors significantly increased root length while 7 decreased root lengths under mock conditions (**Figure 3.15A**). *Arabidopsis* lines expressing *SIE44* and *SIE120* showed the highest increase in root length with 12.6% and 12.2% as compared to control lines, respectively. Under ABA conditions only, 5 effectors significantly increased while 6 significantly repressed root growth (**Figure 3.15B**). *SIE94* significantly increased root growth whereas *SIE5*, *SIE10*, *SIE35*, *SIE56* and *SIE81* repressed root growth on both ABA and mock- treated conditions. With respect to root sensitivity to ABA, 4 effectors significantly altered the response in *Arabidopsis* plants (**Figure 3.15C**); *SIE69* and *SIE10* decreased root sensitivity to ABA by 30 and 12 %, respectively, while *SIE39* and *SIE44* increased the sensitivity by 11 and 8 %, respectively.

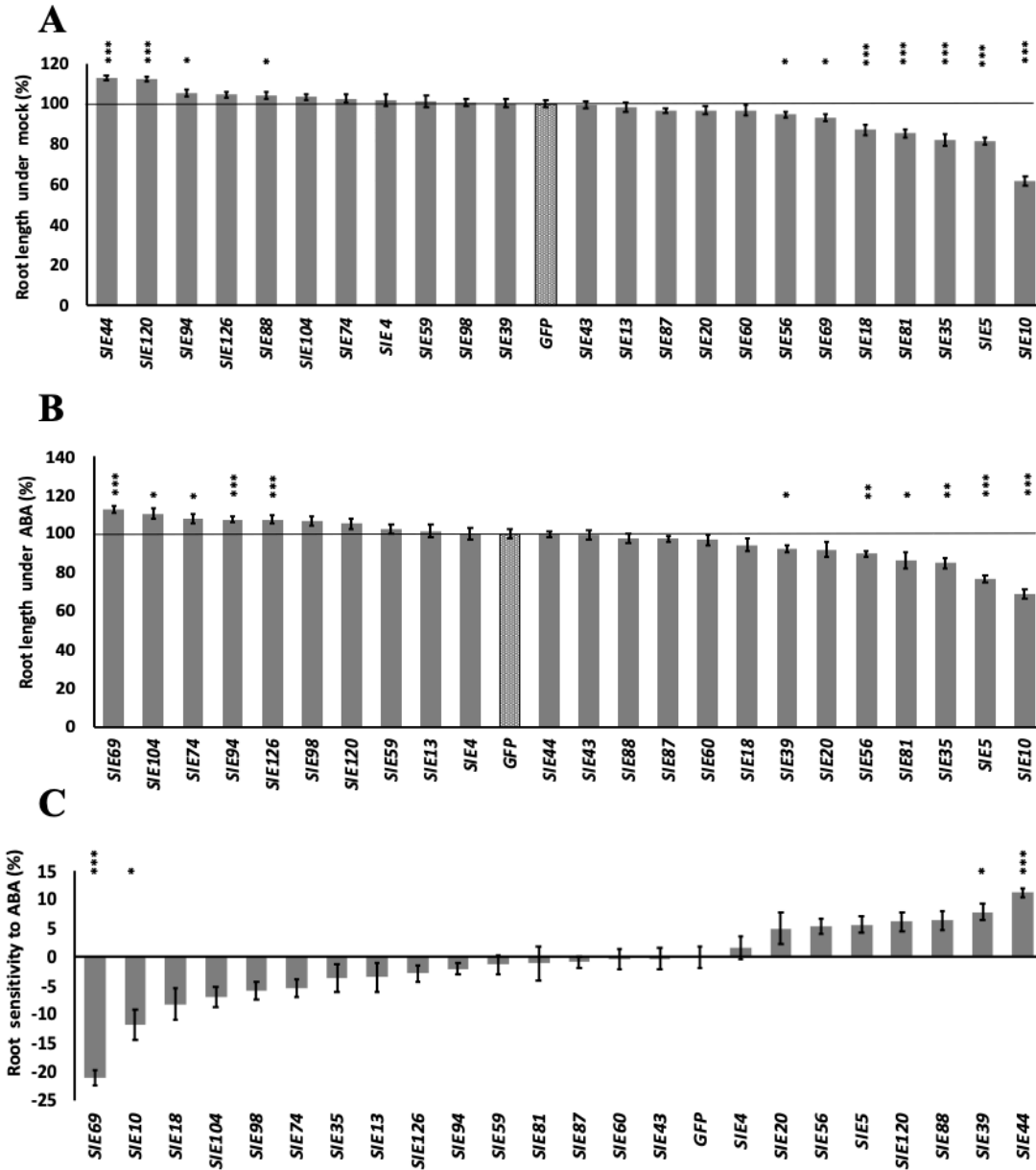


Figure 3.15: Root growth of *Arabidopsis thaliana* lines expressing *S. indica* (SIE) effectors.

Each SIE line represents a pool consisting of three independent lines. 7-day old seedlings were treated with sterile H₂O (mock) and 10 μM abscisic acid (ABA) by transferring the seedlings to mock and ABA containing media. After 7 days of treatment, the primary root length of each line was measured. Data from at least 42 plants per treatment is shown with standard errors. **A:** Root length of SIE-expressing lines relative to 100 % of control lines (*35S::GFP/Col-0*) under mock condition. **B:** Root length of SIE-expressing lines relative to 100 % of control lines upon ABA treatment. **C:** Root growth sensitivity of SIE-expressing lines to ABA treatment relative to the control line. Differences (root length of SIE lines + ABA / root length + mock) are shown as percentages (adjusted relative to 0% of control lines). Number of plants per line =45. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

Six effectors (*SIE10*, *SIE44*, *SIE69*, *SIE88*, *SIE94* and *SIE120*) were selected from the ABA screening of SIE pool lines for further studies (**Figures 3.16-3.21**). As observed from the SIEs pools, all the independent lines of *SIE10* significantly repressed root growth (compared to the control line) under mock and ABA treated conditions. The pool and independent lines expressing *SIE44* showed significant increase in root length under mock condition. Similar to the pool, *SIE88* significantly increased root length under mock treated condition. However, seeds from two independent lines of *SIE88* did not germinate. Also, the independent lines of *SIE94* increased root growth under mock, but the increase in root growth of one independent line did not differ significantly from the control line. *SIE120* significantly promoted root growth under mock and ABA treated conditions.

In relation to the ABA root sensitivity, all the independent lines of *SIE10* showed reduced sensitivity similar to the pool though this was not significant in all cases. All the independent lines of *SIE44*, *SIE88* and *SIE120* increased sensitivity to ABA as observed in their SIEs pools. *SIE94* pool showed slight decrease in ABA root sensitivity but all the independent lines exhibited significant increase in root sensitivity to ABA.

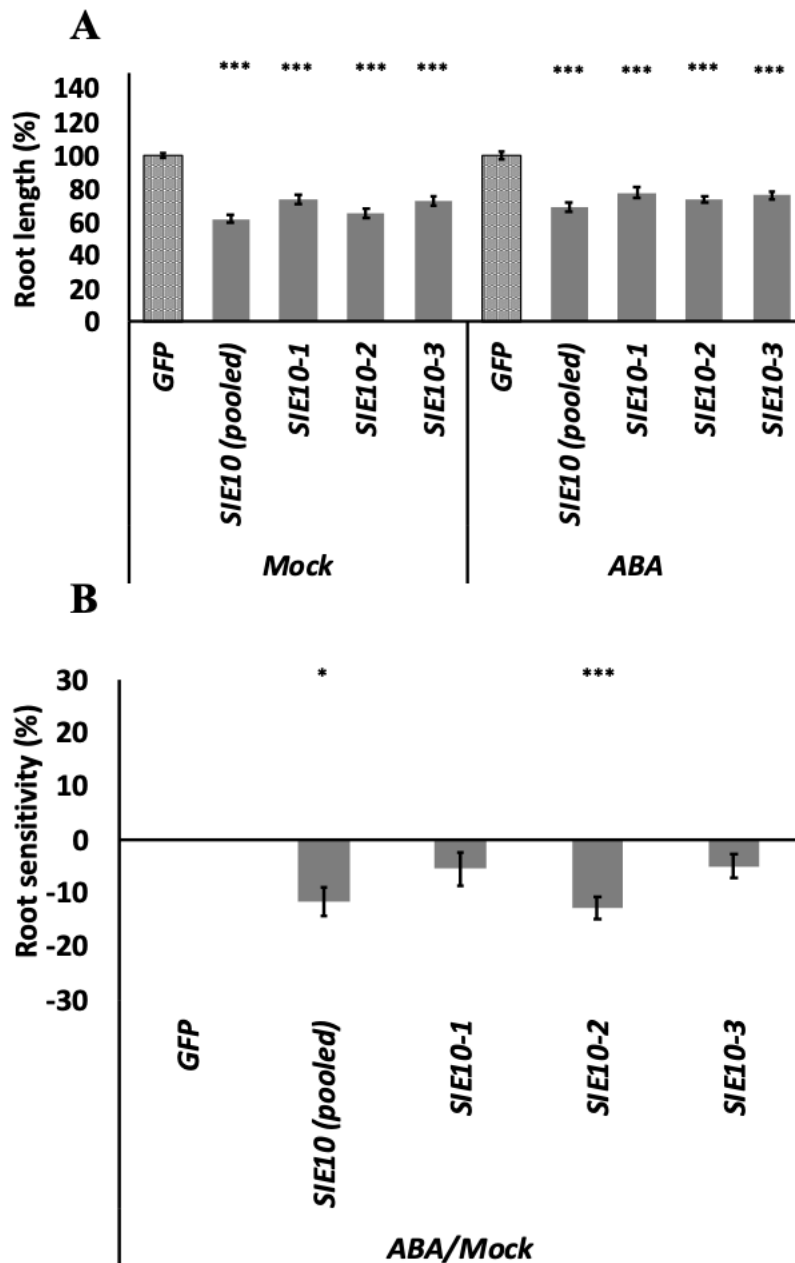


Figure 3.16: Phenotypic analyses of *A. thaliana* lines expressing *SIE10*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE10* relative to 100% of the control line (*35S::GFP*) under mock and abscisic acid (ABA)-treated conditions. 7-days old seedlings were treated with sterile H₂O (mock) or 10 μ M ABA. 7 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to ABA treatment of *SIE10* lines relative to the control line. Obtained differences in root length were adjusted relative to 0% of the control line. Number of plants per line =45. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. ***=significant at p-value of <0.001.

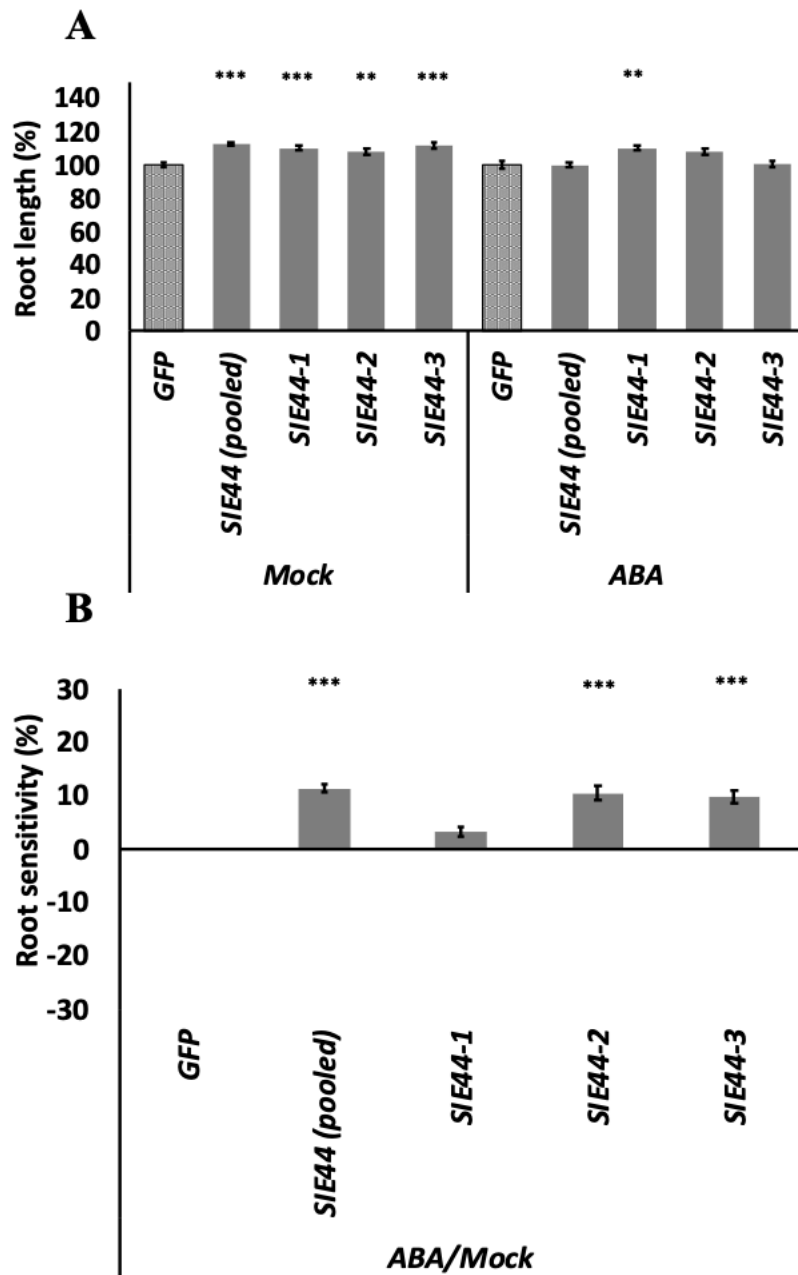


Figure 3.17: Phenotypic analyses of *A. thaliana* lines expressing *SIE44*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE10* relative to 100% of the control line (*35S::GFP*) under mock and ABA-treated conditions. 7-days old seedlings were treated with sterile H₂O (mock) or 10 μM abscisic acid (ABA). 7 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to ABA treatment of *SIE44* lines relative to the control line. Obtained differences in root length were adjusted relative to 0% of the control line. Number of plants per line =45. T-test was used to compare the lines expressing effectors with the control lines. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

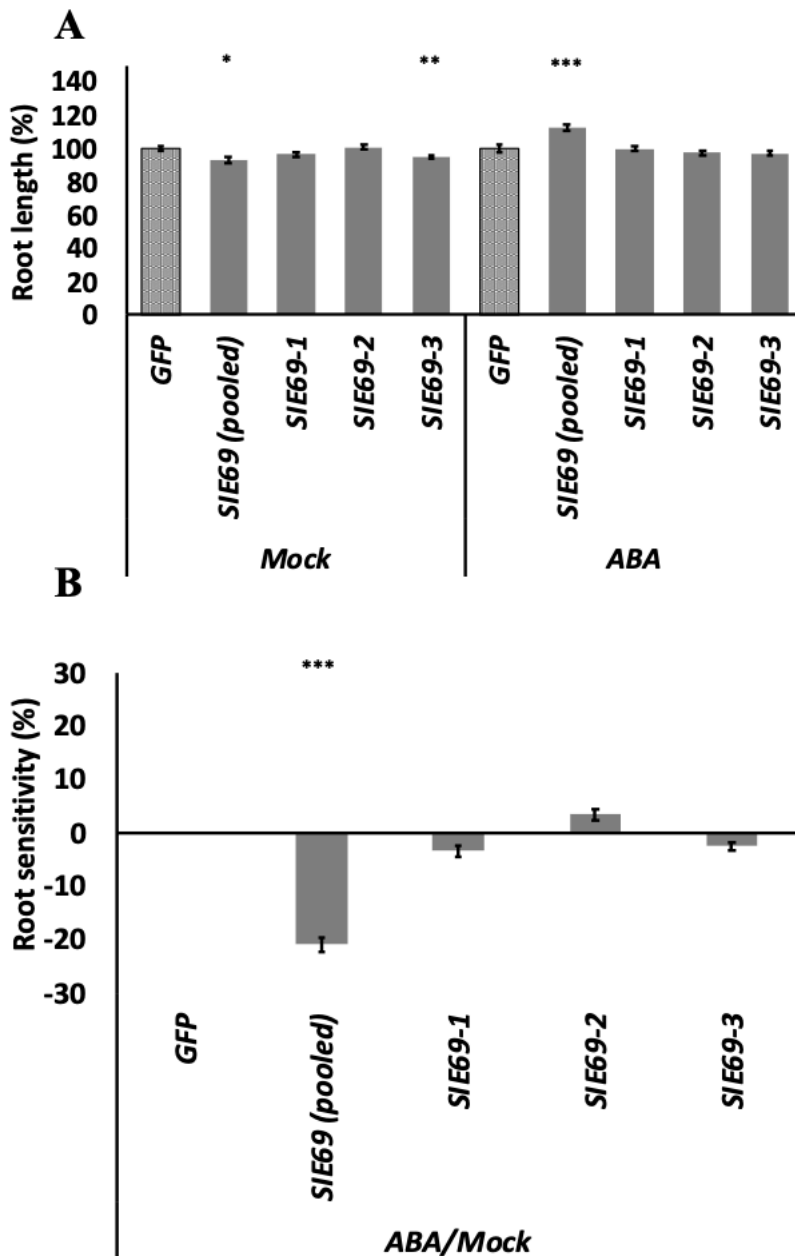


Figure 3.18: Phenotypic analyses of *A. thaliana* lines expressing *SIE69*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE10* relative to 100% of the control line (*35S::GFP*) under mock and ABA-treated conditions. 7-days old seedlings were treated with sterile H₂O (mock) or 10 μM abscisic acid (ABA). 7 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to ABA treatment of *SIE69* lines relative to the control line. Obtained differences in root length were adjusted relative to 0% of the control line. Number of plants per line =45. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

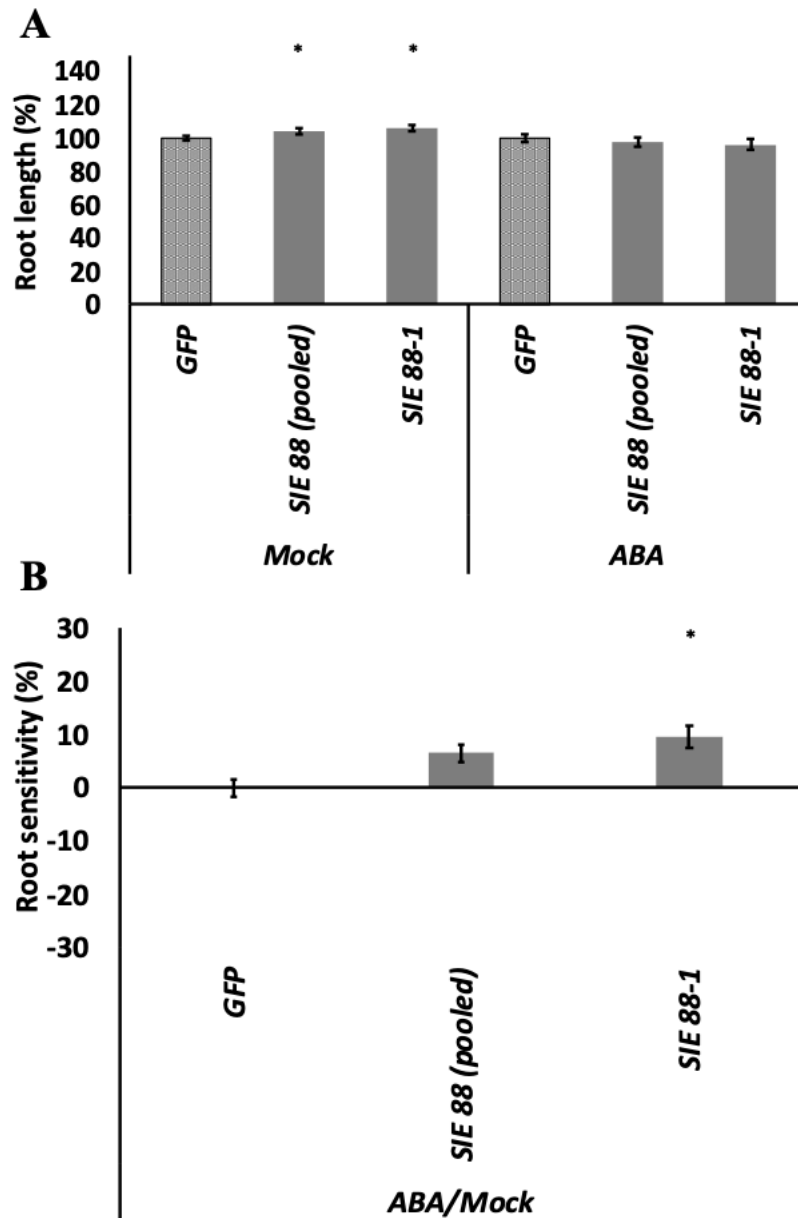


Figure 3.19: Phenotypic analyses of *A. thaliana* lines expressing *SIE88*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE88* relative to 100% of the control line (*35S::GFP*) under mock and ABA-treated conditions. 7-days old seedlings were treated with sterile H₂O (mock) or 10 μM abscisic acid (ABA). 7 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to ABA treatment of *SIE88* lines relative to the control line. Obtained differences in root length were adjusted relative to 0% of the control line. Number of plants per line =45. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05.

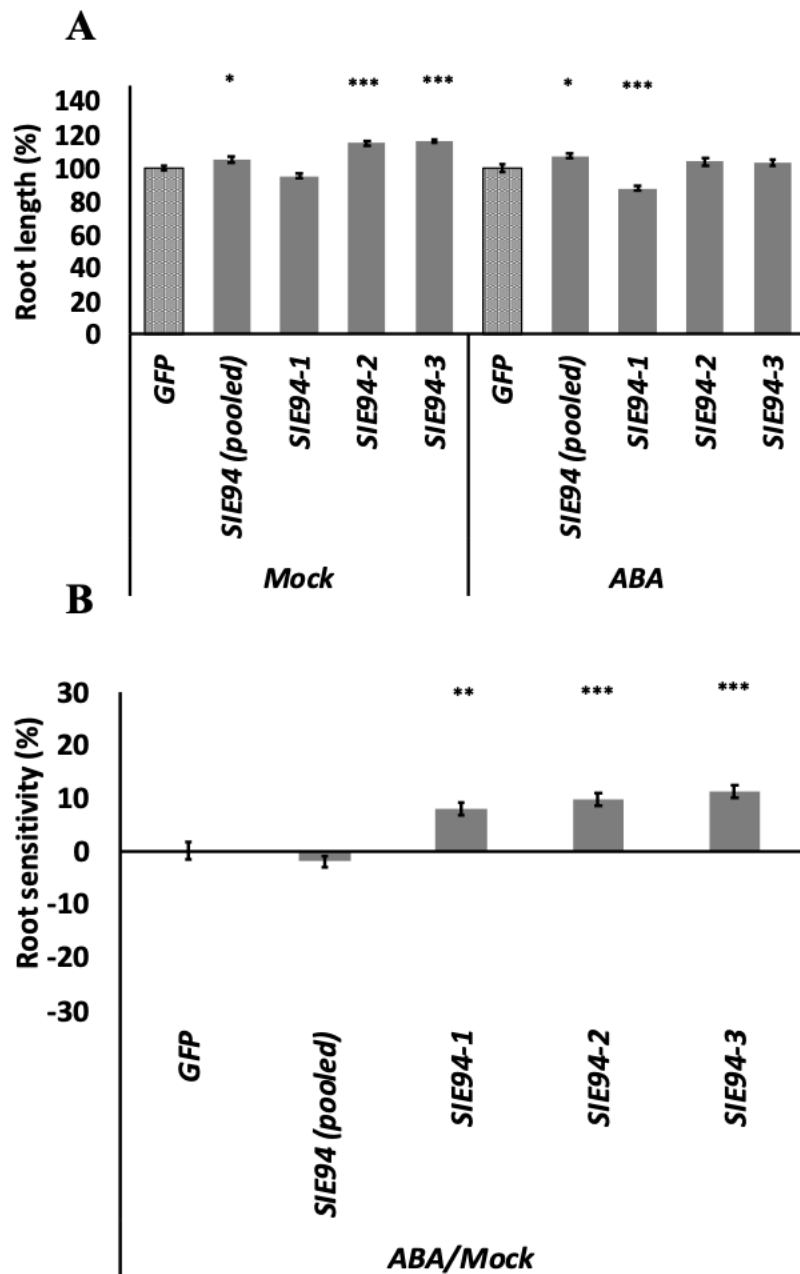


Figure 3.20: Phenotypic analyses of *A. thaliana* lines expressing *SIE94*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE94* relative to 100% of the control line (*35S::GFP*) under mock and ABA-treated conditions. 7-days old seedlings were treated with sterile H₂O (mock) or 10 μM abscisic acid (ABA). 7 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to ABA treatment of *SIE94* lines relative to the control line. Obtained differences in root length were adjusted relative to 0% of the control line. Number of plants per line =45. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

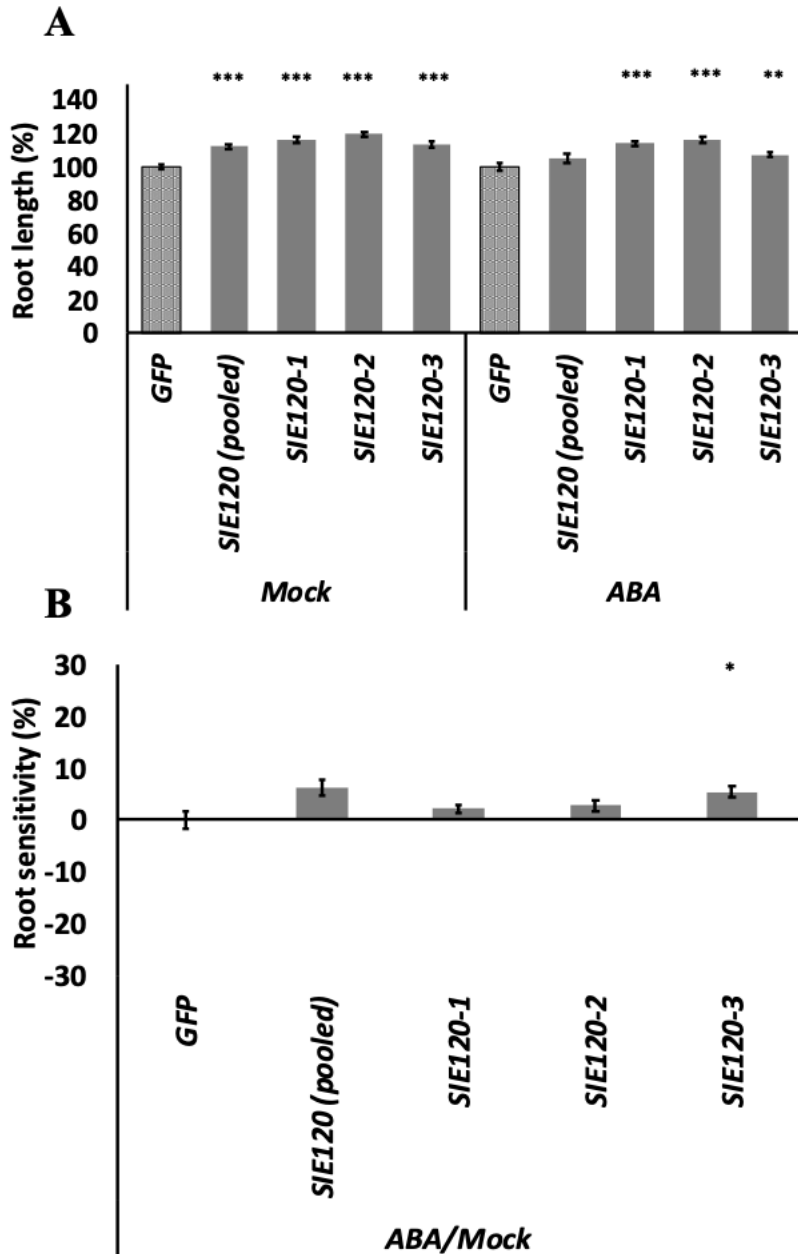


Figure 3.21: Phenotypic analyses of *A. thaliana* lines expressing *SIE120*.

A: Root length of pooled (combination of three independent lines) and independent lines expressing *SIE120* relative to 100% of the control line (*35S::GFP*) under mock and abscisic acid (ABA)-treated conditions. 7-days old seedlings were treated with sterile H₂O (mock) or 10 μM ABA. 7 days after treatment, primary root length was measured and mean values relative to the control line with standard errors are presented. **B:** Root growth sensitivity to ABA treatment of *SIE120* lines relative to the control line. Obtained differences in root length were adjusted relative to 0% of the control line. Number of plants per line =45. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

As reported, SMART was used to identify any known protein domains in six selected effectors (*SIE10*, *SIE44*, *SIE69*, *SIE88*, *SIE94* and *SIE120*). Two domains; inhibitor_I9 (proteinase propeptide inhibitor) and peptidase_S8 were found in *SIE10* (**Figure 3.22A**) and methyltransf_11 was detected in *SIE120* (**Fig. 3.25A**).

To further investigate effector functions associated with plant growth and abiotic stress tolerance, transcriptional expression of three ABA responsive marker genes *RAB18*, *RD29A* and *RD29B* was analysed in Arabidopsis lines expressing *SIE10*, *SIE44*, *SIE69* and *SIE120* (**Figures 3.22 - 3.25**). These lines were chosen mainly based on their altered root sensitivity to ABA and respective pools were used for the analysis. The hypothesis was that the effectors that altered root sensitivity to ABA could be through modulation of expression of ABA response markers. As might be expected, expression levels of these markers were higher under ABA as compared to mock conditions in the control line (**Figures 3.22 - 3.25**). All three ABA markers showed significant reduced expression in *SIE10*-expressing lines as compared to GFP expressing lines (control) under mock condition whereas lines expressing *SIE69* showed significant reduced transcript levels only for *RD29A* (**Figures 3.22 & 3.24**). Transcript levels of ABA marker genes were quantified in all SIE-expressing lines upon ABA treatment. In *SIE10*-expressing lines, *RD29A* and *RD29B* markers were upregulated (**Figure 3.22**). The lines expressing *SIE44* and *SIE120* showed reduced expression of *RD29A* in both replicates but differed significantly from the control line in one replicate (**Figures 3.23 & 3.25**).

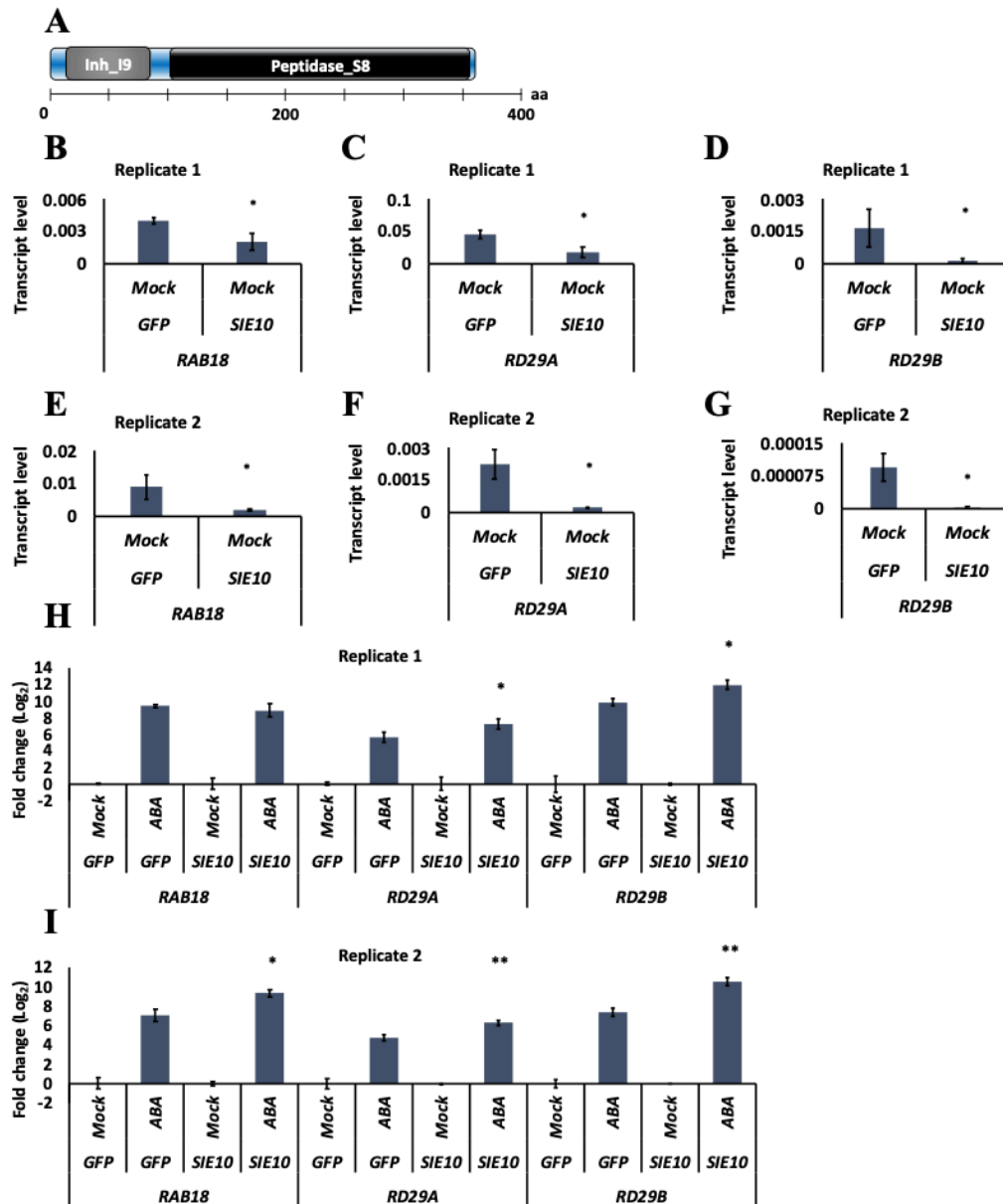


Figure 3.22: Functional analyses of *A. thaliana* lines expressing *SIE10*.

A: Schematic representation of Inhibitor_I9 (e-value = 1.4×10^{-11}) and Peptidase_S8 (e-value = 2.6×10^{-28}) protein domains identified in protein sequence of *SIE10* using Simple Architecture Research Tool (SMART). Inhibitor_I9 domain (amino acid (aa) position 14-87) is represented in gray while Peptidase_S8 (amino acid (aa) position 105-359) is represented in black. Blue indicates the total length of *SIE10*. **B-G:** Transcriptional expression of ABA markers *RAB18*, *RD29A* and *RD29B* in *A. thaliana* lines expressing *SIE10* on mock condition. 11-day old seedlings comprising pool of three independent lines were treated with sterile H₂O (mock) or 50 μ M ABA and the whole seedlings were harvested after 5 hours for qRT-PCR. *UBQ5* was used as a housekeeping gene. **H, I:** Fold change of ABA marker expression in control and *SIE10*-expressing lines upon ABA relative to mock treatment. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01.

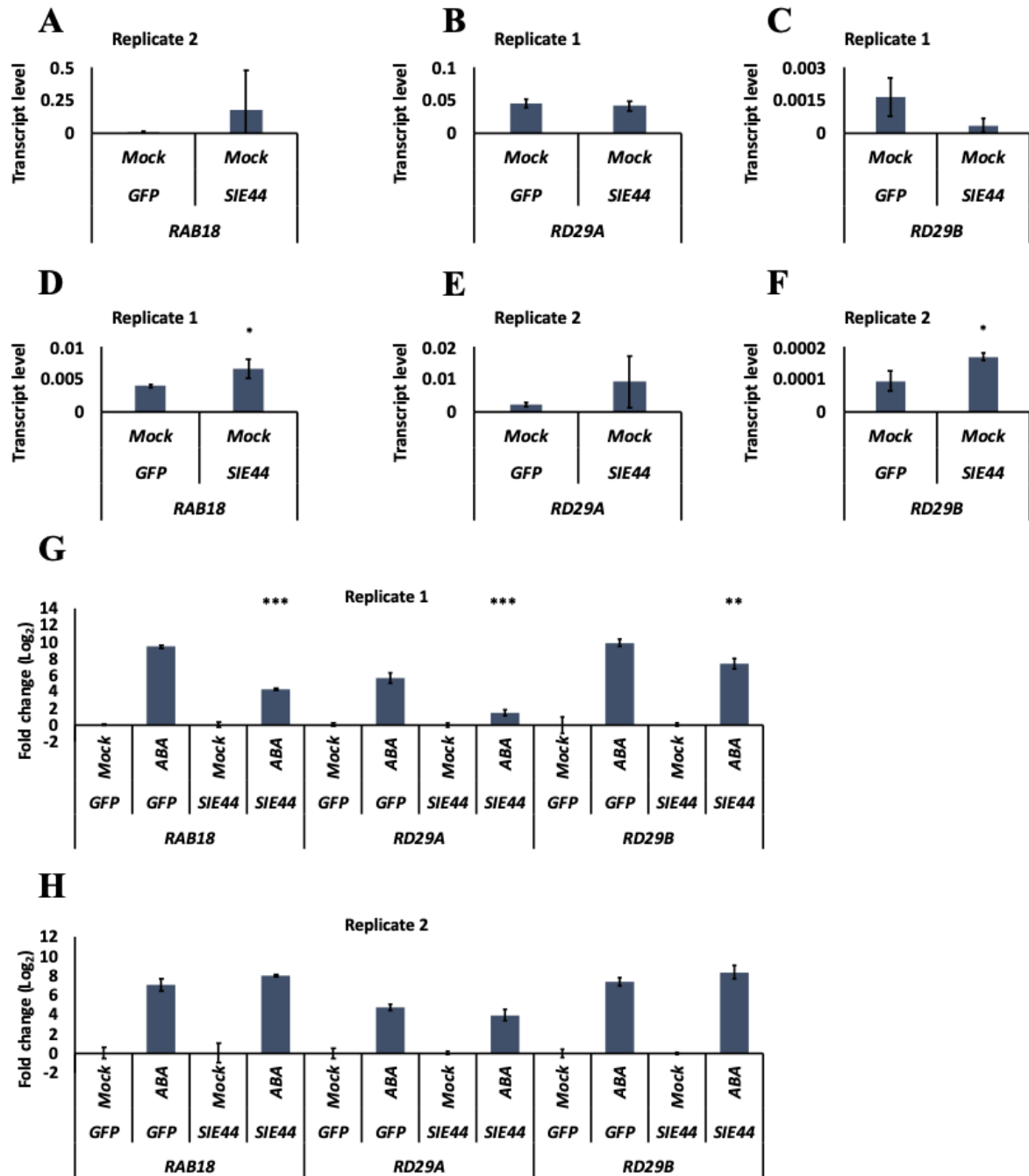


Figure 3.23: Functional analyses of *A. thaliana* lines expressing *SIE44*.

A-F: Transcriptional expression of ABA markers *RAB18*, *RD29A* and *RD29B* in *A. thaliana* lines expressing *SIE44* on mock condition. 11-day old seedlings comprising pool of three independent lines were treated with sterile H₂O (mock) or 50 μM ABA and the whole seedlings were harvested after 5 hours for qRT-PCR. *UBQ5* was used as a housekeeping gene. **G, H:** Fold change of ABA marker expression in control and *SIE44*-expressing lines upon ABA relative to mock treatment. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

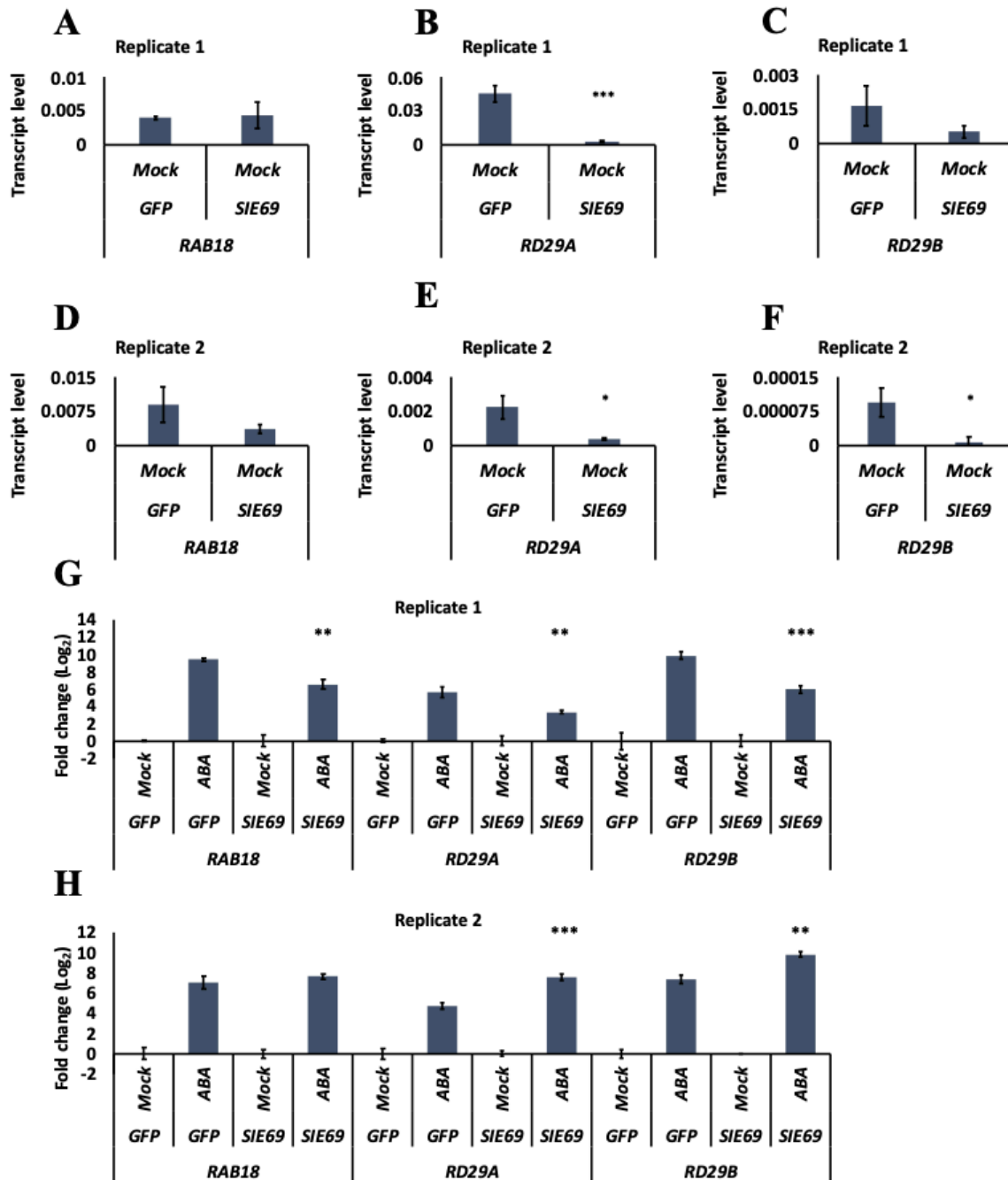


Figure 3.24: Functional analyses of *A. thaliana* lines expressing *SIE69*.

A-F: Transcriptional expression of ABA markers *RAB18*, *RD29A* and *RD29B* in *A. thaliana* lines expressing *SIE69* on mock condition. 11-day old seedlings comprising pool of three independent lines were treated with sterile H₂O (mock) or 50 μ M ABA and the whole seedlings were harvested after 5 hours for qRT-PCR. *UBQ5* was used as a housekeeping gene. **G, H:** Fold change of ABA marker expression in control and *SIE69*-expressing lines upon ABA relative to mock. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. **=significant at p-value of <0.01. ***=significant at p-value of <0.001.

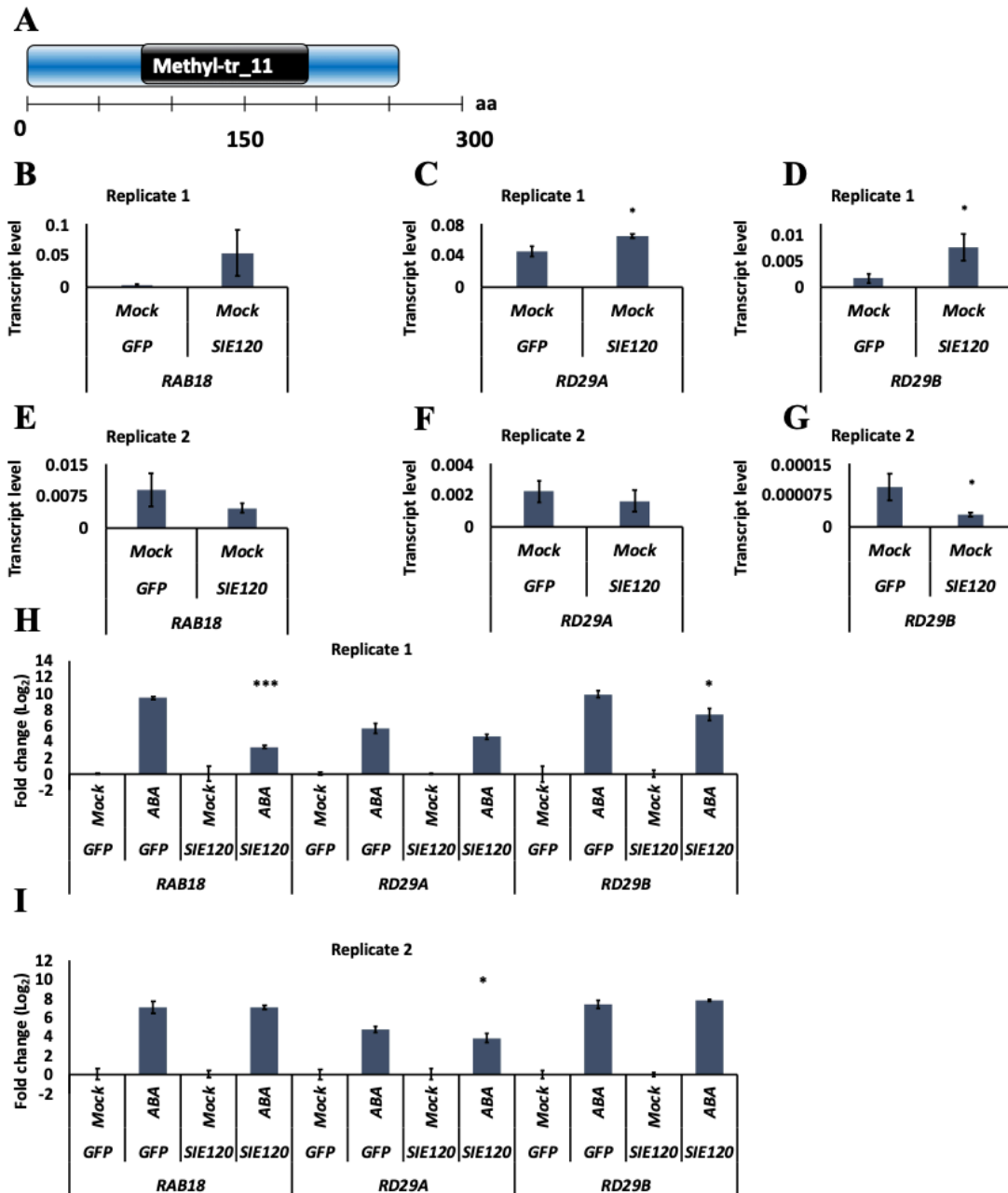


Figure 3.25: Functional analyses of *A. thaliana* lines expressing *SIE120*.

A: Schematic representation of Methyltransf_11 protein domain (E- value of 8.9e-0.9) identified in protein sequence of *SIE67* using Simple Architecture Research Tool (SMART). The domain (amino acid (aa) position 80-185) is represented in black while blue indicates the total length of *SIE120*. **B-G:** Transcriptional expression of ABA markers *RAB18*, *RD29A* and *RD29B* in *A. thaliana* lines expressing *SIE120* on mock condition. 11-day old seedlings comprising pool of three independent lines were treated with sterile H₂O (mock) or 50 μ M ABA and the whole seedlings were harvested after 5 hours for qRT-PCR. *UBQ5* was used as a housekeeping gene. **H, I:** Fold change of ABA marker expression in control and *SIE120*-expressing lines upon ABA relative to mock treatment. T-test was used to compare the lines expressing effectors with the control lines. *=significant at p-value of <0.05. ***=significant at p-value of <0.001.

3.4 Discussion

For the first time, the roles of large sets of effectors from a beneficial microbe were broadly studied in plants. Hassing et al. (2019) stressed that only a small number of effectors from plant beneficial microbes have been known and functionally described. This wide gap in knowledge has hindered the exploitation of effectors from beneficial microbes in boosting plant growth, yield and stress resistance as an alternative to the application of the live microbes as bio-fertilizers in the soil. Mutualistic (beneficial) microbes such as *Serendipita indica* might use their effectors to interact and confer the various benefits observed in their colonized host plants. In order to rapidly investigate the roles of effectors from *S. indica*, a protoplast-based assay was used to screen 150 *S. indica* effectors in *Arabidopsis thaliana*. Transient expression of effectors in protoplasts has proven to be a rapid and reliable technique for studying the functions of microbial effector proteins (Li et al., 2005; He et al., 2006). It provides the opportunity to test large sets of candidates in a medium to high-throughput manner (Zheng et al., 2014).

In our research group, various stresses are currently studied. The focus of this work is on the immunity elicitor flg22 and abscisic acid stress responses. 10 effectors that induce and 10 that suppress flg22 or ABA responses in the protoplast assay were identified by Dr Silke Lehmann in our research group. These experiments could support the previous studies that effectors from beneficial microbes have the ability to adapt plant growth under biotic or abiotic stresses. Interestingly, only two effectors (*SIE10* and *SIE87*) were identified to alter flg22 (biotic stress) and ABA (abiotic stress) responses. This suggests that as known from pathogen effectors (Sharpee et al., 2017; Huang et al., 2019); mutualistic effector proteins may alter specific pathways in host plants; i.e. they might affect only biotic stress or abiotic stress signalling.

Arabidopsis thaliana has been an important and commonly used model organism for studies of plant stress and growth physiology (Meyerowitz and Somerville, 1994; Meinke et al., 1998; Pyke, 1994). The candidate effectors selected from protoplasts screen were stably transformed and tested again in the model plant, *Arabidopsis thaliana*. The candidates selected for flg22 screening bioassay were firstly evaluated for growth- promoting activities. Some effectors improved root growth in *Arabidopsis* under both mock (up to 38 % increase) and flg22 conditions. This implies that some *S. indica* effectors could confer enhanced growth and yield in host plant, and that these growth enhancements still occur during stress.

Akum et al. (2015) showed that Arabidopsis lines expressing *S. indica* effector *PIIN_08944* exhibited a reduced expression of marker genes of flg22-induced PTI and the salicylic acid defence pathway. In terms of root sensitivity to flg22, using pooled lines of 9 effectors significantly altered the sensitivity as compared to the control line. However, when independent lines for 4 SIEs (*SIE76*, *SIE67*, *SIE99* and *SIE146*) were screened, only *SIE67* and *SIE76* confirmed the results. It might indicate that certain lines dominate phenotypes observed for the pools. That is why use of homozygous lines (T3) is much better than T2 used in this study. There is possibility that 25 % of wild type from the T2 seeds contributed to the dissimilarities between phenotypes observed in pools vs. individual lines, thus a pool effect. In addition, position of the effectors on the chromosome that could change their expression and number of gene copies might contribute to slight dissimilarities. It further highlights the significance to use individual lines for the functional analyses of effector activities.

Effector proteins are highly polymorphic, which might reflect their constant evolution to avoid plant recognition (Sharpee et al., 2017; Huang et al., 2014; Yang et al., 2008). As a result, effectors often do not have any clear protein domain homology. In my analysis of six effectors aforementioned for flg22 assay, DJ-1_PfpI was found in *SIE67*. The DJ-1/ThiJ/PfpI superfamily represents a diverse group of proteins found in almost all organisms (Wei et al., 2007) (Smith and Wilson, 2017). However, only a few members of this superfamily have been functionally described (Zhan et al., 2014) and those that have been characterized seemed to be involved in stress resistance. For instance, the PH1704 protease found in *Pyrococcus hoikoshii* OT3 has hyper-thermophilic activities. In addition, *Escherichia coli* heat-shock protein 31 (*Hsp31*) that is described as a chaperone and peptidase also belongs to this superfamily (Lee et al., 2003; Mujacic and Baneyx, 2007; Sastry et al., 2002; Shendelman et al., 2004) as is *Escherichia coli* *YhbO*, which is involved in hyperosmotic or acid stress responses. Expression of *DJ-1* transcripts in rice (*OsDJ-1*) was increased in response to different abiotic stresses such as salinity, drought, cold, heat and oxidative stresses (Ghosh., et al 2016). Likewise, the transcript level of *AtDJ-1A* in Arabidopsis was induced in response to different external stresses including strong light, CuSO₄, H₂O₂ and methyl viologen (Xu, et al., 2010). Presence DJ-1_PfpI domain in *SIE67* might play a vital role in enhancing stress resistance in plants. In addition, the expression of three immunity marker genes (*FRK1*, *PHI-1* and *NHL10*) was analysed in four effector lines (*SIE67*, *SIE76*, *SIE99* and *SIE146*) to assess their function in altering PTI. However, expression of all the immunity markers tested in line expressing *SIE67*

did not show consistent pattern in the two replicates. More studies on *SIE67* are needed to understand its roles in plant stress resistance.

The functions of *S. indica* effectors were further investigated for their potential roles in abiotic stress integration in plants. Abscisic acid (ABA) was used to stimulate abiotic stress response in *Arabidopsis thaliana* transformed with different effectors. For this purpose, we employed a protoplast-based assay and studied plants constitutively expressing the effectors that strongly altered ABA pathway. Some effectors consistently promoted or suppressed root growth in *A. thaliana* plant under mock and ABA-treated conditions. It is likely that not all effectors from beneficial microbes mediate beneficial effects in plants and those that suppress growth, for instance, might be part of a process that facilitates host colonisation and accommodation. In turn, other beneficial effectors could be recruited thereafter to strengthen host plants against different environmental cues or support plant development under stress. Six effectors (*SIE10*, *SIE44*, *SIE69*, *SIE88*, *SIE94* and *SIE120*) expressing lines were selected for further studies. *SIE44*, *SIE88* and *SIE120* significantly promoted growth under mock conditions while growth reduction was observed in lines expressing *SIE10*. The former effector candidates might therefore represent a resource to decipher processes regulating growth and yield in plants. *SIE10* also showed reduced root sensitivity to ABA whereas *SIE44*, *SIE88* and *SIE120* increased it suggesting *SIE10* could be involved in integrating development with stress adaptation. *SIE10* might therefore be an interesting candidate to study and understand the interplay of stress and developmental pathways in plants and which processes actually disturb plant growth under stress. In this respect it is important to highlight the ability of *S. indica* to uncouple growth-immunity trade-offs (Jacobs et al. 2011). The uncoupling of the growth-immunity antagonism will enhance plant immunity and growth concomitantly.

Protein domain searches with *SIE10*, *SIE44*, *SIE69*, *SIE88*, *SIE94* and *SIE120* revealed known domains only in *SIE10* and *SIE120*. Two domains; inhibitor_I9 (proteinase propeptide inhibitor) and peptidase_S8 were found in *SIE10*. The I9 family of protease inhibitors have been found in fungi (Hohl et al., 2017) and specifically inhibit the S8 family of serine peptidases, which comprise subtilisins in bacteria and plants, kexin in yeast and related mammalian proprotein convertases (secretory mammalian serine proteinases close to subtilisin-like enzymes in bacteria) (Schaller et al., 2012). The peptidase_S8 family (also called subtilisin or subtilase family) is the second largest family of serine proteases (Rawlings et al., 2010). They are among the large group of degrading enzymes found in almost all organisms

(Muszewska et al., 2011). Fungi use them to penetrate and colonize their host (Varshney et al., 2016). Li et al. (2017) further reported that subtilases are generally present in fungi and are thought to have unique functional molecular abilities that could enable the adaptation of fungi to new environments and hosts.

Considering the functions of peptidase_S8 protein domain (though under control of inhibitor_I9) identified in *SIE10* and the reduced root length observed in *SIE10* expressing lines under mock and ABA-treated conditions, the results might suggest that *SIE10* has a function in host colonization. Plett et al. (2014) shown that effector protein (*MiSSP7*) from mycorrhiza is essential for colonization of host trees by modulating the host protein *PtJAZ6* that is a negative regulator of jasmonic acid signalling. Schäfer et al. (2009) emphasized that *S. indica* relies on cell death of host roots at the late stage of interaction to enable colonization. However, this cell death-linked colonization does not cause root necrosis as seen in hemibiotrophic or necrotrophic fungi colonization with their hosts (Schäfer et al., 2009). In addition, the reduced expression of the three ABA markers (*RAB18*, *RD29A* and *RD29B*) observed in *SIE10* expressing under mock condition hinted its modulating capacity. This possibly explains the reduced root growth observed in both the pool and independent lines of this effector under mock and ABA treated conditions.

A methyltransf_11 protein domain was identified in *SIE120*. Methyltransferases are enzymes that are responsible for catalysing the transfer of a methyl group from S-adenosyl-l-methionine to different substrates such as DNA, RNA, proteins, and small-molecule metabolites (Cai, 2016). Lines expressing *SIE120* and *SIE44* upon ABA treatment that showed reduced induction of *RD29A* compared to the line expressing GFP in both replicates could explain the increased root sensitivity to ABA in the pool and independent lines of those two effectors.

In conclusion, these analyses demonstrated that some *S. indica* effectors promoted growth in *Arabidopsis thaliana*. *SIE44*, *SIE76*, *SIE106* and *SIE120* increased root growth when stably expressed in *Arabidopsis*. Thus, the findings represent a first important step for further studies of those effectors in order to exploit their potential function in improving crop performance (e.g. growth and yield). In addition, *SIE10* might play an essential role in host colonisation. These studies revealed the presence of a peptidase_S8 family domain in *SIE10*, a domain that has been shown to facilitate pathogenic and mutualistic associations with host plants. In addition, the reduced expression of the ABA markers observed in *SIE10* expressing lines under

mock compared to the control line further showed that it could have a modulating capacity. This points to *SIE10* as promising for future work to further understand its roles in host colonization and more attention will be on its modulation on immunity markers. In addition, the lines expressing this effector can be tested with bacterial pathogen such as *Pseudomonas syringae*.

It will be interesting to see which function the DJ-1_PfpI protein domain plays in *SIE67* as it has been reported to be involved in microbial stress resistance. Although further studies are needed, *SIE67* could be an important genetic source for boosting plant stress resistance. Towards these goals a barley root transformation platform has been established (see next chapter) that will be used in future to study the function of these effectors in a monocot crop. Barley was chosen because it is a model crop that is fairly easy to genetically transform, and one of the most important global crops.

4 Chapter 4

Optimization of a barley scutella transformation system for rapid genetic studies

4.1 Introduction

Barley has been one of the most important global cereal crops, the fourth most abundant and consumed cereal crop after wheat, rice and maize in the world (Bartlett et al., 2008). Because of being used for food, brewing and animal feed (Hisano and Sato, 2016), it has been used as a model crop in cereal genetic studies for many years (Kumlehn et al., 2006); especially for crops such as bread wheat with its larger and more complex genome. With its diploid genome, barley is much more amenable to transformation than hexaploid bread wheat. As such, a highly efficient and reliable barley transformation system is required. Efficient, high-throughput, and cost-effective transformation systems are very essential to enable the functional analysis of important genes of potential interest in crop breeding programmes. Most common, transformation system are employed for the analysis of single gene functions by either over-expressing the gene of interest or by silencing it using RNA interference-based approaches. Particle bombardment of immature embryo was the first method successfully used to produce transgenic Golden promise barley plants. (Wan and Lemaux, 1994). Attempts have been made to transform other varieties/genotypes but Golden promise is the most responsive cultivar for transformation (Harwood et al., 2009). Other techniques such as DNA uptake, electroporation and microinjection have been used to transform this barley variety but at low rate (El-Assal et al., 2011). In the struggle to improve transformation efficiency, *Agrobacterium tumefaciens*-mediated transformation technique has been established in the past (Tingay et al., 1997). In contrast to other techniques, particle bombardment in particular, *Agrobacterium*-mediated method is more efficient, introduces a lower number of copies per transgene, is more stable over generations and is less prone to gene silencing (Travella et al., 2005). Immature embryos are the preferred target tissue for transformation because of the high transformation efficiency (Harwood et al., 2009) though other tissues such as androgenetic pollen (Jaehne et al., 1994), shoot meristematic tissues (Zhang et al., 1999), protoplasts (Funatsukiet al., 1995) and isolated zygotes (Holm et al., 2000) have been used. *A. tumefaciens*-mediated transformation has produced over 10,000 transgenic barley plants (Marthe et al., 2015).

Despite the substantial progress that has been made in barley transformation, a number of challenges still remain (Harwood, 2012). Following transformation of immature embryo or pollen, it takes at least 1 year to generate homozygous transgenic lines that can be used for genetic studies. In addition to being exceedingly time consuming, the maintenance of transformed lines is very laborious (Imani et al., 2011). The absence of more rapid, high-throughput transformation systems in cereal crops that can keep pace with ‘omics’-derived knowledge from transcriptome, proteome, or metabolome studies is a major drawback and limits our abilities to functionally examine and develop novel traits in crops (Imani et al., 2011). Imani et al. (2011) developed a more appropriate method in this respect by generating stably transformed roots directly from calli that can be used for rapid functional studies of gene functions in abiotic and biotic resistance in barley. They termed the method Stable Root Transformation System (STARTS). This system is more time efficient and as regenerated roots can be used for functional analyses, and it is also meant to precede and complement conventional transformation systems. In other words, only those genes that have been confirmed to have a valuable function will enter conventional transformation procedures to produce homozygous plants while lines with no phenotype can be discarded at an early stage. The procedure has thus helped in facilitating and accelerating the identification of promising candidate genes to whole plant analyses.

Imani et al. (2011) showed that this method is suitable for rapid functional analysis of genes that underlie root development, pathogenesis and symbiosis. They provided evidence that the method was also suitable for gene silencing using RNA interference and protein localization studies. With the rapid advance in next-generation-sequencing technology, more crop genomes will be available but efficient, rapid and large-scale examination of gene/protein functions in plant will be indispensably needed (Imani et al., 2011). STARTS is a very promising tool to enable more rapid pre-screening of gene/protein functions. However, there are still some challenges; not all parts of an embryo are initially transformed resulting in a chimeric tissue of transformed and untransformed cells. Consequently, roots generated from such tissues will also have both transformed and untransformed fragments interlaced together. This will lead to a variation in the expression of genes of interest. Therefore, more uniformly transformed calli and, thus, homogeneously expressing roots will ensure accurate and uniform expression of genes of interest. Thus, accurate examination and interpretation will be facilitated.

For these reasons, the objective of this work was to optimize STARTS and develop it as a stable root/calli transformation system that combines high transformation efficiency with a high uniformity in the expression of transgenes across calli and regenerated root tissue. In this study, calli and roots that showed high uniformity in transformation patterns were obtained, which was not reported in STARTS developed by Imani et al. (2011). The ultimate aim was to employ the optimised system for the functional analyses of *Serendipita indica* effectors and other potential genes of interest in a crop-based system rather than only in model *Arabidopsis thaliana*.

4.2 Results

4.2.1 Generation of transformed scutella and calli

Barley transformation was carried out based on the protocol published by Imani et al. (2011) and some modifications were systematically introduced. The steps that enabled generation of calli and roots with uniformity of transformation patterns were optimised. The immature embryos (scutella) were carefully extracted from sterilized barley seeds as shown in **Figure 4.1**. The scutella extracted were carefully dissected to remove the embryonic axis to avoid calli formation from the axis tissue (that cannot be transformed) (**Figure 4.1D**). This is the most critical stage of embryo preparation and has significant impact on the transformation success. Any damage of the embryo or delay in dissection can significantly reduce transformation efficiency and intensity.

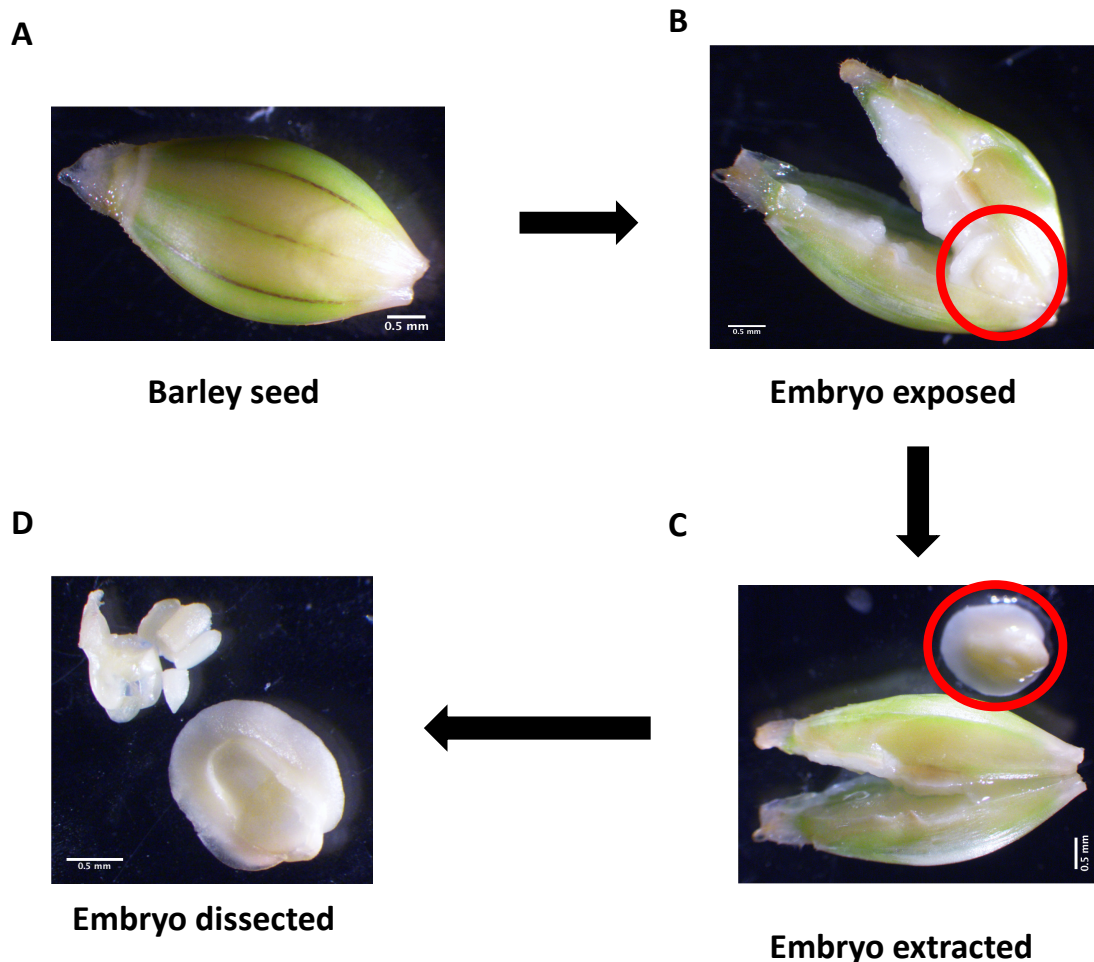


Figure 4.1: Preparatory stages of barley seed embryos (scutella) for transformation.

A: Sterilized immature barley seed. **B:** Exposed embryo after careful removal of the seed coat husk using tweezers. The red circle shows the embryo. **C:** Embryo detached from the seed. **D:** Dissection of the embryo by removing the embryonic axis from the scutella.

To monitor transformation success, scutella were transformed with *pUBII::GFP* (Figure 4. 2). Thus, *GREEN FLUORESCENT PROTEIN (GFP)* expression indicates all transformed cells and tissue areas (Figure 4.2A-C). All potentially transformed scutella were also examined for autofluorescence (Figure 4.2D-F) and bright field to detect any tissue browning as an indicator of tissue damage or necrosis (Figure 4.2 G-I) visualization used for selection of transformed and healthy scutella. In general, transformed scutella varied in the expression of *GFP*, thus scutella were categorised into three groups based on their *GFP* intensity: high, medium and low intensity as shown in Figure 4.3. The transformed scutella with high *GFP* intensities showed green fluorescence in almost all the boundaries where the embryonic axis was removed, while those with low *GFP* intensity expressed fluorescence in a small portion of the boundaries.

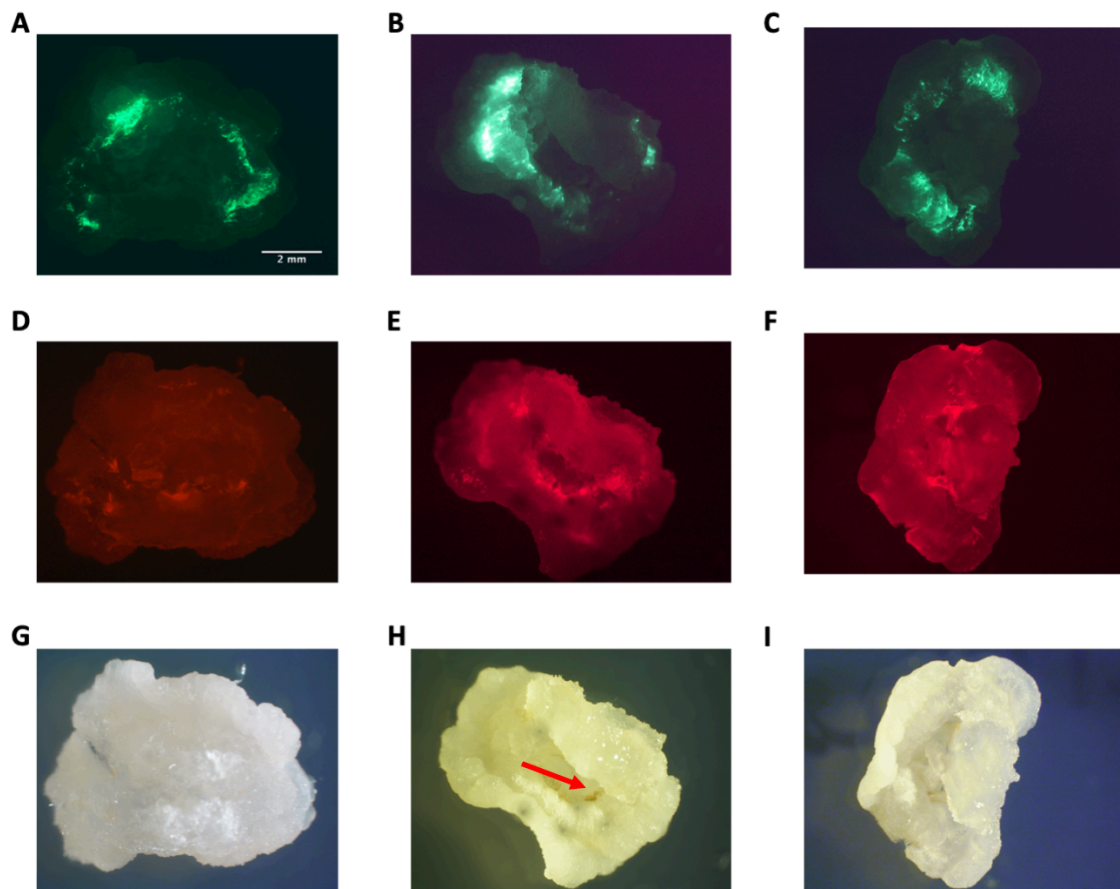


Figure 4.2: Representative images of scutella at 7 days after transformation.

Scutella were transformed with *pZmUBI5::GFP*. **A-C**: The transformed scutella were selected on hygromycin containing media. Scutella cells expressing *GFP* were identified with epifluorescence microscopy (excitation: 488 nm, emission: 509 nm). **D-F**: Scutella analyzed for unspecific autofluorescence (excitation: 561 nm, emission: 575 nm). **G-I**: Scutella images taken under bright field conditions. No autofluorescence was detected. The red arrow points to the browning on the scutellum (**H**). The scale bar shown (2mm) are the same for all the images.

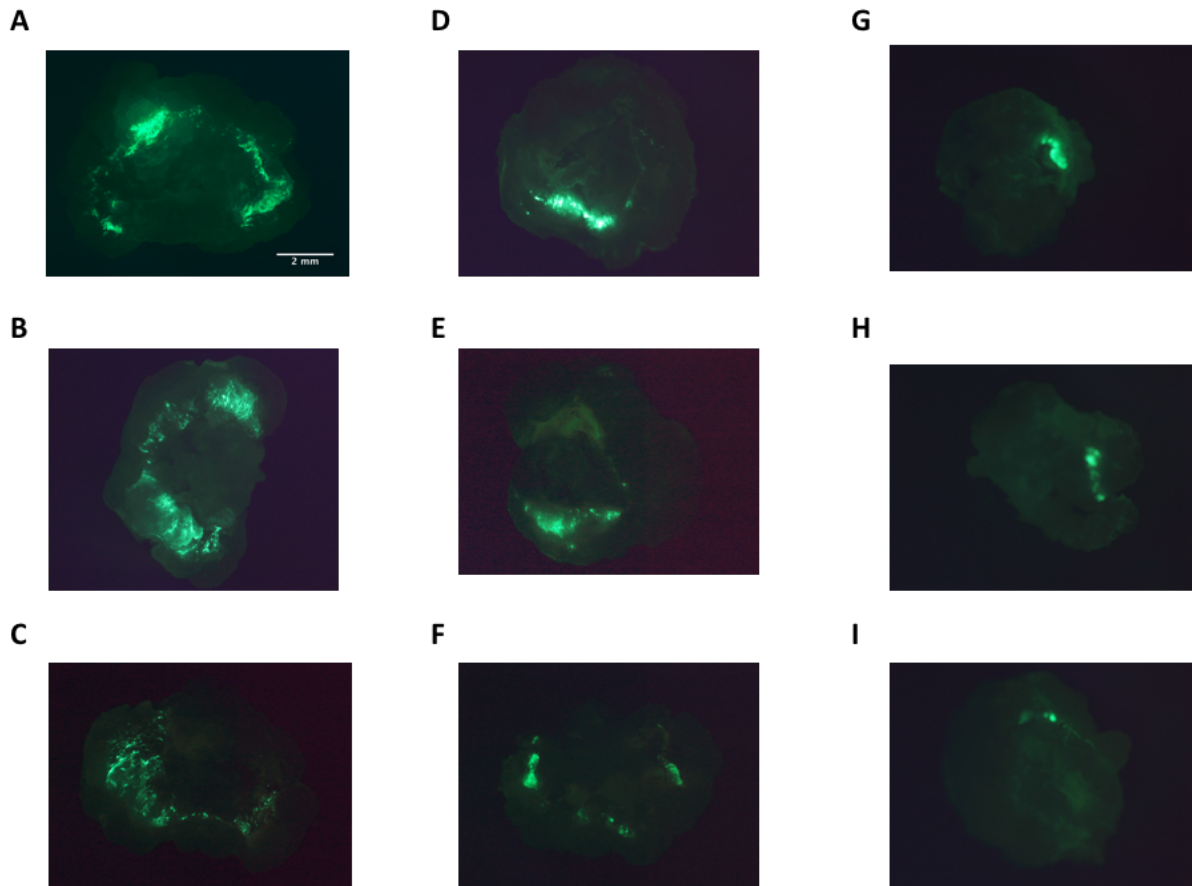


Figure 4.3: Degrees of *GFP* expression used for classifying transformation intensity of scutella.

A-I: Scutella with high (**A-C**), medium (**D-F**) and low transformation intensity (**G -I**). Transformation intensity is a measure of transformed area relative to total area of the scutella intended to be transformed. All images were taken 9 days after transformation. *GFP* were identified with epi-fluorescence microscopy (excitation: 488 nm, emission: 509 nm). The scale bar shown (2mm) are the same for all the images.

High transformation efficiency (70-100 %) and expression of transgene intensity are very crucial in any transformation experiment. High transformation expression of transgene shows that almost all the periphery where the embryonic axis was removed from the scutella is transformed (**Figure 4.3A-C**). However, in the case of calli, high transformation intensity is also a measure of a very large area of each callus being uniformly transformed compared to its untransformed parts. After a series of resilient efforts, the transformation efficiency, and intensity (scutella with high green fluorescence intensity) were progressively optimised up to 100% and 70%, respectively (**Figure 4. 4A**). The transformation intensity took more work to rise appreciably as compared to the transformation efficiency. The percentage of transformed

scutella with lesions was not found to be a widespread issue (**Fig. 4.4A**). The progressive increase was a result of continual improvement in technical expertise required for barley transformation. In order to ensure high transformation efficiency and intensity, two different concentrations (12.5 mg/l and 25 mg/l) of hygromycin (antibiotics used for selection of positive transformants) were tested. High transformation efficiency and intensity were obtained with both hygromycin concentrations, (**Fig. 4.4B**). Although, there was no significant difference in transformation efficiency or intensity, scutella exposed to higher concentrations (25mg/l) appeared to show higher transformation results. On average, transformation efficiency of 96 % was obtained from scutella selected on 25 mg/l hygromycin containing media, while 87 % was observed on those on 12.5 mg/l hygromycin containing media. The transformation intensity was ~74% for 25 mg/l and 51% for 12.5 mg/l hygromycin.

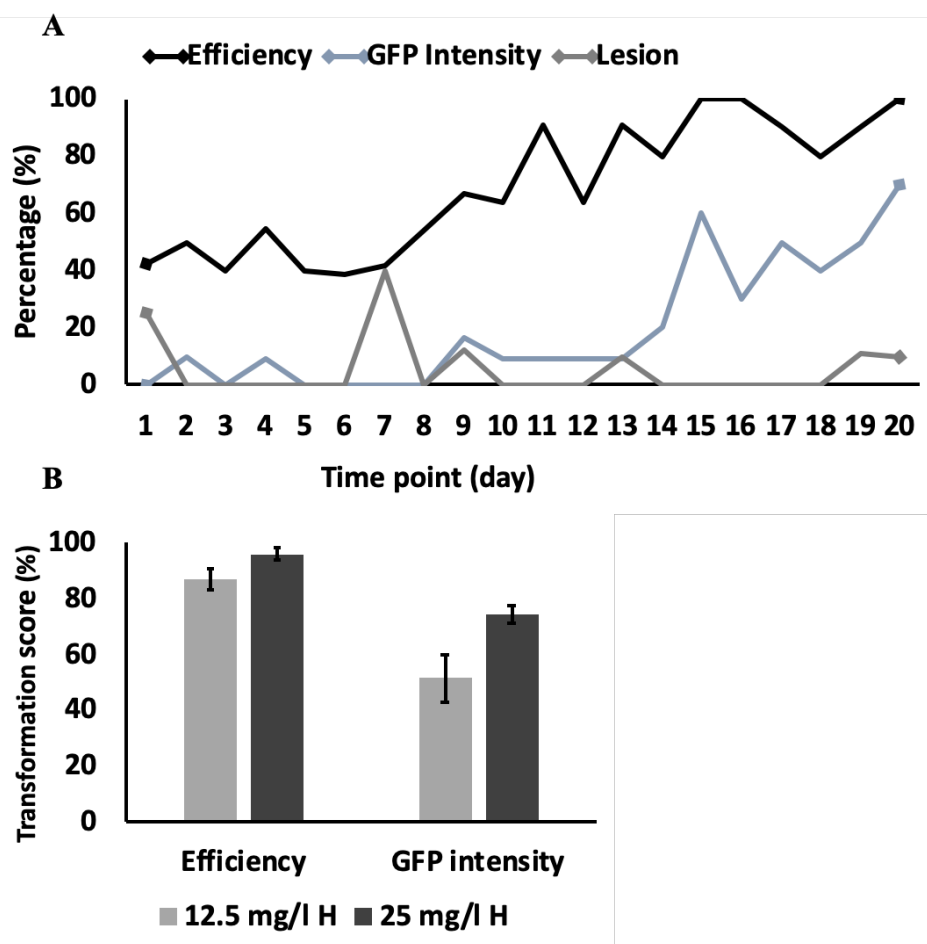


Figure 4.4: Transformation efficiency, intensity and the occurrence of lesions of scutella.

A: Improvement in transformation efficiency, intensity and lesion percentage after many attempts. **B:** Transformation efficiency and intensity of scutella under different hygromycin (H) concentrations at 7 days after transformation. T-test was used to compare the scutella under different hygromycin concentrations.

Scutella were transferred to callus-inducing media to produce calli (**Figure 4.5**). However, some portions of the calli formed were not transformed. The transformed area was found to be much smaller than the non-transformed portion (**Figure 4.6**). In an attempt to understand why the calli formed had both transformed and non-transformed parts, the stability transformation efficiency of transformed scutella/calli were monitored on a weekly basis (**Figure 4.7**) for 3 weeks. These analyses revealed that the transformation efficiency was approximately stable over time, but all the calli produced had both transformed and non-transformed portions intermingled together as previously shown in **Figure 4.6**.

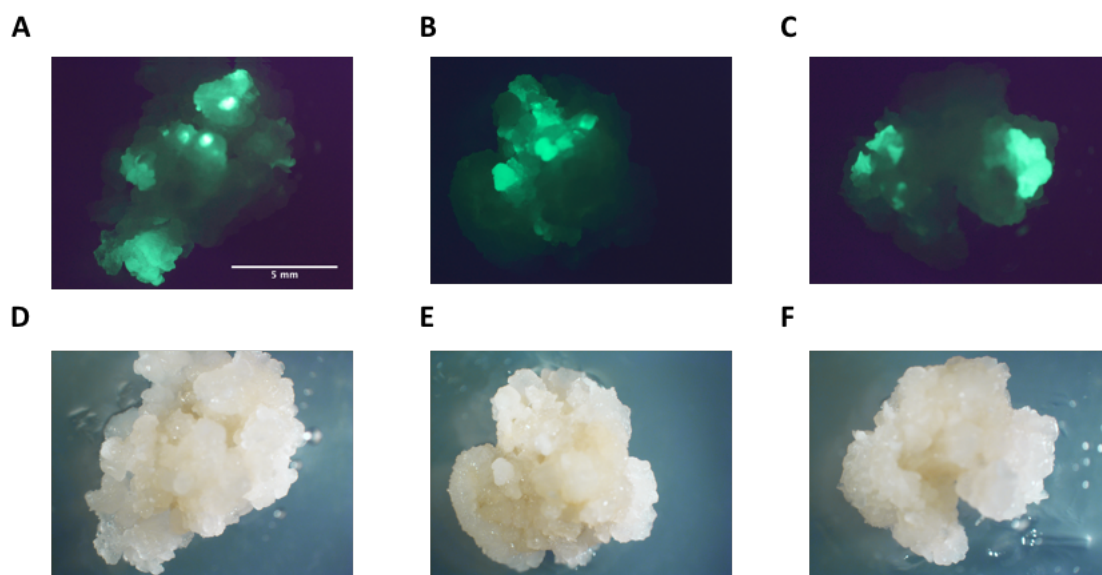


Figure 4.5: Calli generated from the transformed scutella.

The calli were generated from scutella that showed high transformation intensity. **A-C**: Representative calli expressing *GREEN FLUORESCENT PROTEIN (GFP)* were monitored using epi-fluorescence microscopy (excitation: 488 nm, emission: 509 nm). **D-F**: Bright field images of calli shown in **A-C**. All images were taken 4 weeks after transformation. The scale bar shown (5mm) are the same for all the images.

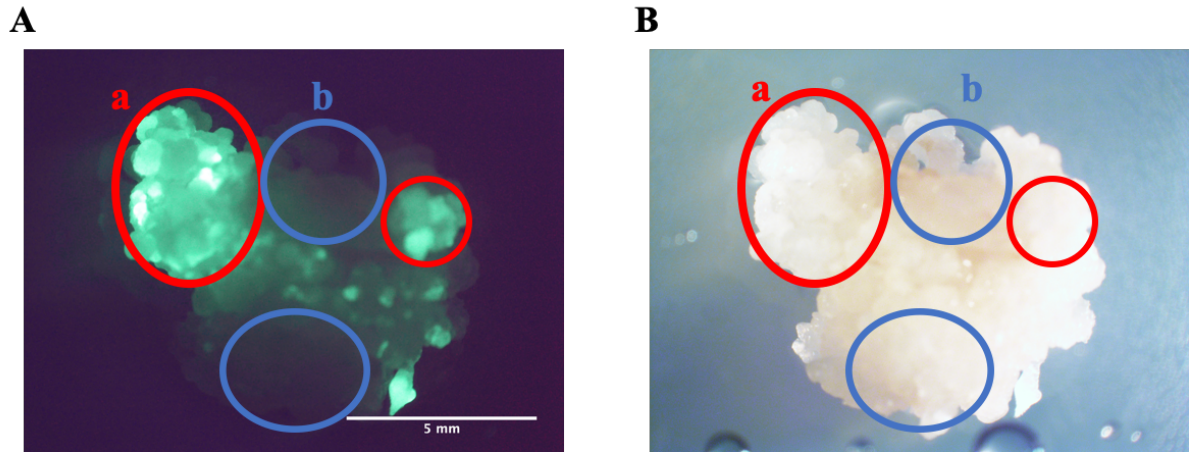


Figure 4.6: Callus generated from the transformed scutella.

A: The transformed parts are represented with red circles (a) while the non-transformed portions are designated with blue circles (b). **B:** Bright field image of A. Both images were taken 4 weeks after transformation. *GFP* were identified with epi-fluorescence microscopy (excitation: 488 nm, emission: 509 nm). The scale bar shown (5mm) are the same for all the images.

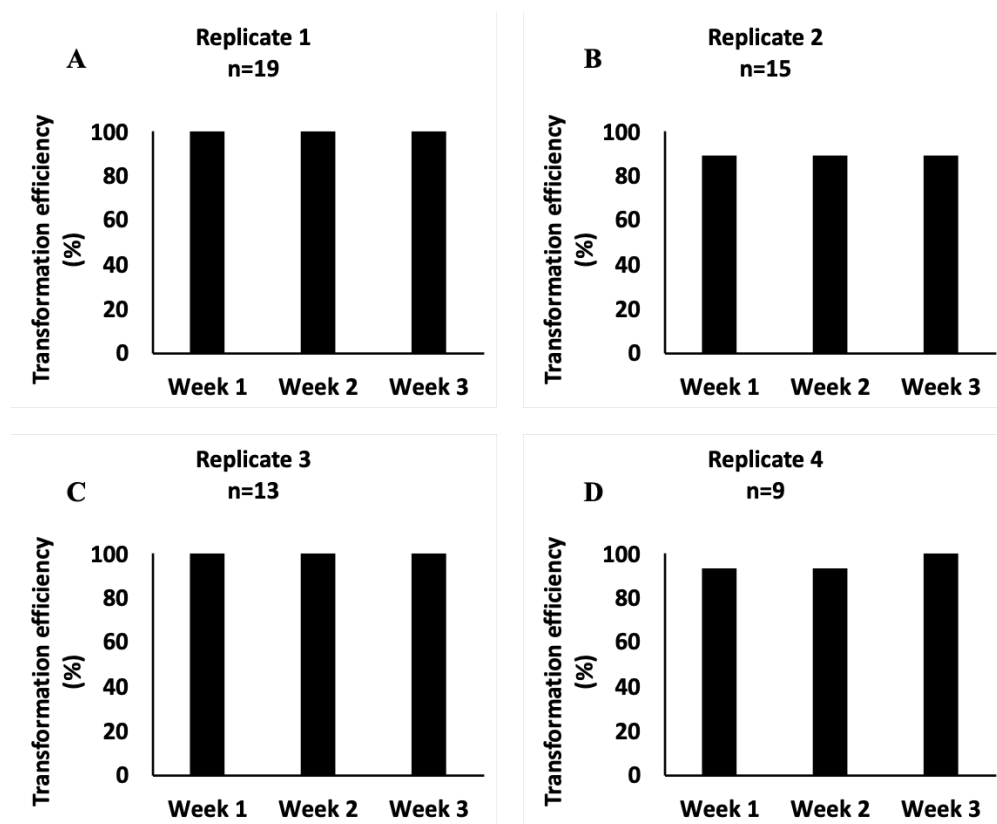


Figure 4.7: Transformation efficiencies of developing calli over period of time.

The number of scutella (n) used was 19, 15, 13 and 9 in A, B, C, & D, respectively.

4.2.2 Generation of transformed roots from the calli

Calli produced from scutella on 12.5 mg/l and 25 mg/l hygromycin were transferred to RIM containing the same hygromycin concentrations. Roots generated from calli (**Figure 4.8**) were categorized according to their length into three groups 14 days after transfer: long (> 3 cm in length), medium (1-2 cm in length) and short roots (< 1cm in length). Although use of higher hygromycin concentration seemed to result in a higher number of roots in all categories, observed differences were not statistically significant (**Figure 4.9A**). Each callus produces approximately 12 roots on 12.5 mg/l containing media. In terms of root categories, each callus produced ~3 small, ~4 medium and ~5 long roots. Callus placed on 25 mg/l containing media yielded ~ 17 roots with root categories of ~5 small, ~5 medium, and 7 long roots.

To validate root transformation efficiencies, the number of GFP transformed roots per callus was quantified. A callus produced approximately 5 and 12 GFP expressing roots on 12.5 and 25 mg/l containing media, respectively (**Figure 4.9B**). This could be further categorised into 1 small, 1, medium and 2 long, transformed roots on RIM containing 12.5 mg/l hygromycin, calli grown on RIM with 25 mg/l hygromycin generated on average 3 small, 3 medium, and 5 long, transformed roots per callus. This shows that a callus is likely to produce more transformed long roots than medium/short roots. The percentage of the transformed roots out of the total roots generated was 40.4 in 12.5 mg/l containing media (**Figure 4.9C**). The percentage of the transformed roots produced from 25 mg/l containing media was 68.5 % (**Figure 4.9C**). Although relatively homogenous roots were obtained, generating ~ 100 % transformed roots with uniform transformation (fluorescence) intensity would be more suitable for functional gene studies.

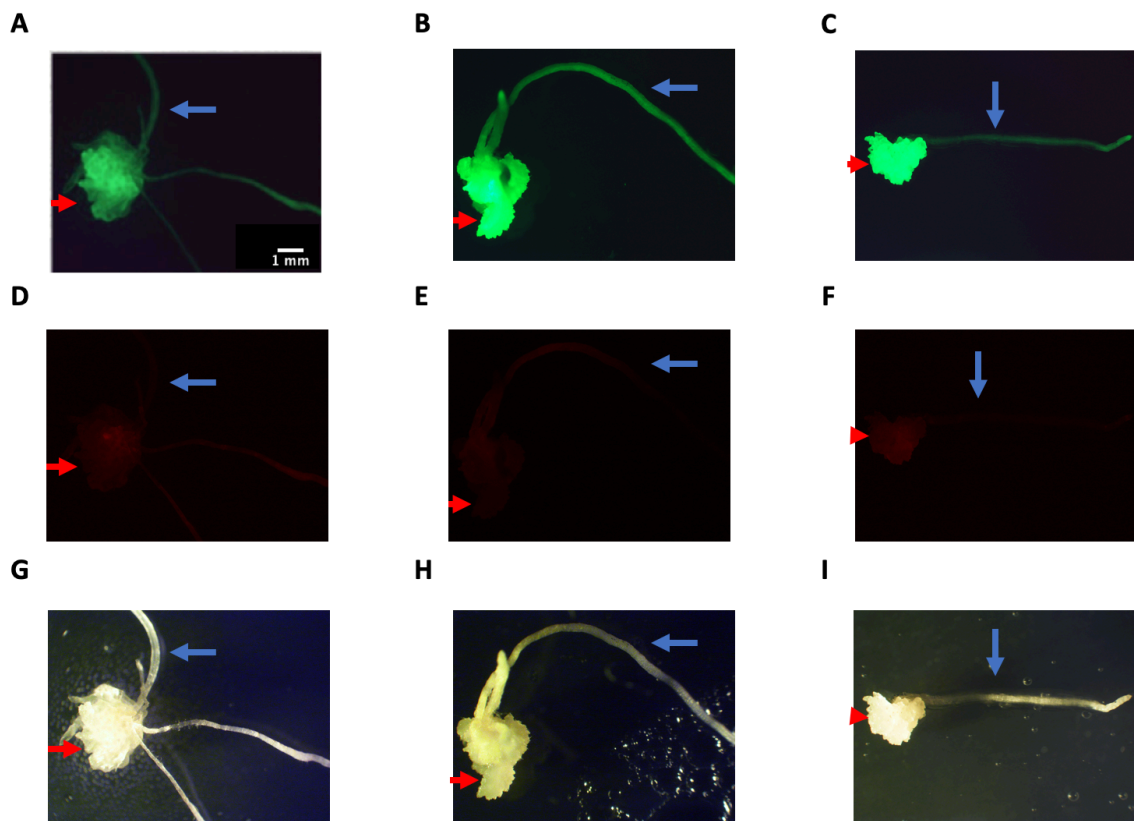


Figure 4.8: Representative images of transformed roots generated from calli.

A -C: Roots expressing GFP were identified with epi-fluorescence microscopy (excitation: 488 nm, emission: 509 nm). **D-F:** Respective roots under red filter condition analyzed for unspecific autofluorescence (excitation: 561 nm, emission: 575 nm). **G-I:** Respective Root images taken under bright field condition. The scale bar shown (1mm) are the same for all the images. The red arrows showed the calli, while the blue arrows indicated the roots.

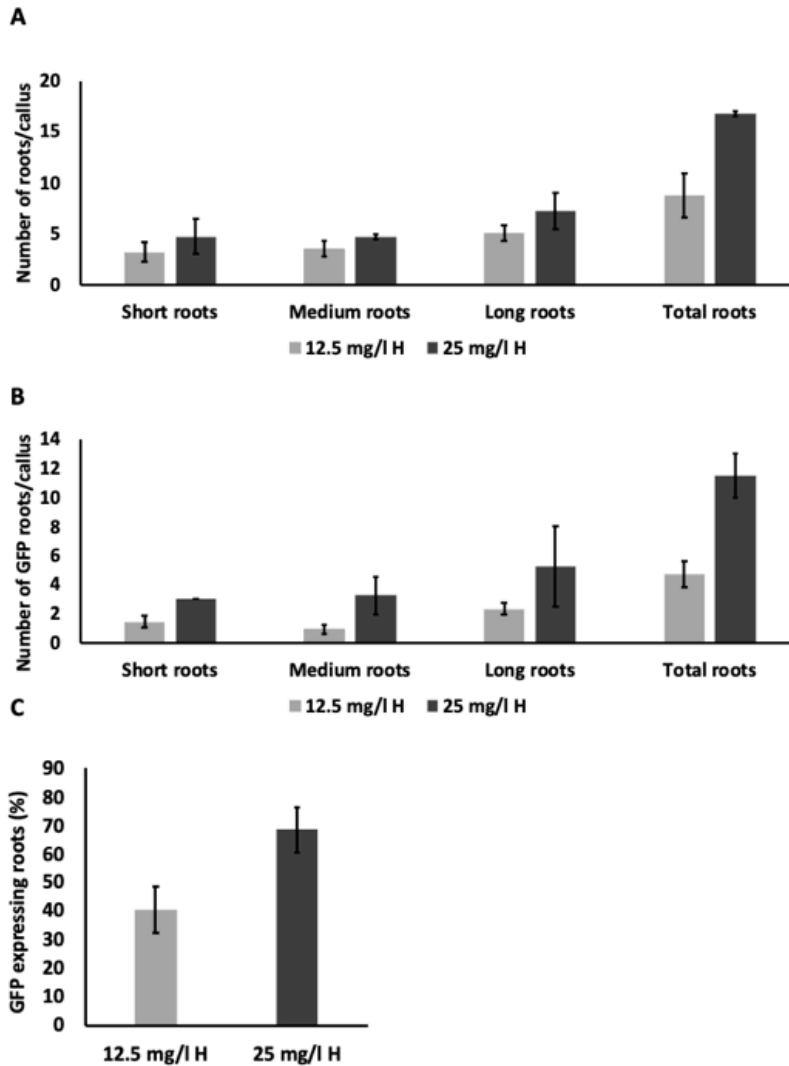


Figure 4.9: Roots lengths and distribution generated from transformed calli.

A: Number of roots generated per callus under different hygromycin (H) concentrations. **B:** Number of transformed roots generated per callus under different hygromycin concentrations. Roots were categorized into three groups: short (< 1cm in length), medium (1-2 cm in length) and long roots (> 3 cm in length). **C:** Percentage of GFP-transformed roots under different hygromycin concentrations. T-test was used to compare the scutella under different hygromycin concentrations.

An optimised barley root transformation system for functional studies is urgently needed. A major drawback in the generation of transformed roots from calli is that not all areas of a callus are transformed. Consequently, not all roots that develop from a callus are transgenic (**Figure 4.9C**). To elevate the number of transformed roots requires a more homogeneous transformation of calli. Therefore, those parts of calli that were uniformly transformed and showed a stable high GFP expression were separated from their ‘mother’ calli (**Figure 4.10**).

These ‘separated’ parts of calli were regenerated for 3 weeks to form mature calli that were transferred to RIM. All roots regenerated from these calli were evenly transformed based on their uniform GFP expression patterns. This approach of separating transformed parts of calli for the regeneration of transformed roots might be highly suitable for functional analyses. It might enhance the robustness and reproducibility of phenotypic and genetic studies. In summary, the optimized barley calli/root transformation system for functional studies is summarized in **Figure 4.11**.

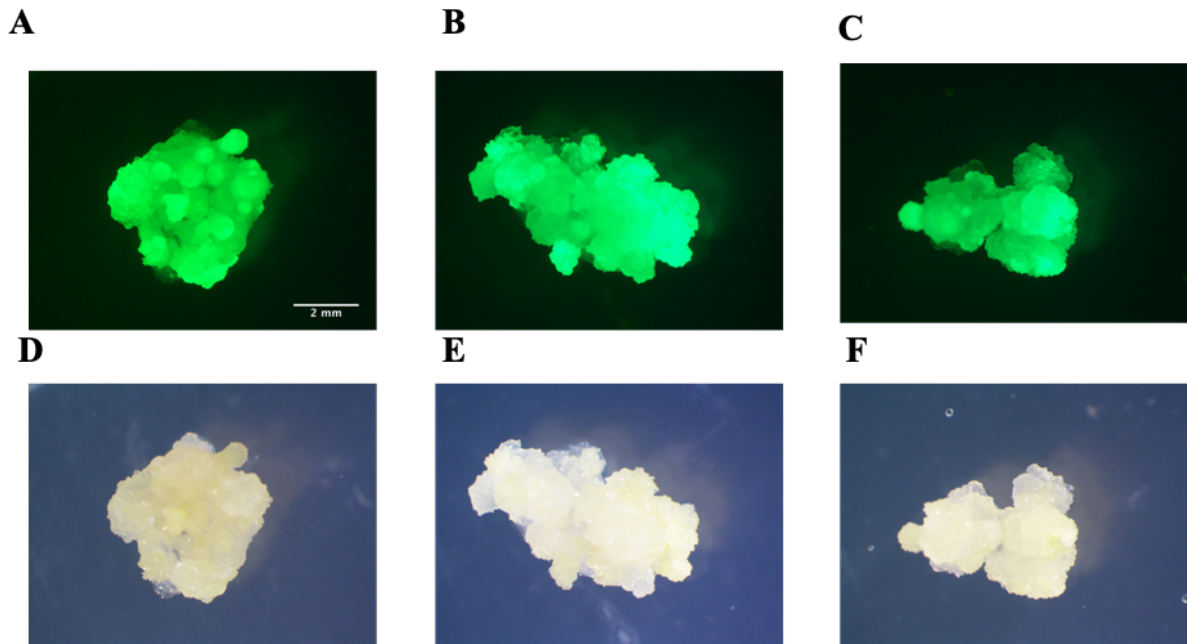


Figure 4.10: Uniformly transformed calli parts under regeneration to form mature calli.

A-C: Representative calli parts expressing *GFP* were monitored using epi-fluorescence microscopy (excitation: 488 nm, emission: 509 nm). **D-F:** Bright field images of calli shown in **A-C**. All images were taken 8 days of regeneration. The scale bar shown (2mm) are the same for all the images.

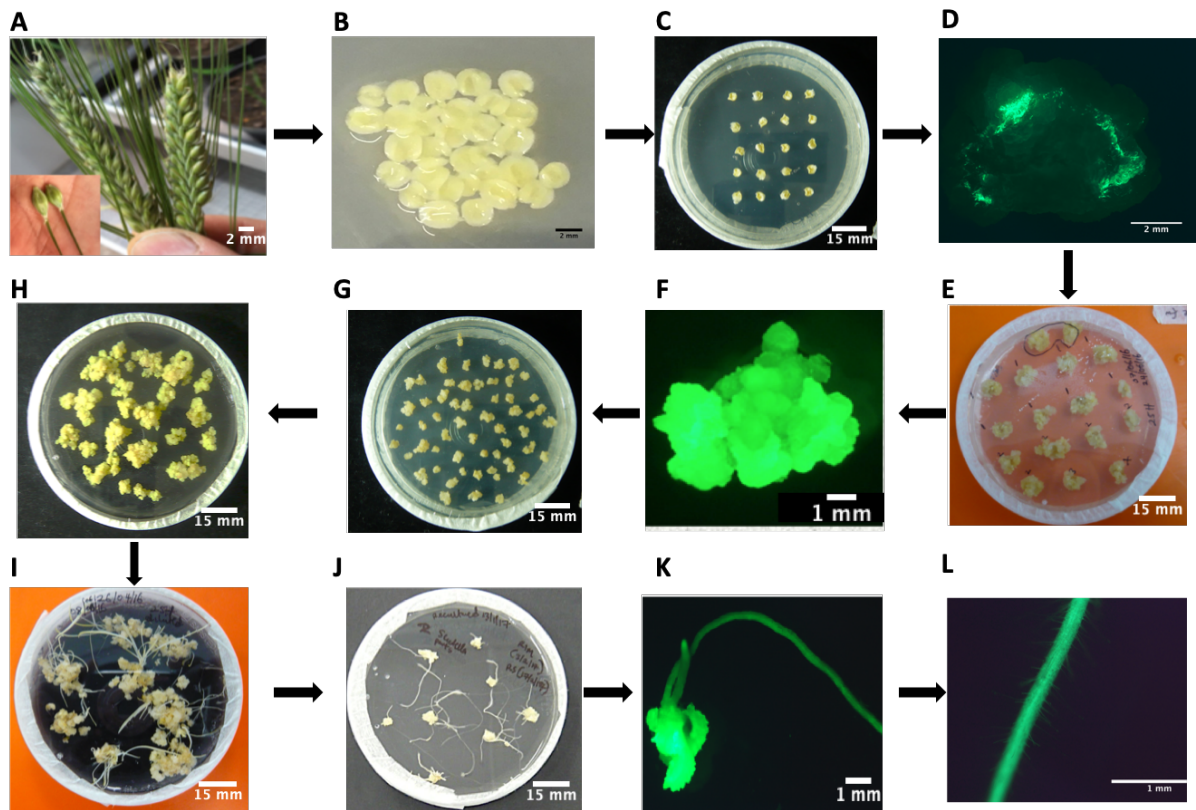


Figure 4.11: Revised barley root transformation system for functional studies.

A: Barley seeds (1.5–2 mm in diameter). **B:** Scutella prepared for transformation. **C:** Transformed scutella on CIM. **D:** Transformed scutellum expressing *GFP*. **E:** Calli developed from transformed scutella (4 weeks after transformation). **F:** A piece of calli with uniform expression of *GFP*. **G:** Calli parts harvested for regeneration and functional analyses. **H:** Fully regenerated calli from calli parts. **I:** Roots developed from regenerated calli. **J:** Individual roots separated from calli are used for functional analyses. **K, L:** Transformed roots on 10 cm² diameter round plates expressing *GFP*. The improved specific changes introduced since Imani et al. (2011) were **F, G, J** and **K**.

4.3 Discussion

Although barley transformation systems have been substantially improved, there are many limitations associated with current systems that have hindered its effective use as part of post-genomic research studies. Transformation efficiency is still one of the limiting factors in the production of large numbers of genetically engineered barley plants (Bartlett et al., 2008). Recently, transformation efficiency of 25% or more have been reported in barley (Bartlett et al., 2014, Hensel et al., 2008; Harwood, 2014). Efficient and high-throughput transformation systems are essential for functional analyses in crop breeding programmes (Harwood, 2012). Imani et al. (2011) further stressed that the absence of rapid high-throughput transformation systems in cereal crops for ‘omics’ studies such as transcriptome, proteome, or metabolome is a major drawback to effective functional examination and development of novel traits in crop systems. The conventional barley transformation system currently in use requires at least 1 years to generate homozygous lines expressing genes of interest. It is not surprising that most of large-scale molecular studies intended for cereal crops improvement program are usually carried out in model plants (such as *Arabidopsis thaliana*, *Nicotiana benthamiana*) that are easy and fast to transform and generate transgenic homozygous lines. However, there is still high possibility that outcome of research studies from model plant can be totally different when tested in the intended crops. Barley, being an important world crop that attracts a lot of research attention and also a model crop for studying other economic vital cereal crops (Bartlett et al., 2008), requires a rapid and simple transformation system that will permit effective large- scale omics studies.

Imani et al. (2011) established a new approach for barley called Stable Root Transformation System (STARTS). It involved the same process as in the conventional method but transformed calli were used to generate roots instead of whole plants. This new system could be a powerful and rapid tool for large-scale genetic studies in barley. However, some improvements were needed in order to obtain calli with a uniform transformation pattern since using calli that are not uniformly transformed results in a chimeric tissue that varies in the expression of genes of interest. Consequently, roots regenerated from heterogeneous tissue would show qualitative and quantitative differences in transgene expression levels. High transformation efficiency combined with uniform transgene expression patterns is however essential for functional studies of genes to be utilized in molecular breeding (Li et al., 2017). To compensate this lack of homogeneity and to obtain a representative number of transgenic roots for functional

analyses would therefore require an increase in scutella transformation, the regeneration of more transgenic roots from those calli and careful monitoring of transgene expression levels in transgenic roots (e.g. by qRT-PCR). For this reason, the aim was to identify parameters that might limit the generation of calli and roots with a higher homogeneity in transgene expression.

Nadolska-Orczk et al. (2000) observed that one of the most important advantages of *Agrobacterium*-based transformation systems is the relatively high transformation efficiency. Despite culturing calli on selective media with hygromycin, calli generated from transformed scutella were composed of transformed and untransformed fragments that are randomly interlaced. Moreover, untransformed regions were much more extended than the transformed parts on each callus. This pattern can be explained by the transformation process since only the periphery of scutella where the axes were removed will be initially transformed, resulting in the observed chimeric transgenic state of calli. In order to understand the reason of having both transformed and untransformed portions on calli, the stability of transformation efficiency and intensity of the transformed scutella were monitored during calli development. While transformation efficiencies remained stable the transformation intensities altered over time. In support of this, it was observed that scutella grown on higher hygromycin concentrations tend to increase transformation intensities in calli (e.g. increasing the homogeneity in transgene expression) and, as a result, increased the number of transgenic roots. Hence, optimization of the precise concentration of selective antibiotics might be a critical parameter to maximise transformation outputs.

In a second step, it was attempted to further 'enrich' transformation intensities to obtain calli with homogeneous transgene expression patterns. By separating transformed from untransformed calli areas (without damaging calli tissue), an increase in the homogeneity and transformation intensity was finally achieved. These fully transformed parts obtained from 'mother' calli were free of any necrosis. This is because every part of the fully transformed calli expresses the selective marker (hygromycin gene) that is able to inactivate the hygromycin antibiotics in the media. Sub-culturing these selected calli parts on higher concentrations of selective antibiotics (as used for selecting transformed scutella) reduced the growth of any untransformed tissue. Moreover, calli obtained by this procedure resulted in regeneration of ~100 % stably transformed roots per callus. From these studies, it was also found out that calli with non-uniform transformation patterns being used for the generation of the plants is the main reason transformation efficiency has not been increased substantially. Hence, plantlets or

seedlings develop from the non-transformed parts of the calli will not be transformed. This means that for large-scale studies involving screening hundreds or thousands of genes, for instance, a lot of plant seeds will be needed to be screened to compensate for the relatively low transformation efficiency associated with the transformation system. Space and labour to propagate and transform the plants, and cost of running the entire transformation processes will be prohibitively high.

In conclusion, the changes introduced to the formerly published barley root transformation system (Imani et al. 2011) allowed the generation of homogeneously transformed calli and generation of roots that were transformed. By reducing input and handling parameters (e.g. number of scutella for transformation, scale of calli cultures), these modifications will facilitate the use of the system for more large-scale functional genetic studies as required in our post-genome era. Importantly, the flexibility of the system allows generation of plants from calli that carry the functionally characterized genes of interest. Plantlets and mature plants that will be developed from such uniformly transformed calli will enable the generation of homogeneous transgenic plants. With this the optimised STARTS, in complementary to the conventional barley transformation systems, functional genomic studies and trait improvements in barley can be fast-tracked. In addition, the technical adjustments introduced here to optimize barley scutella-based transformation could be applicable to other cereal crops (especially wheat that embryos are also used for transformation) and can help in establishing large-scale genetic screening systems.

5 Chapter 5

Deciphering the functions of *Serendipita indica* effectors in stress resistance in barley crop

5.1 Introduction

Barley is one of the most widely cultivated cereal in the world; it is ranked fourth among cereals and in top ten of most important crops in the world (FAO, 2010; Bartlett et al., 2008). It is currently cultivated in about 70 million hectares with a global production of 160 million tons (FAO, 2014). Global barley production has been projected to increase by 54 % compared to the level it was in the year 2000 to be able to meet the projected demand for food, feed, and industrial purposes by 2050 (Kruse, 2011). Presently, there has been a renewed interest in barley crop (*Hordeum vulgare* L) food uses due to its versatile benefits (Haque et al., 2017). About 75 % of world production of barley is used for making animal feed, 20 % is malted for producing alcoholic and non-alcoholic beverages, and 5 % as ingredients in different kinds of food products (Blake et al., 2011). In addition, barley is a model crop for cereals for different kinds of plant improvement studies (Hockett and Nilan, 1985; Hogberg, 1987). In order to elevate its global yield production, biotic and abiotic stresses that severely limit barley crop growth and productivity have to be effectively and sustainably tackled. One of the world most devastating fungal pathogen in barley and wheat is *Fusarium graminearum*, which is the causal agent of economically important diseases such as root, crown, floral and seed rot, and Fusarium head blight (FHB) (Machado et al., 2018, Taheri, 2018) (Yang et al., 2013). Accumulation of mycotoxins in the harvested seeds or plant remains caused by the attack of the pathogen can be toxic to humans and animals and this poses problems in malt production and brewing quality (Chehri and Godini, 2017).

In terms of abiotic stresses, soil salinity is one of the most severe environmental threats for of all economically important crops including barley (FAO, 2009; Flowers, 2004). Salt stress affects plants at different developmental stages and cellular levels, e.g. interrupting cell membranes, reducing enzymatic and photosynthetic activities, and decreasing nutrient uptake (Hasegawa et al., 2000). As a result, salt stress leads to drastic reductions in plant development and yield. Zhang et al. (2010) indicated that out of over 900 million hectares of agricultural land world-wide, approximately 20 % are impoverished by soil salinity. The continuous increase in salinization of agricultural land is a major serious threat to global food security

(Oyiga et al., 2016). Annually, 27.3 billion US dollars are spent to ameliorate irrigation-triggered salinity (Qadir et al., 2014). Witzel et al. (2014) stressed that sustaining food security for the growing world population requires an improvement of crop salinity tolerance. It has been projected that by 2050, above 50 % of the global arable land will be salinized (Jamil et al., 2011).

Improvement in biotic and abiotic stress resistance of crops is a major breeding goal (Ashkani et al., 2015). Research studies have proposed that small proteins (effectors) that plant mutualistic microbes secrete during host colonization could provide new opportunities for improving biotic and abiotic stress resistance in elite crops such as barley (Bhattacharyya et al., 2016; Singh et al., 2011; Khalid et al., 2009). *Serendipita indica*, a plant beneficial endophytic fungus that can colonize all cultivated plants has been widely documented for its ability to confer abiotic stresses tolerance in host plants including barley (Baltruschat et al. 2008; Schäfer et al. 2009; Unnikumar et al., 2013). The ability of the fungus to confer resistance to *Fusarium* and salt stresses in barley has been studied (Waller et al., 2005). It has been established that the microbe secretes effectors similar to those of pathogenic microbes (Rovenich et al., 2014), but the functions of *S. indica* effectors in mediating plant growth and stress resistance still remains little characterised. *S. indica* effectors might represent a currently untapped resource to identify plant traits that can improve host growth and stress tolerance (e.g. against salinity and *Fusarium*).

5.2 Aims and objectives

S. indica effectors that increased growth and stress resistance in the model plant, *Arabidopsis thaliana* were identified in Chapter 1. The objective in this chapter was to investigate the roles of *S. indica* effectors in improving plant growth and stress resistance in barley. More specifically, the aim was to identify those effectors that can increase plant resistance against *Fusarium graminearum* and/or salt stress in barley.

5.3 Results

5.3.1 Establishment of an assay to screen *Fusarium graminearum* resistance in barley roots regenerated from calli

The ability of *Fusarium graminearum* to cause root rot and browning disease symptoms was tested in barley seedlings. The pathogen had the ability to infect and cause rot on the roots of 2-day old barley seedlings (**Figure 5.1**). Barley roots were dip-inoculated with different concentrations (250 conidia/l and 500 conidia/l) of *F. graminearum* spore solution based on optimization. Root rot was observed 3 days after inoculation. As expected, root rot was much more pronounced in roots inoculated with higher spore concentrations.

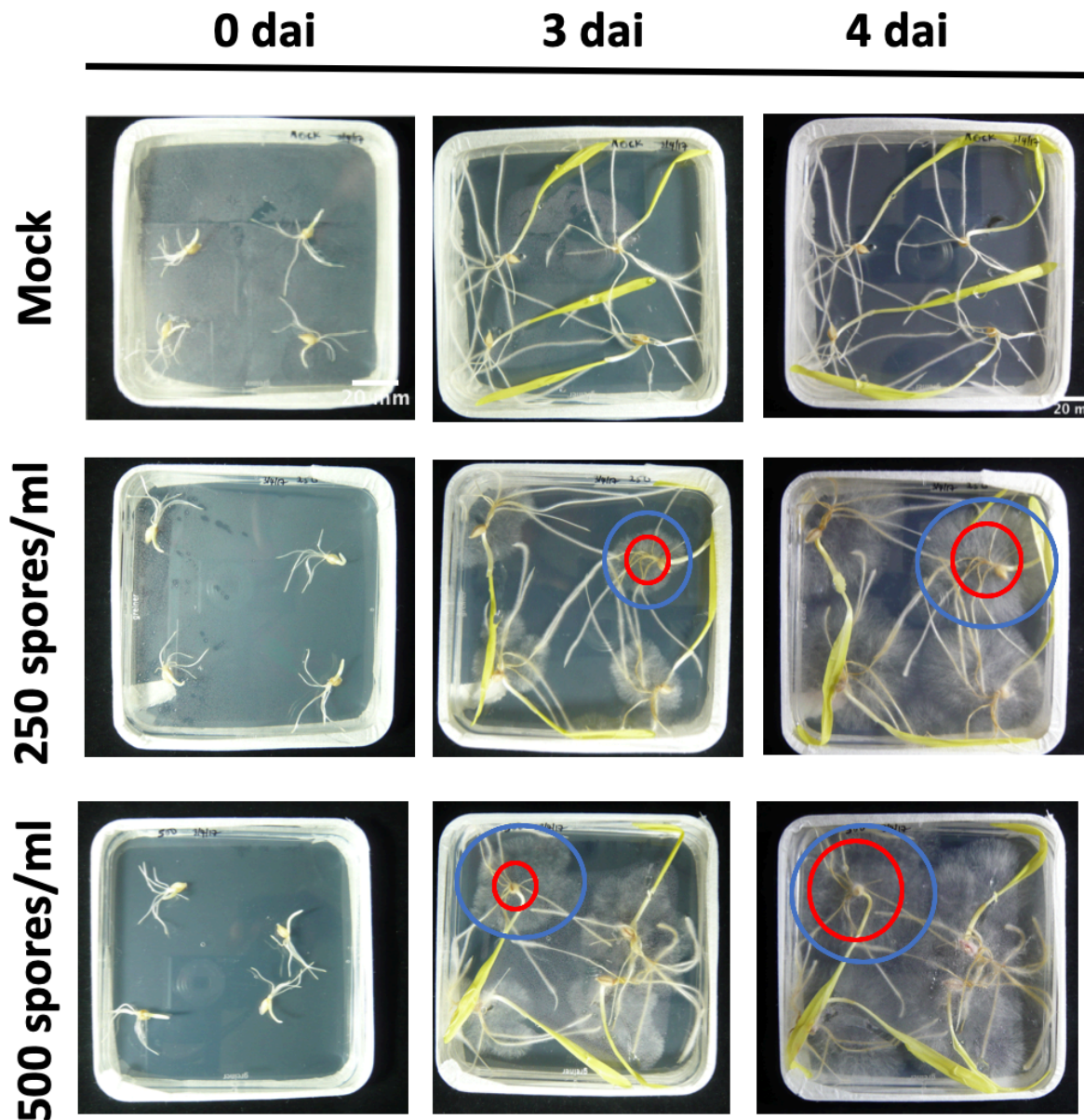


Figure 5.1: Inoculation of roots of barley seedlings with *Fusarium graminearum*.

Roots of 2-day old barley seedlings were dipped in *F. graminearum* spore suspension; mock (0 spores/ml), 250 spores/ml and 500 spores/ml. Images were taken at 0, 3 & 4 days after inoculation (dai) to monitor *Fusarium* infection, as seen as root rot and browning. The media used contained 30 g/l maltose. The red circle showed the brown areas while the blue circle indicated the *Fusarium* growth. The scale bar (20 mm) showed on plate containing roots on mock (0 dai) are same for all the images.

The same dipping method was applied to roots transformed with *pPS2015::GFP* and regenerated from barley calli. However, the pathogen was unable to consistently infect roots (**Figure 5.2**). Therefore, different methods of inoculation were tested. Applying 50 spores on the agar media before placing the roots resulted in a reproducible infection pattern and development of disease symptoms (**Figure 5.3**).

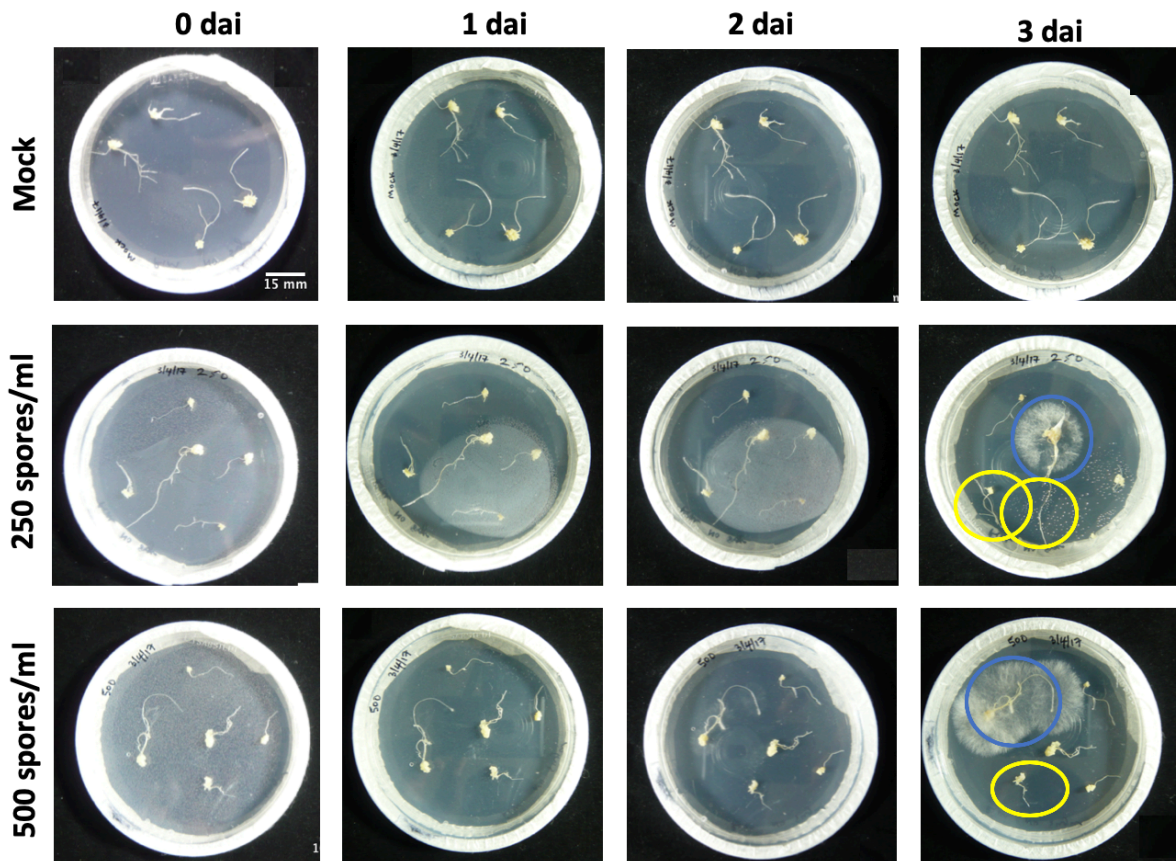


Figure 5.2: Inoculation of roots from calli with *Fusarium graminearum*.

Roots were dipped in *F. graminearum* spore culture; mock (0 spores/ml), 250 spores/ml and 500 spores/ml. Images were taken at 0, 1, 2 & 3 days after inoculation (dai) to monitor root rot and browning. The media contained 30 g/l maltose. The blue circle showed the roots that were successfully inoculated with *Fusarium*. Yellow circle indicated the roots that were not successfully inoculated with *Fusarium*. The scale bar showed (15 mm) are same for all the images.

The optimal number of spores (12, 25, 50, 100 spores) was then identified in combination with the right concentration of maltose (0, 2, 3, 7.5, 30 g/l) in the growth media to be used for screening calli-regenerated roots (**Figure 5. 4**). Optimizing maltose concentration is very important in order to get the right minimum concentration that will enable the fungus to proliferate and still cause disease symptoms on the roots. Establishing the right quantity of fungal inoculum (e.g. number of spores) in combination with the sugar concentration will provide reproducible conditions for effective fungal proliferation and infection without inducing media-related stress to calli and roots. 50 spores was found to be enough to cover the plate surface and was chosen for the root assays.

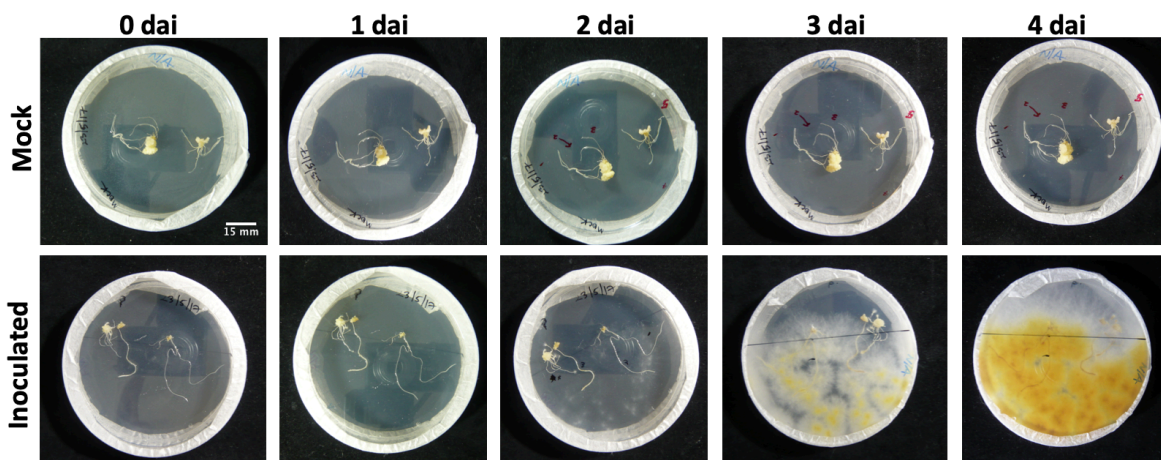


Figure 5.3: Inoculation of roots from barley calli with *Fusarium graminearum*.

Sterile H₂O containing 50 *F. graminearum* spores was evenly distributed on the plates before roots were placed on the inoculated media. Mock contained sterile H₂O only. Images were taken at 0, 1, 2, 3 & 4 days after inoculation (dai) to monitor the development of disease, as measured by root rot and browning. Inoculated plates at 3 and 4 dai were inverted (turned upside down) for better imaging of the infected roots. The media contained 30 g/l maltose. The scale bar showed (15 mm) are same for all the images.

In terms of maltose concentrations, 30, 7.5 and 3 g/l showed good spore germination and hyphal growth. There were pinkish exudates (secondary metabolites that pathogenic fungus produced during host interaction) the spores produced on media containing a lower maltose concentration (2 g/l). There was no spore growth on media without maltose. Based on the positive result above, the same maltose concentrations (0, 2, 3, 7.5, 30 g/l) were used to test the transformed barley roots (**Figure 5. 5**). All the media were inoculated with 50 spores except

in the media with 30 g/l of maltose that was inoculated with 12.5 spores because of high maltose content. The results of the fungal proliferation as influenced by combination of different levels of maltose concentration and number of conidia were similar to what initially observed when the roots were not tested. Media containing 7 g/l maltose was effective to support even proliferation of the spores and therefore for testing the roots regenerated from calli. This was similar to the result obtained earlier (**Figure 5. 4**) when the roots were not used. The pinkish exudates were also observed in the media with lower maltose concentration (2 g/l). was strongly observed in the media containing 2 g/l as observed earlier. Small patches of pink colour were observed in the media with 2 g/l of maltose. The spores showed little proliferation with pinkish exudates in the media without maltose. To further optimize the best minimum maltose concentration to use, 7.5, 5 and 3 g/l maltose containing media were inoculated 50 spores were used to test the roots from calli against *F. graminearum* (**Figure 5. 6**).

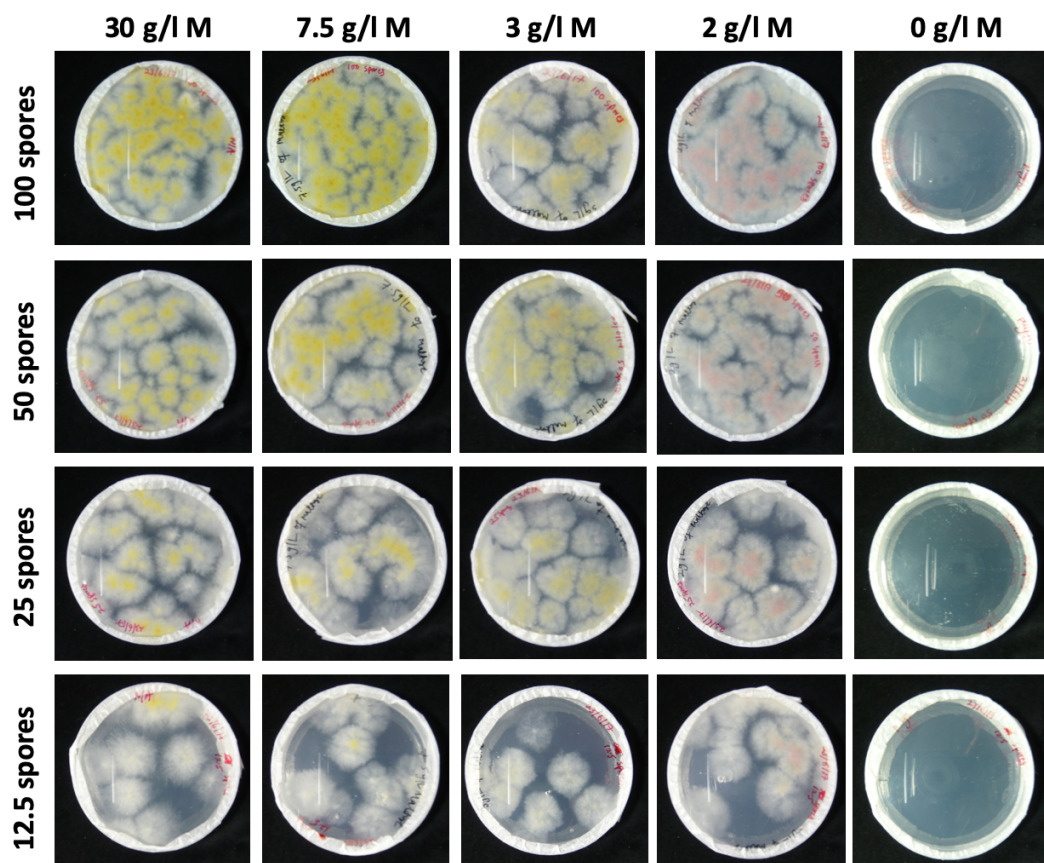


Figure 5.4: Proliferation of *Fusarium graminearum* under different concentrations of maltose and numbers of *F. graminearum* spores.

Images were taken at 3 days after spore application to study the growth of fungal hyphae. Different maltose (M) concentrations were used: 30 g/l, 7.5 g/l, 3 g/l, 2 g/l and 0 g/l, while the number of *F. graminearum* spores was 100, 50, 25 and 12.5.

There were few pinkish exudates on plates containing 3 g/l of maltose. Media containing 7.5 or 5 g/l of maltose showed consistent spore germination of hyphal growth (**Figure 5.6**).

To confirm that the browning observed on the roots was due to the fungal colonization, roots were stained with wheat germ agglutinin (WGA)-AF488 and observed under the microscope using bright field and green fluorescence protein (GFP) settings (**Figure 5.7**). The analyses revealed mycelia to coincide with browning in inoculated root parts.

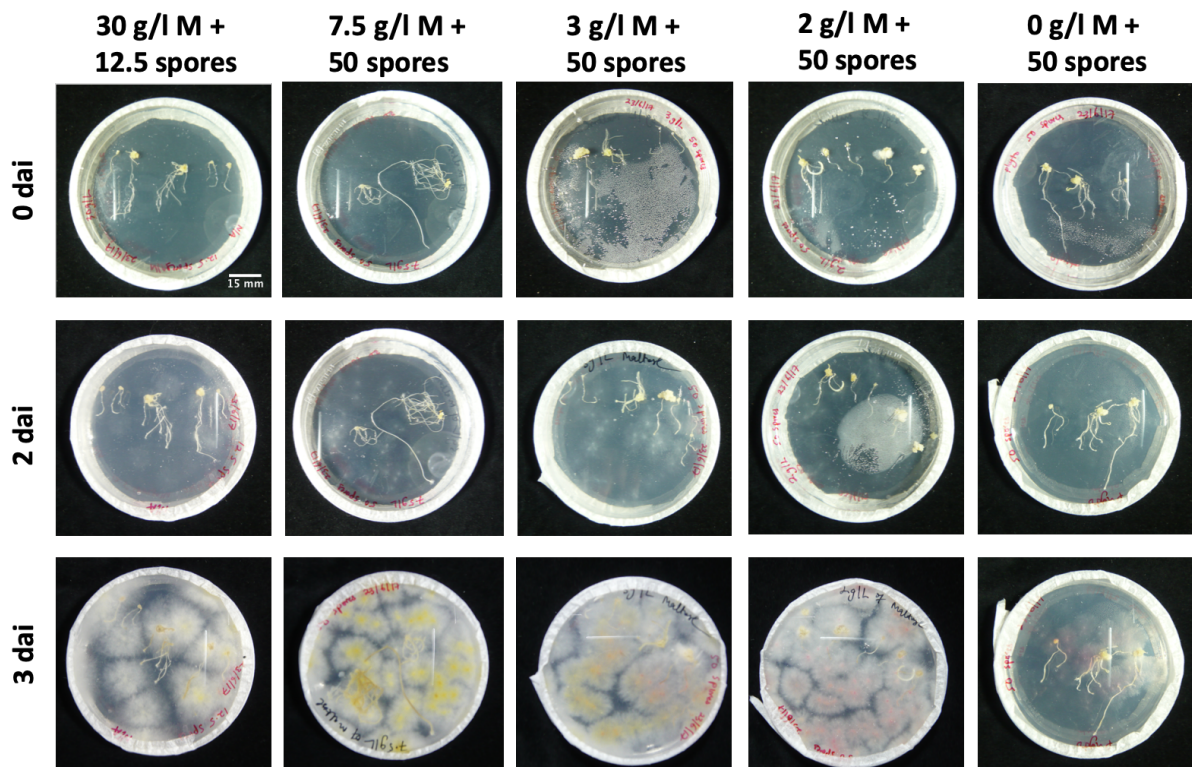


Figure 5.5: Fusarium root rot disease on GFP expressing roots regenerated from barley calli under different maltose concentrations.

Inoculation of sterile H₂O containing 50 or 12.5 *F. graminearum* spores was evenly distributed on the media before the roots were placed on the inoculated media. Different maltose (M) concentrations (30 g/l, 7.5, 3 g/l, 2 g/l and 0 g/l) were tested. Images were taken on 0, 2 and 3 days after inoculation (dai) to monitor development of disease, as measured by root rot/browning disease. The scale bar showed (15 mm) are same for all the images.

Having identified the optimal concentration of maltose (7.5 g/l) and numbers of spores (50) to be used for Fusarium infection of barley roots allowed screening of GFP transformed roots. Intensities of green fluorescence of both *F. graminearum* inoculated and non-inoculated roots were monitored until 3 days after treatment (**Figure 5.8**). This is to test if the GFP intensity of the transformed roots inoculated with *F. graminearum* will change compared to the GFP intensity of non-inoculated roots. It was speculated that Fusarium disease could alter the GFP intensity of the inoculated roots compared to the non-inoculated. If that happens, fluorescence intensity will be used as basis for screening the SIE-expressing roots for *F. graminearum* resistance. There was no noticeable reduction in fluorescence intensity of either inoculated or non-inoculated roots. The roots were further examined under bright field conditions (**Figures 5.9 and 5.10**). The browning of the inoculated roots was clearly observed under bright field (**Fig. 5.9**) while the non-inoculated roots showed no symptoms of browning (**Fig. 5.10**). Thus, confirming Fusarium infection of seedlings. The analyses revealed that further optimisation is required to use GFP intensities as indicator for disease progress and to test the effect of *S. indica* effectors on Fusarium root rot. Because of time constraint, this was not achievable in this work. However, these analyses build the basis for establishing such a screen in future.

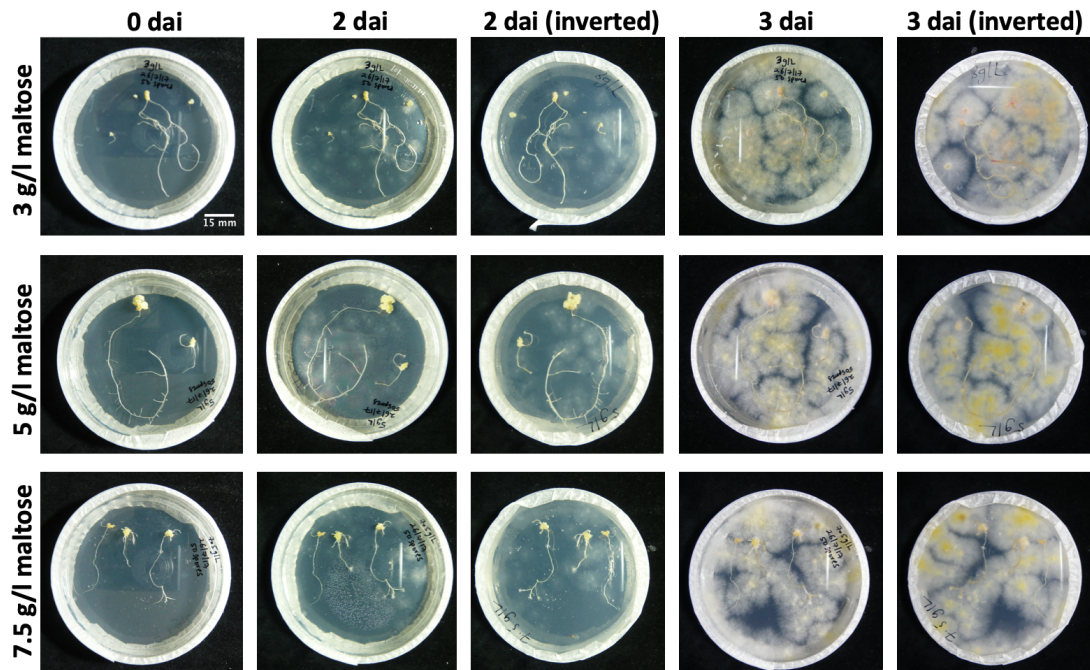


Figure 5.6: Root rot disease on *Fusarium graminearum* -inoculated roots from barley calli under different maltose concentrations.

Inoculation of sterile H₂O containing 50 *F. graminearum* spores was evenly distributed on the media using glass beads before the roots were placed on the inoculated media. Different maltose (M) concentrations; 7.5 g/l, 5 g/l & 3 g/l were tested. Images were taken on 0, 2 & 3 days after inoculation (dai) to monitor development of disease, as measured by root rot/browning disease. Inverted =the plate was turned upside down for imaging of the roots. The scale bar showed (15 mm) are same for all the images.

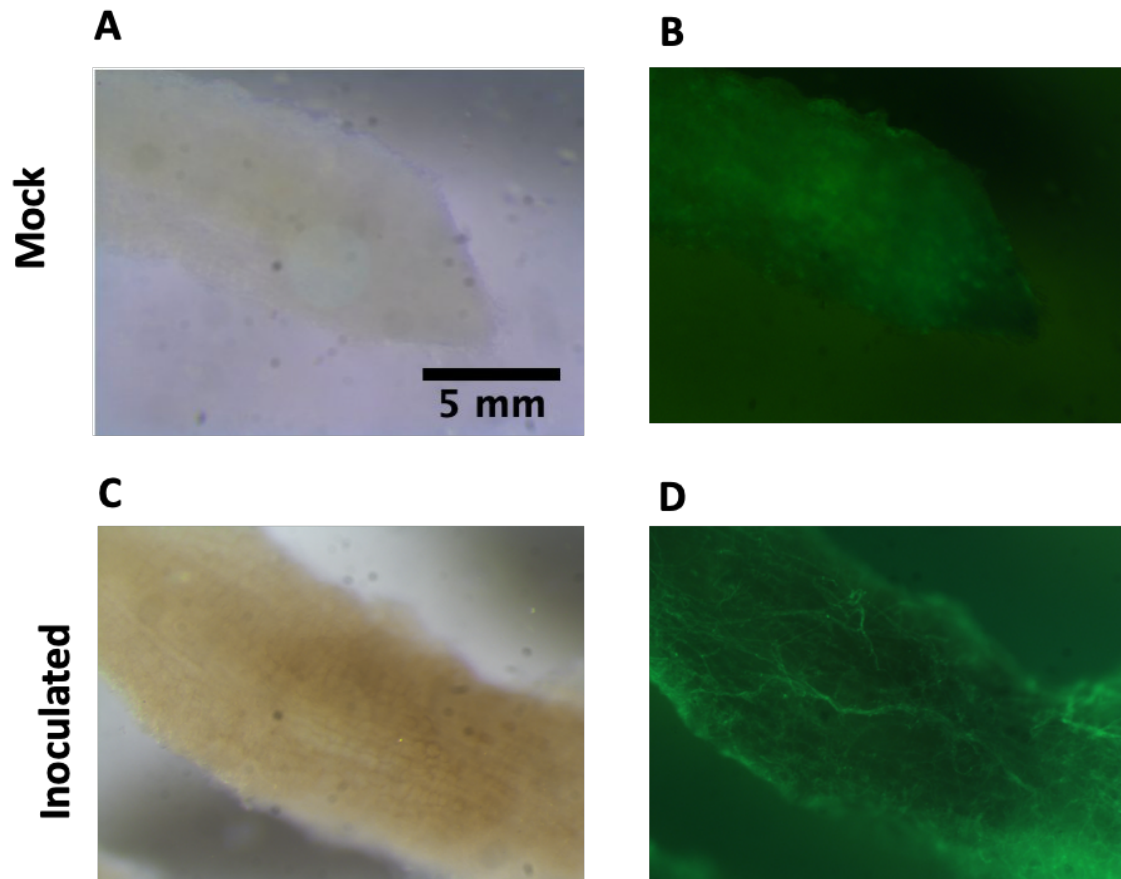


Figure 5.7: Visualization of *Fusarium graminearum* colonization of roots regenerated from barley calli.

The roots were visualized 3 days after inoculation. Roots were stained with wheat germ agglutinin (WGA)-AF488 and visualized under bright field (A & C) and GFP (B & D) settings (excitation: 488 nm, emission: 509 nm). Bar size = 5 mm for all the images.

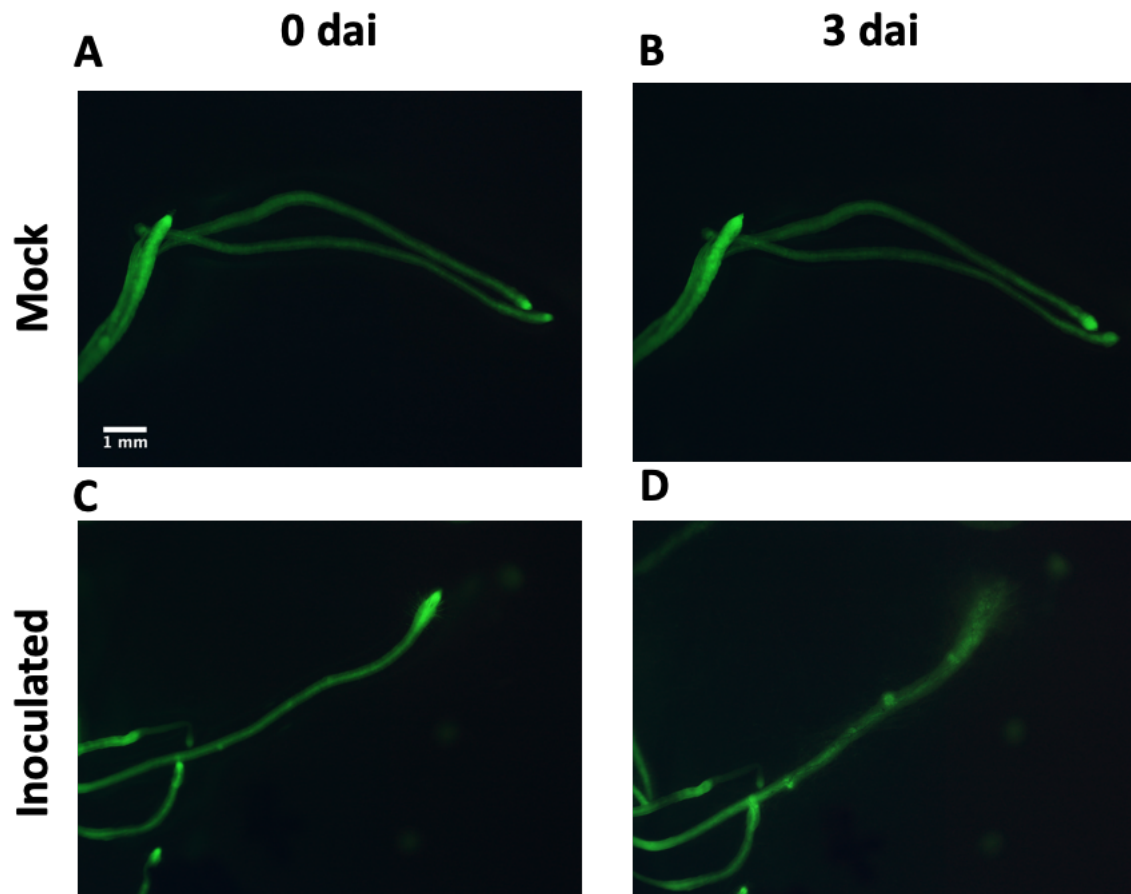


Figure 5.8: Green fluorescence of *Fusarium graminearum*-inoculated and non-inoculated roots.

Roots were regenerated from calli transformed with GFP. GFP was detected using GFP setting (excitation: 488 nm, emission: 509 nm). Images were taken at 0 (A & C) and 3 days (C & D) after inoculation (dai). Bar size = 1 mm for all the images.

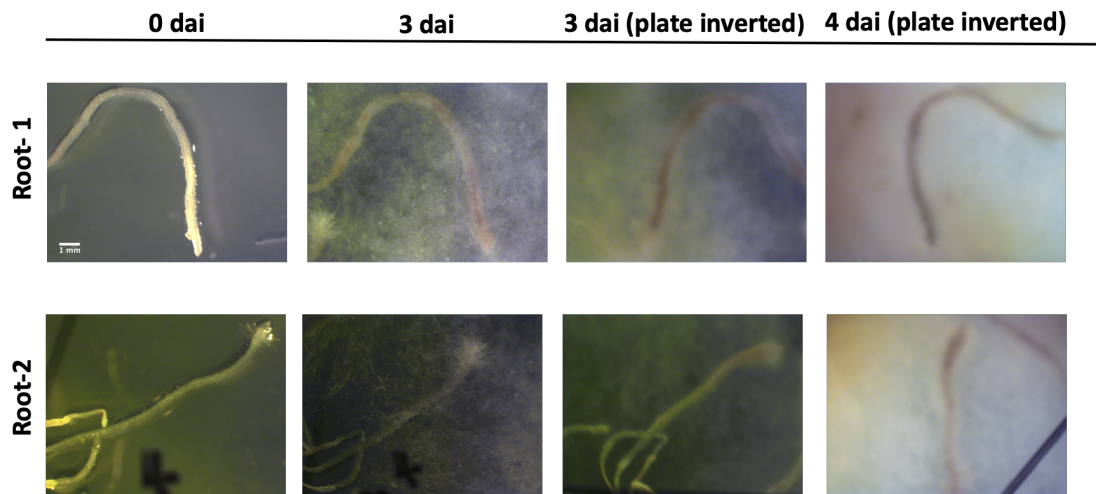


Figure 5.9: *Fusarium graminearum*-inoculated roots generated from barley calli.

50 spores were uniformly distributed on 7.5 g/l maltose-containing media using glass beads before the roots were placed on the inoculated media. Images were taken on 0, 3 and 4 days after inoculation (dai) to monitor development of root rot and browning symptoms. Size bars = 1 mm for all the images. Plate inverted = the plate was turned upside down for imaging of the roots.

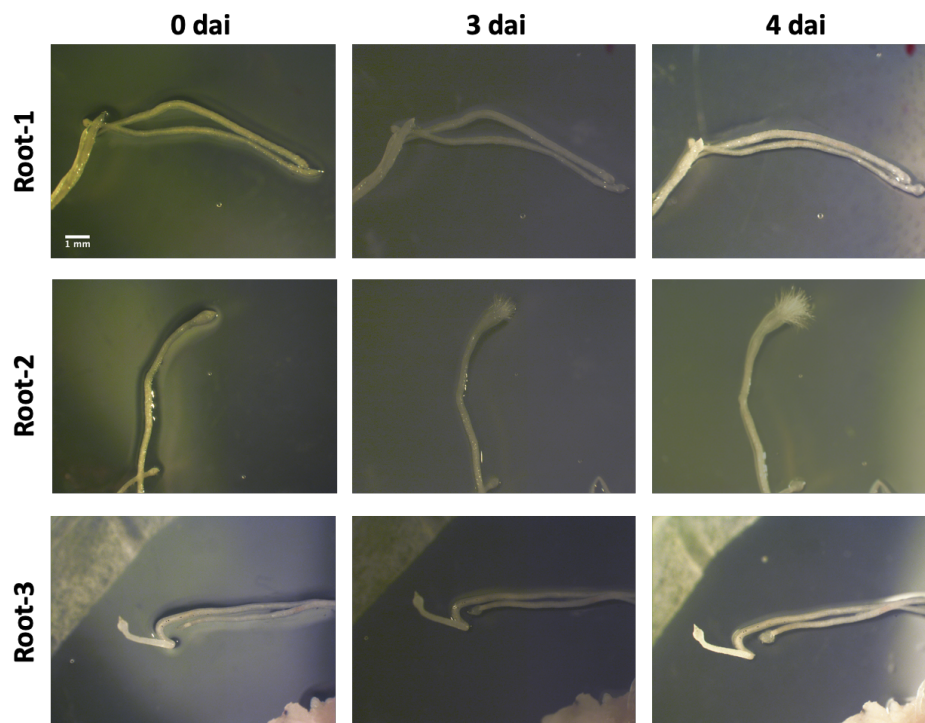


Figure 5.10: Non-inoculated roots generated from barley calli.

Roots were placed on mock-treated media. Bright images of the roots were taken on 0, 3 and 4 days after H₂O treatment (dai). Bar size = 1 mm for all the images.

5. 3. 2. Investigating the functions of *S. indica* effectors in improving salt stress tolerance in barley

To test the ability of *S. indica* effectors transformed into barley to enhance salt tolerance, parameters to establish a barley calli-based salt stress assay were established. In addition to determining the concentration of sodium chloride, the age of scutella/calli, and the timepoint for detecting salt stress, a method for quantifying salt stress in calli was identified. After testing different parameters, 300 mM NaCl were found to reproducibly induce salt stress at 14 days after salt treatment. This was seen as 14-day old scutella fresh weight was significantly reduced (**Figure 5.11A**). Similarly, the fresh weight of regenerated calli were also significantly reduced (**Figures 5.11B & C**).

As a next step, the suitability of use of intensity of GFP to monitor salt stress tolerance in GFP-expressing calli was determined. The green fluorescence intensity of the calli on control and salt stressed media was monitored from day 0 to day 14. A significant reduction in green fluorescence intensity was detected in calli subjected to salt stress as compared to the control-treated calli (**Figure 5.12**). Based on the result of optimization, callus weight and GFP intensities were effective to test for salt tolerance. Therefore, these two parameters were found to be suitable for screening for *S. indica* effectors that have the ability to alter salt tolerance in barley calli.

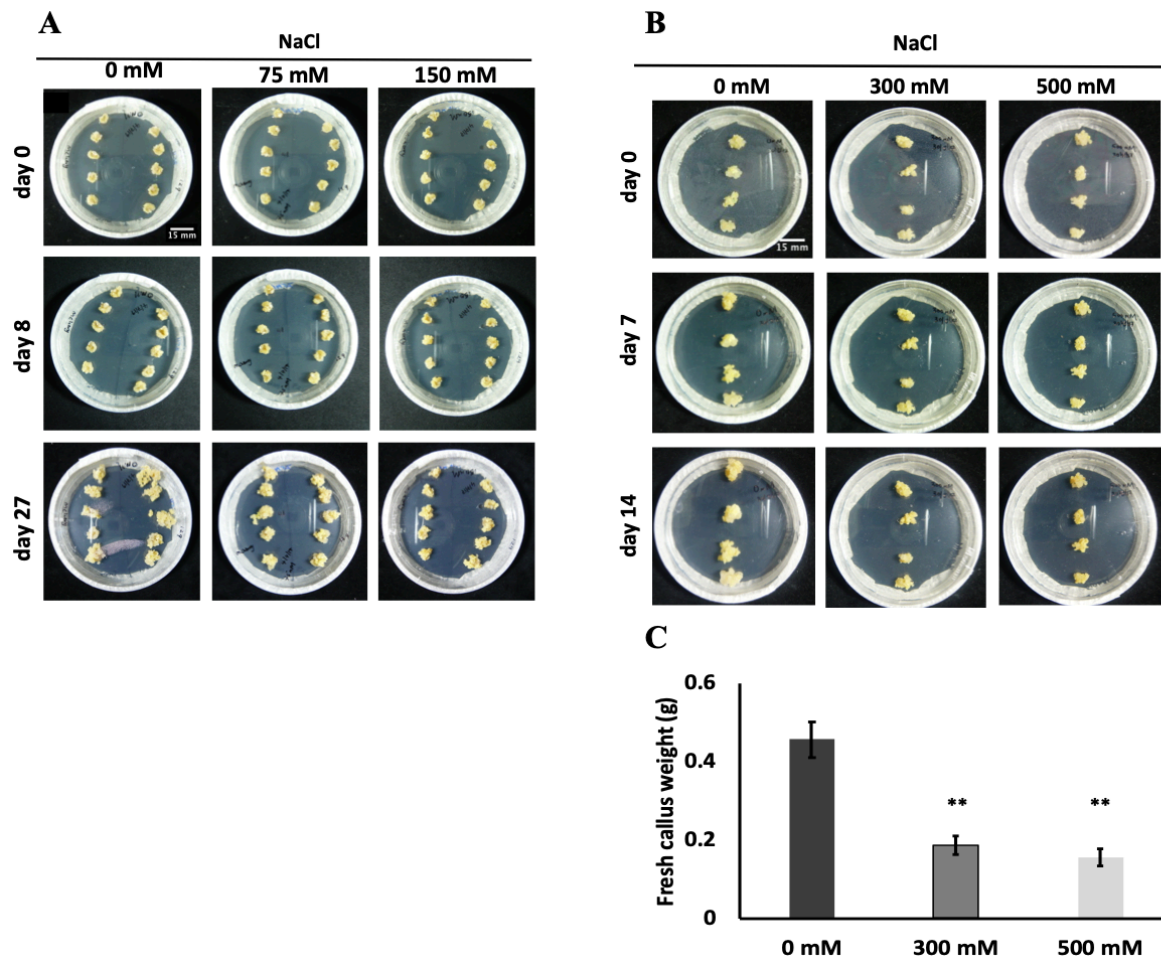


Figure 5.11: Barley scutella/calli parts exposed to different levels of salt stress.

A: 14-days-old scutella exposed to 75 and 150 mM NaCl (moderate salt stress). Pictures were taken at 0, 8 and 27 days after treatment **B:** Calli exposed to 300 and 500 mM NaCl (high salt stress). Pictures were taken at 0 and 14 days after treatment. **C:** Impact of different concentrations of salt stress on fresh calli weight (in g). Calli from **B** were weighed after 14 days of stress and mean calli weight with standard errors of biological experiments are shown. T-test was used to compare the fresh weight of calli on salt stress media to those on control media. **=significant at p-value of <0.01.

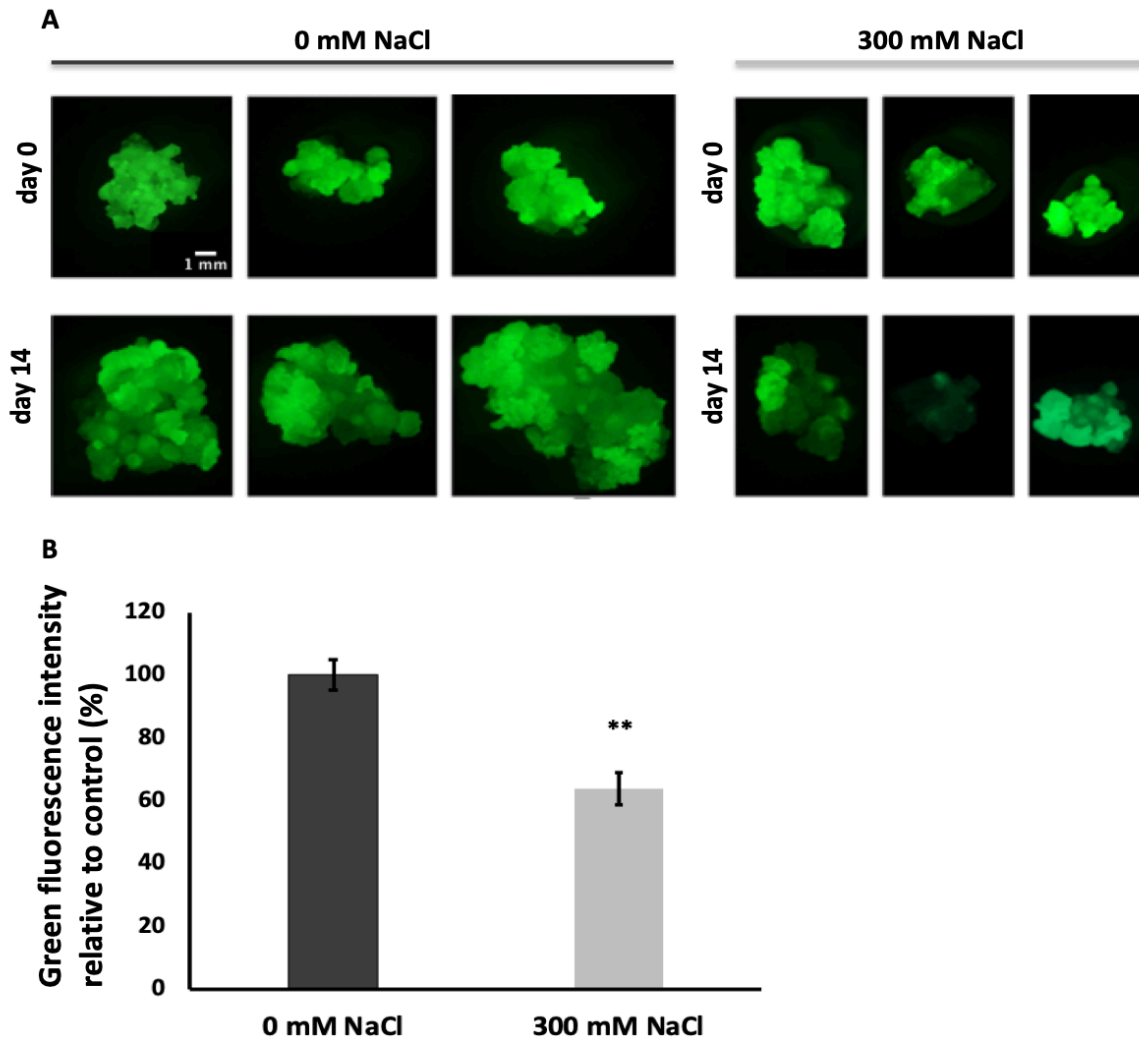


Figure 5.12: Salt stress reduces green fluorescence intensity in *GFP*-expressing calli.

Calli uniformly expressing GFP were used for the assay. **A:** Salt stress reduces green fluorescence intensity in GFP-expressing calli (GFP detection under 488 nm [excitation] and 509 nm [emission]). **B:** Salt stress reduces GFP intensity of calli exposed to 300 mM NaCl. Mean values with standard error of GFP intensities of GFP-expressing calli grown on mock or NaCl-containing media were determined using Image J software at 0 and 14 days after treatment. The mean gray value (the sum of the gray values of all the pixels in the selection divided by the number of pixels) was selected in the software for the measurement of the green fluorescence intensities. The changes in GFP intensities before and after treatment were calculated. The average GFP intensities of calli exposed to salt stressed conditions were plotted relative to the average GFP intensities of calli exposed to mock conditions. Bar size = 1 mm for all the images. T-test was used to compare the GFP intensity of calli on salt stress media to those on control media. **=significant at p-value of <0.01.

Having defined the conditions for the salt stress assay, scutella were transformed with a construct generated from the vector in use, *pPS2015::GFP* with mCherry fluorescent protein (as the negative control). This confirmatory check is to be sure the effectors will be expressed in this vector (*pPS2015::GFP*) used for the optimization. Unexpectedly, only GFP that serve as a transformation control was detectable in transformed scutella. The scutella were expected to express both green (GFP) as a transformation control and red (mCherry) fluorescence to test for functionality of the promoter (*ZmUbi1*) in driving the gene of interest. It was assumed that *ZmUbi1* might not be effective to drive or fully drive the expression of the effector in calli using *pPS2015::GFP* vector. Another vector, *pANIC 10A::RFP* that constitutively also expresses red fluorescent protein was tested. GFP was cloned into *pANIC 10A::RFP* to generate a gene construct that was used to transform the scutella. Both RFP (transformation control) and GFP (to test for functionality of the promoter (*ZmUbi1*) in driving the gene of interest) were detected in all the transformed scutella/calli (**Figures 5.13 & 5.14**). Thus, *pANC 10A::GFP* was used as the vector expressing the effectors for the salt stress assay.

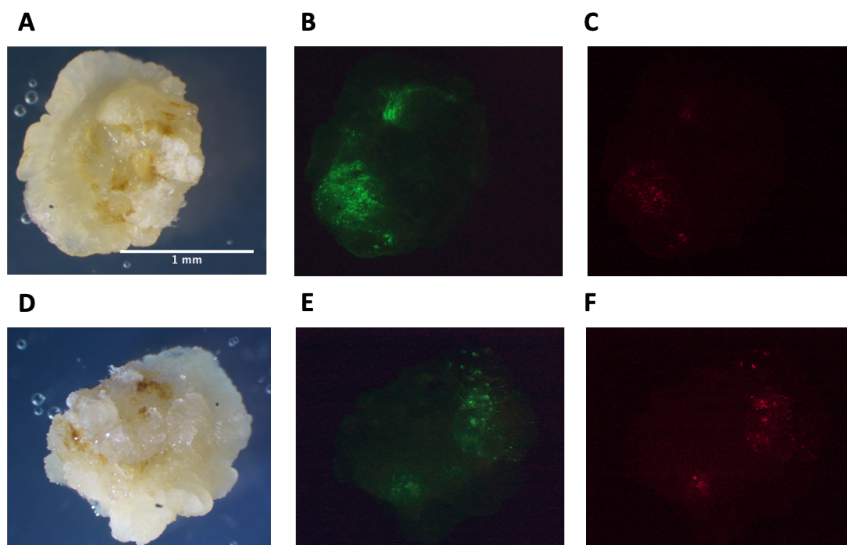


Figure 5.13: Scutella transformed with *pANIC 10A::RFP-GFP*.

The vector; expresses red fluorescence proteins serving as a transformation control (C & F) while the GFP was detected was to test if the promoter (*ZmUbi1*) was capable of driving the expression of the gene of interest (B & E). Images were taken under bright field (A & D); GFP (B & E), and RFP (C & F) conditions after 7 days of transformation. Bar size = 1 mm for all the images. GFP was detected using GFP setting (excitation: 488 nm, emission: 509 nm). RFP was detected using RFP setting (excitation: 561 nm, emission: 575 nm).

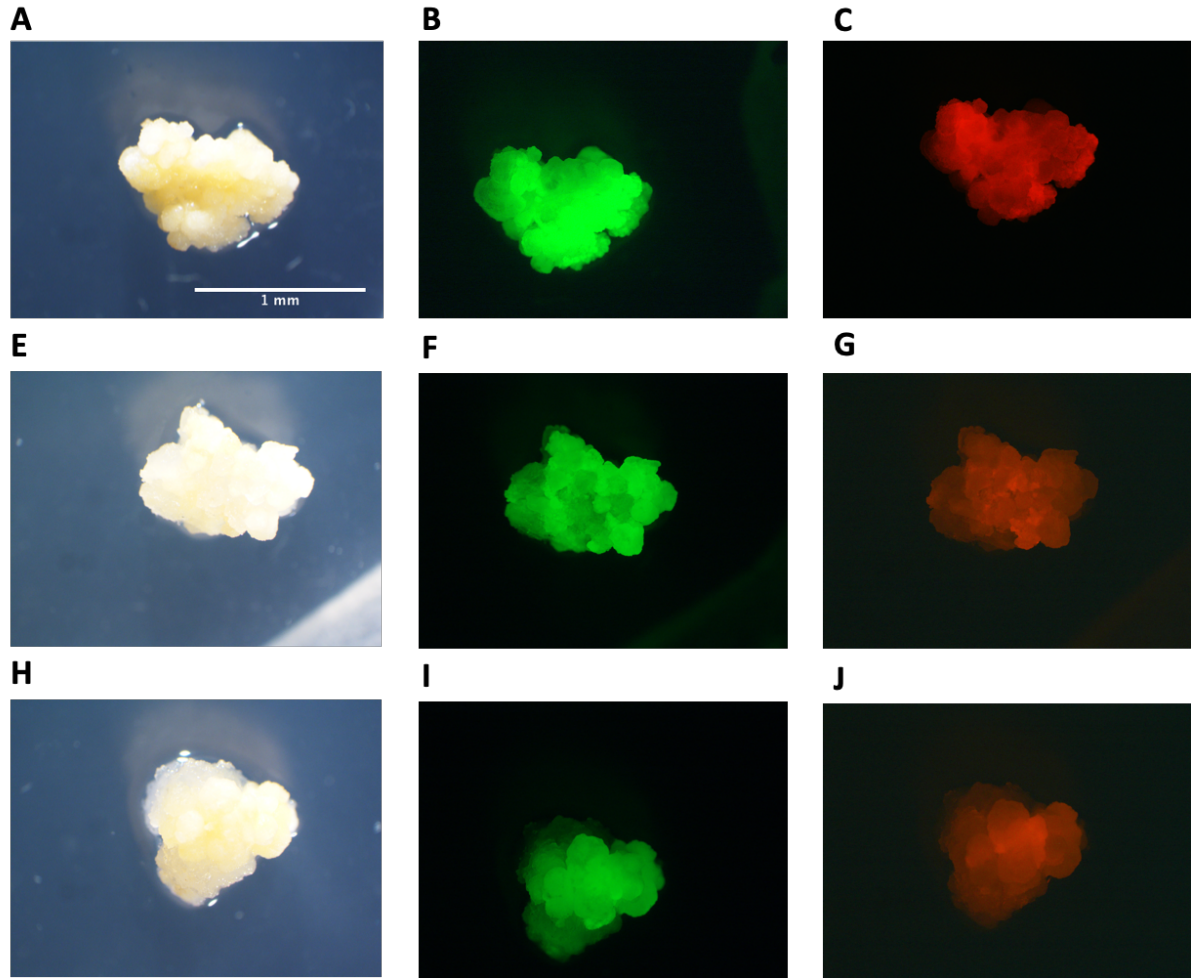


Figure 5.14: Calli parts fluorescent expression following scutella transformation with the construct *pANIC 10A::RFP-GFP*.

The vector; constitutively expresses red fluorescence proteins (C, G & J) while the cloned gene; green fluorescence protein (GFP) was detected (B, F & I). Images were taken under bright field (A, E & H); GFP (B, F & I), and RFP (C, G & J). Bar size = 1 mm for all the images. GFP was detected using GFP setting (excitation: 488 nm, emission: 509 nm). RFP was detected using RFP setting (excitation: 561 nm, emission: 575 nm).

To test the effect of *S. indica* effectors in improving salt stress tolerance, the percentage increase in callus weight was used as a screening parameter. 8 *S. indica* effectors (SIEs) were tested: *SIE13*, *SIE39*, *SIE69*, *SIE76*, *SIE88*, *SIE108*, *SIE120* and *SIE129*. Any change in calli weight expressing SIEs was compared to any change in weight of calli transformed with GFP as a control. Weight of calli expressing *SIE13*, *SIE69* and *SIE108* did not differ significantly from the calli expressing GFP (Figures 5.15, 5.16 & 5.17), while *SIE76* transformation significantly reduced calli weight only in one instance (Figure 5.18). *SIE120* and *SIE129*

appeared to repress growth of barley calli substantially in mock and salt stress conditions; although differences were not significant in all instances (**Figures 5.19 & 5.20**). *SIE120* reduced calli weight 36.5 % (replicate 1) and 75.5 % (replicate 2) under mock conditions and by 67 % (replicate 1) to 109.9 % (replicate 2) under salt stress conditions relative to calli expressing GFP. Also, *SIE129* altered calli weight 83.1 % (replicate 1) and 84.7 % (replicate 2) under mock conditions and by 42.9 % (replicate 1) to 114.4 % (replicate 2) under salt stress conditions relative to calli expressing GFP.

Conversely, *SIE 39* and *SIE88* seemed to promote growth in both mock and salt stress conditions in two independent experiments (**Figures 5.21 & 5.22**). Overall, *SIE88* altered calli weight 22.2 % (replicate 1) and 25.5 % (replicate 2) under mock conditions and by 30.74 % (replicate 1) to 32.27 % (replicate 2) under salt stress conditions relative to calli expressing GFP. In turn, *SIE39* altered calli weight by 86.4 % (replicate 1) to 24.9 % (replicate 2) on mock conditions and by 49.1 % (replicate 1) to 29.4 % (replicate 2) under salt stress conditions relative to calli expressing GFP.

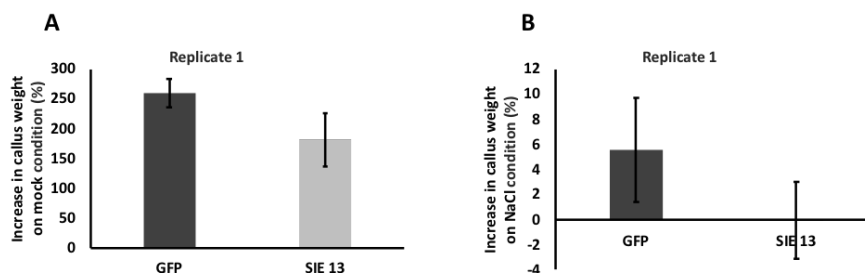


Figure 5.15: Change in weight of calli expressing *S. indica* effector *SIE13* under mock and salt stress conditions.

Calli were exposed to media containing 0 (A) and 300 mM NaCl (B) for 14 days. The initial and final weight of each callus was obtained at 0 and 14 days after treatment, respectively. Increase in callus weight (in percent) was calculated and the mean values with standard errors of two technical experiments are shown. Calli expressing GFP served as control. T-test was used to compare the weight of calli expressing *SIE13* to those expressing GFP.

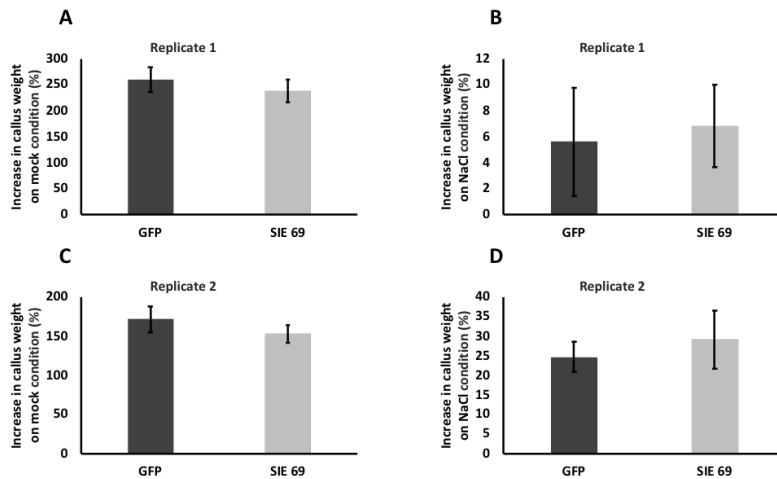


Figure 5.16: Change in weight of calli expressing *S. indica* effector *SIE69* under mock and salt stress conditions.

Calli were exposed to media containing 0 (A, C) and 300 mM NaCl (B, D) for 14 days. The initial and final weight of each callus was obtained at 0 and 14 days after treatment, respectively. Increase in callus weight (in percent) was calculated and the mean values with standard errors of two technical experiments are shown. Calli expressing GFP served as control. T-test was done to compare the weight of calli expressing effector to those expressing GFP. T-test was used to compare the weight of calli expressing *SIE69* to those expressing GFP.

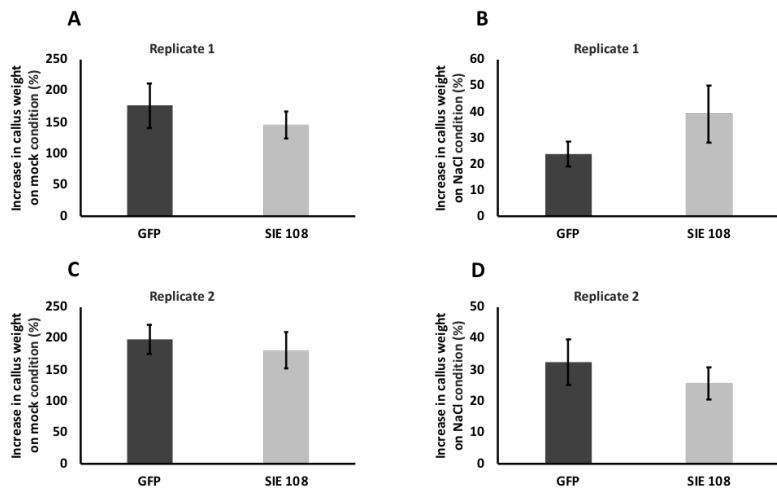


Figure 5.17: Change in weight of calli expressing *S. indica* effector *SIE108* under mock and salt stress conditions.

Calli were exposed to media containing 0 (A, C) and 300 mM NaCl (B, D) for 14 days. The initial and final weight of each callus was obtained at 0 and 14 days after treatment, respectively. Increase in callus weight (in percent) was calculated and the mean values with standard errors of two technical experiments are shown. Calli expressing GFP served as control. T-test was used to compare the weight of calli expressing *SIE108* to those expressing GFP.

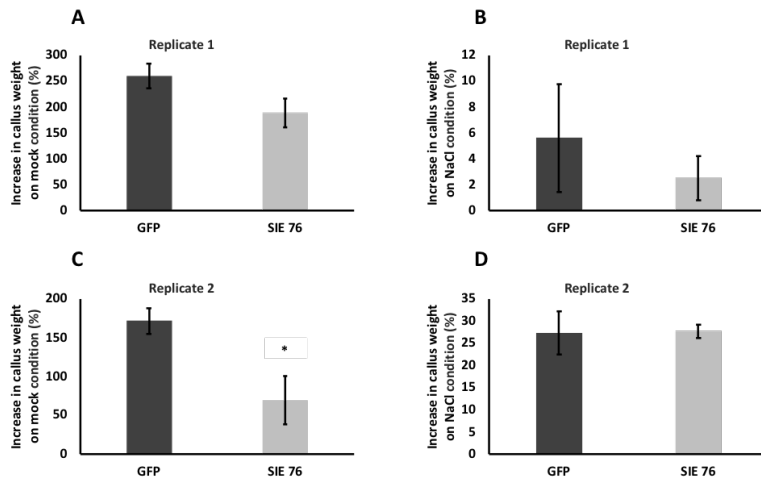


Figure 5.18: Change in weight of calli expressing *S. indica* effector *SIE76* under mock and salt stress conditions.

Calli were exposed to media containing 0 (A, C) and 300 mM NaCl (B, D) for 14 days. The initial and final weight of each callus was obtained at 0 and 14 days after treatment, respectively. Increase in callus weight (in percent) was calculated and the mean values with standard errors of two technical experiments are shown. Calli expressing GFP served as control. T-test was used to compare the weight of calli expressing *SIE69* to those expressing GFP. *=significant at p-value of <0.05.

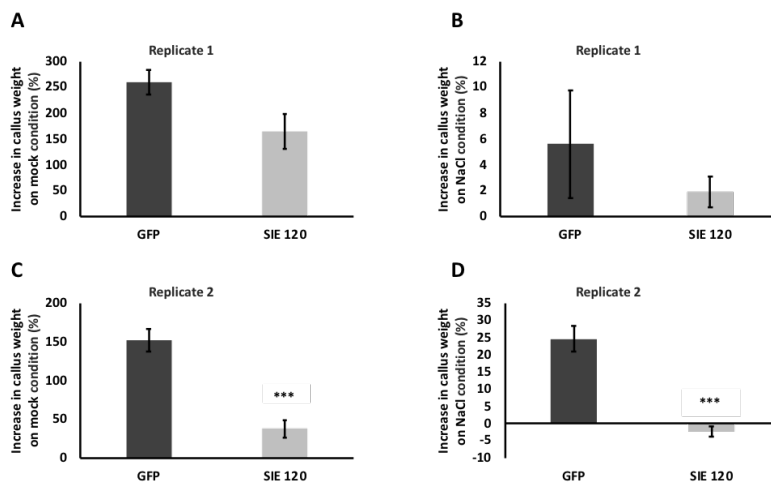


Figure 5.19: Change in weight of calli expressing *S. indica* effector *SIE120* under mock and salt stress conditions.

Calli were exposed to media containing 0 (A, C) and 300 mM NaCl (B, D) for 14 days. The initial and final weight of each callus was obtained at 0 and 14 days after treatment, respectively. Increase in callus weight (in percent) was calculated and the mean values with standard errors of two technical experiments are shown. Calli expressing GFP served as control. T-test was used to compare the weight of calli expressing *SIE120* to those expressing GFP. ***=significant at p-value of <0.001.

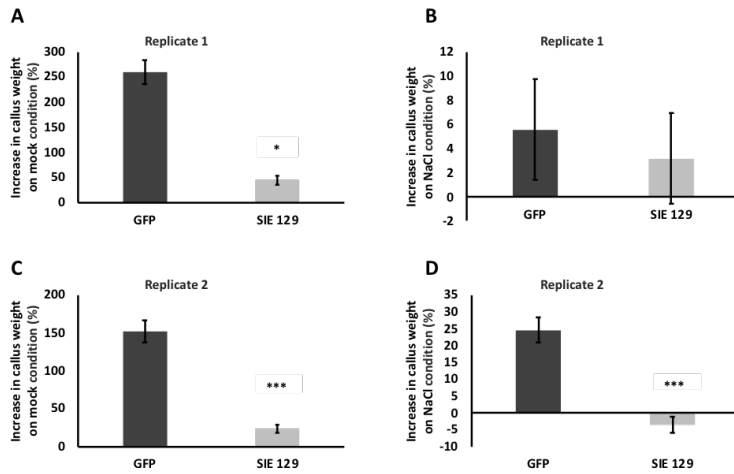


Figure 5.20: Change in weight of calli expressing *S. indica* effector *SIE129* under mock and salt stress conditions.

Calli were exposed to media containing 0 (A, C) and 300 mM NaCl (B, D) for 14 days. The initial and final weight of each callus was obtained at 0 and 14 days after treatment, respectively. Increase in callus weight (in percent) was calculated and the mean values with standard errors of two technical experiments are shown. Calli expressing GFP served as control. T-test was used to compare the weight of calli expressing *SIE129* to those expressing GFP. *=significant at p-value of <0.05. ***=significant at p-value of <0.001.

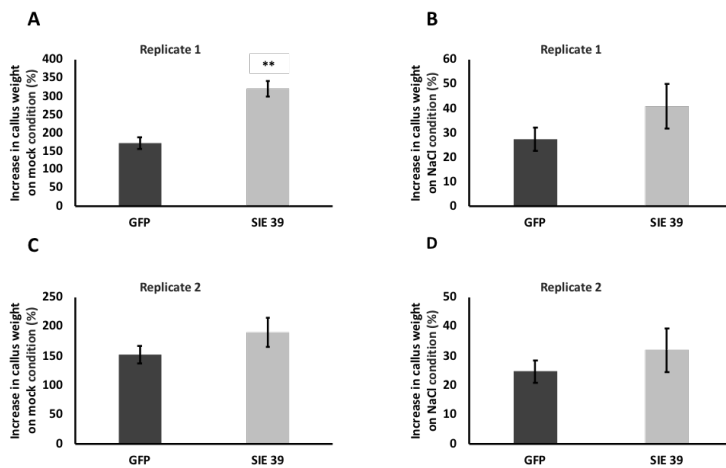


Figure 5.21: Change in weight of calli expressing *S. indica* effector *SIE39* under mock and salt stress conditions.

Calli were exposed to media containing 0 (A, C) and 300 mM NaCl (B, D) for 14 days. The initial and final weight of each callus was obtained at 0 and 14 days after treatment, respectively. Increase in callus weight (in percent) was calculated and the mean values with standard errors of two technical experiments are shown. Calli expressing GFP served as control. T-test was used to compare the weight of calli expressing *SIE39* to those expressing GFP. **=significant at p-value of <0.01.

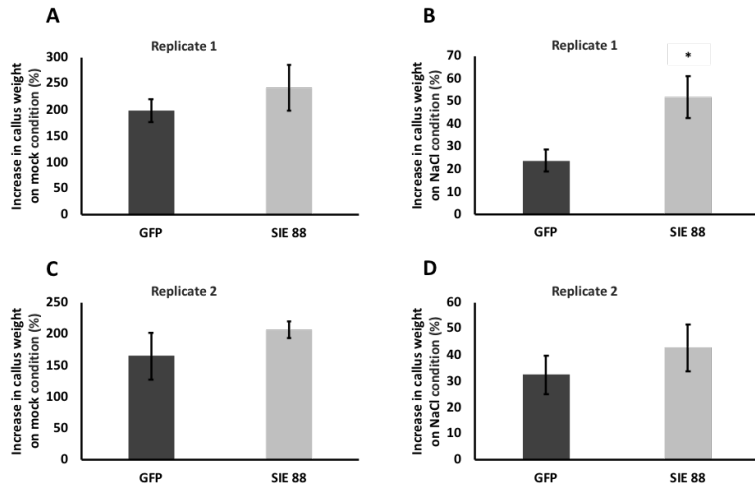


Figure 5.22: Change in weight of calli expressing *S. indica* effector *SIE88* under mock and salt stress conditions.

Calli were exposed to media containing 0 (A, C) and 300 mM NaCl (B, D) for 14 days. The initial and final weight of each callus was obtained at 0 and 14 days after treatment, respectively. Increase in callus weight (in percent) was calculated and the mean values with standard errors of two technical experiments are shown. Calli expressing GFP served as control. T-test was used to compare the weight of calli expressing *SIE88* to those expressing GFP. *=significant at p-value of <0.05.

5. 4. Discussion

The purpose of these studies was to investigate and translate the functions of *Serendipita indica* effectors identified in stress assays in the model plant *Arabidopsis thaliana* to barley as a model crop system. The long-term aim is to exploit effectors for crop improvement programmes. Model plants such as *Arabidopsis thaliana* have played a pivotal role in functional gene studies, but applying findings in a crop is very important (Nelissen et al., 2014). In terms of applications, the relevance of information obtained from studies in *Arabidopsis* plant for the improvement of cereals to secure food security is still questionable (Nelissen et al., 2014). Hence, barley was chosen in this study not only because it is one of the most important crops in the world (FAO, 2010), but also a versatile model crop that have been widely and successfully used for different kind of trait improvement studies in cereals or other closely related crops (Schulte et al. 2009; Hockett and Nilan, 1985; Hogberg, 1987). Bo et al. (2015) reported that for functional gene studies, barley serves as an ideal model for polyploid bread wheat because of its high transformation efficiency and lower genome complexity. An improved barley scutella transformation system that can be employed for more large-scale genetic studies (as established in the preceding chapter) allows researchers to investigate the roles of a large number of *S. indica* effectors in a crop-based system. The primary focus here was to test the transformation system suitability for stress resistance assays of transformed gene products. Thus, the conditions for analysing roots generated from barley calli for *Fusarium graminearum* resistance as well as for screening transgenic calli for salt tolerance were defined and optimized. Although *F. graminearum* inoculation methods that result in root rot infections in roots from barley seedlings have been published (Machado et al., 2018, Taheri, 2018), the same dipping method was not effective in causing root rot disease in roots regenerated from calli. Bishop and Cooper (1983) explained that after fungal spore germination, hyphae produced adhere to the host root surface before fungal infection begins. A lack of well-developed root hairs was observed on roots from calli, and such roots might be unable to provide enough points of attachment for spores, unlike roots from whole barley seedlings that have well-advanced root hairs.

Therefore, alternative methods of inoculation were tested. An even application of 50 spores on the surface of media plates either by use of spreaders or glass beads before transferring roots was found to be very effective for the fungus to colonize and invade roots regenerated from calli. To effectively assay for *F. graminearum* disease resistance, a uniform distribution of

spores on the media is therefore recommended, since it results in disease symptoms on roots from calli, which are similar to the symptoms observed on roots of barley seedlings. These observations are in line with Imani et al. (2011) who further showed evidence that analyses of roots generated from barley calli can be used to study microbial (*S. indica* and *F. graminearum*) root interactions.

These studies demonstrated that sugar content in the culture media plays a significant role in the proliferation of fungi. While higher maltose concentrations prevent fungal infection, lower maltose concentrations will force the fungus to start producing a pinkish exudate. Sugar starvation triggers alteration in physiological and biochemical processes (as earlier) with which fungi aim to sustain respiration and other important metabolic processes (Shi et al., 2008). Different environmental stresses can change the nature of secondary metabolites produced by *F. graminearum* (Garcia-Cela et al., 2018). Thus, the pink exudate (secondary metabolite) produced could be a symptom of starvation of carbohydrate. Therefore, optimizing the provision of carbohydrate sources is very important to ensure fungal root infection to enable *in vitro* studies. Apart from the maltose concentration in the culture media, the number of spores is another crucial parameter that needs to be optimised. Too many spores will be too devastating for the plant materials to bear, thus detecting small effect due to effectors will be difficult while lower spore concentrations/numbers will be ineffective as the spores will be too far from target roots. In my study, 50 *F. graminearum* spores was found to be sufficient for effective root infection. In previous studies, cereal roots were immersed in fungal cultures and shaken on a rotary shaker (Wang and Gottwald, 2017; Wang et al., 2015). Shaking of the roots was to aid uniform inoculation as a prerequisite for successful colonization and invasion of the pathogen. However, this shaking is not recommended for roots regenerated from calli, which are very tender and delicate. Those roots should be handled with maximum care and attention to ensure they are not exposed to any unwanted secondary stress that will lead to a biased inference.

The lectin WGA conjugated to a fluorophore or fluorescein isothiocyanate (FITC) is a commonly applied dye for microscopic visualization of fungal infection structures in plant material (Ayliffe et al., 2013). WGA-AF488 was used to confirm that root rot/browning was due to colonization and invasion presumed to be *Fusarium*. The mycelia were observed all over the inoculated roots, indicating that root rot symptoms coincided with fungal pathogenesis. It further confirmed that roots regenerated from barley calli can be used as an

efficient and reliable system to study root-microbe interaction as previously reported by Imani et al. (2011). Importantly, since *F. graminearum* inoculated roots formed from calli appeared to respond in a way as observed for roots from whole barley seedlings, the infection assay described here can be used for screening transformed roots for altered resistance to fungal pathogens such as *F. graminearum*.

In addition, the suitability of using GFP expression as a transgenic indicator for calli and root viability was assessed. However, the intensity of GFP in roots remained unaltered after 3 days of inoculation with *F. graminearum*. In addition to measuring the intensity of GFP, transformed roots were observed under bright field. It revealed different degrees of root browning that might instead be categorized and serve as an accurate assessment of fungal pathogenesis. The intensity and extension/spread of root browning (as indicator of the progress of root rot) can be employed to quantify the ability of *S. indica* effectors to mediate *F. graminearum* resistance in barley. For instance, browning intensity could be scored as follows; from 0 = symptomless, 1 = slightly necrotic, 2 = moderately necrotic, 3 = severely necrotic, and 4 = completely necrotic as published (Beccari et al., 2011) while the extension of brown spots could be scaled from 0 = no lesions, 1 = 1 to 24%, 2 = 24 to 49%, 3 = 50 to 75%, and 4 = >75% (browning of the entire root) as has been established before (Percy et al., 2012). It was observed that mycelia coverage significantly interfered with root imaging and thus further adjustments would be required for accurate quantification of root browning; this becomes clearly visible at 3 days after inoculation.

The functions of *S. indica* effectors were successfully investigated in abiotic stress responses in barley. Salt stress was chosen because it is one of the major abiotic stresses worldwide that threatens the productivity of all economically important crops including barley. It has been predicted that impact of salt stress will worsen with the progress of climate changes (Dahal et al., 2019; Dasgupta et al., 2009). The generation of salt-tolerant crops is and has been a major goal in plant breeding programmes for many years (Shrivastava and Kumar, 2015). Salt tolerant crops have improved tolerance in at least one of the three ways plants try to cope with salt stress: osmotic stress tolerance, Na⁺ or Cl⁻ exclusion, or activation of the innate ability conferred by salt tolerant genes to tolerate higher Na⁺ or Cl⁻ levels (Munns and Tester, 2008). Identification of such key tolerant genes is very important in breeding for robust salt tolerant crops. The barley scutella transformation system established in this study was used to examine the functions of *S. indica* effectors in boosting salt tolerance in barley. Improving the

transformation system by generating fully transformed calli has facilitated the study of effectors in the salt stress assay. Although both roots and calli were suitable for the salt stress studies, calli are much less tedious to generate. This was the reason why calli were used for the salt stress studies. 300 mM NaCl were identified to monitor salt stress responses at 14 days after treatment. Callus weight and intensity of GFP were found to represent sensitive parameters to screen for alterations in salt stress tolerance. In addition, the intensity of GFP could be much more suitable in detecting even minor effects of effector as compared to callus weight. This is because little effect of the effector could result in more measurable change in GFP intensity than in callus weight.

In total, 8 SIEs and *GFP* (as a control) were successfully transformed and screened against salt stress. *SIE39* and *SIE88* needs more replications to know if they can increase callus weight under both mock and salt stress conditions. Conversely, *SIE129* seem to reduce callus weight. The yeast 2 hybrid (Y2H) screen data on some of the effectors in the Schäfer group showed that *SIE129* targets abiotic stress induced genes such as *DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 2A (DREB2A)* and *ETHYLENE RESPONSE FACTOR 1 (ERF1)*. *DREB2A* and *ERF1* are major transcription factors that are involved in plant resilience to abiotic and biotic stresses (Nakashima et al., 2000; Sakuma et al., 2002; Cheng et al., 2013). However, the nature of the interaction of the effector with the target proteins is currently studied (pers. comm. P. Schäfer).

In conclusion, this research demonstrated that an improved the barley transformation system published by Imani et al. (2011) can be efficiently used for genetic studies in a more large-scale approach. Using this transformation system, among eight effectors tested, *SIE39* and *SIE88* might promote growth under both mock and salt stress conditions, which requires further confirmation in additional experiments. However, both effectors might represent a promising resource to enhance salt stress tolerance in barley.

6 General discussion

6.1 Plant genetic engineering will continue to play a significant role in tackling the global food crisis

Food security is a major challenge for humankind in the next decades and beyond. To be able to sustain food security for future generations, the world would have to produce about 50 to 100 % more food than the current global food production in the face of the predicted harsh impacts of climate change (Baulcombe, 2010). Breeding techniques will continue to help in development of climate resilient crops with better adaptability (Raza et al., 2019). Since the advent of plant genetic engineering, tremendous improvements in globally important crops have been achieved which might not have been possible using only traditional breeding methods (Moeller and Wang, 2008). Genetic engineering has been successfully applied to generate transgenic plants with enhanced resistance against different biotic (pathogen and pest attack) and abiotic (environmental factors such as salinity, drought, high and low temperature etc) stresses (Raza et al., 2019) and in improving crop yield. Between 1996 and 1999, the area planted commercially with transgenic crops has increased from 1.7 to 39.9 million hectares (James, 1999). Although there are many concerns, many countries have accepted genetically engineered crops (GECs) because of their significant improvement in economically important traits. Ronald (2011) argued that crops developed either through conventional or genetic engineering methods are not different with respect to possible unintentional hazardous effects on human health and the environment. With the increase in food crisis especially in the developing countries, there is possibility that genetically engineered crops will be accepted globally though with some regulations. These regulations may include obtaining license to produce GECs, certifying that transgenic crops produced pose no risk to consumers and environment before releasing to the public, labelling of GECs to respect customer choice among others.

Identifying genes of interest is very crucial for any molecular breeding to be employed. Many genes that have been used to generate new crop varieties could come from the same or different genera, species, or even from microbes. For instance, genes derived from plant viruses can be considered as safe transgenes provided these viruses are not known to cause disease in humans and animals (Datta, 2013). As well as inserting of genes of interest into the genome of a crop under improvement, host gene overexpression, silencing and gene editing are currently being

used to develop novel and enriched crop varieties. With the application of modern plant breeding tools, plant breeders can create novel genes that can be exploited to engineer economically vital crops with desirable traits of interest (Datta, 2013). Thus, genetic engineering will continue to play a significant role in meeting the demand of feeding about 9 billion world population by year 2050 and beyond. However, the major challenge is finding or creating new key genes that can boost plant yield and stress resistance uncoupling the growth vs resistance trade-off. Exploring all the possible means to generate as many important genes as possible for improving crop yield, quality and stress resistance will be fundamentally crucial in tackling global food crisis.

Pathogens have found to use their effectors to suppress immunity of their host plants. This invasion usually leads to different kinds of plant disease (Toruño et al., 2016) resulting in reduced yield outputs. Functions of effectors from plant pathogens have been extensively studied in different types of pathogens, for example bacteria (Lozano-Durán et al., 2014), fungi (Doehlemann et al., 2009, Ahmed et al., 2015), nematodes (Lilley et al., 2018), and insects (Rodriguez et al., 2017). Understanding the functions and structures of plant pathogenic effectors has significantly increased our knowledge of important molecular components involved in plant–pathogen interactions such as plant hormones, plant receptors, and signal transduction proteins (Win et al., 2012). The ultimate aim of understanding the molecular mechanisms underlying plant-pathogen interaction is to identify the key molecular components that can be exploited for generating disease-resistant crops. These molecular components could be genetically manipulated through small interfering RNAs (siRNAs) and host-induced gene silencing of virulent domains of pathogen effectors or through gene/genome editing using the (recent technique such as clustered regularly interspaced short palindromic repeats [CRISPR] and CRISPR-associated protein 9 [Cas9]) of the target host proteins. Genetically engineered crops through transgene-based Host-Induced Gene Silencing (HIGS) or Virus Induced Gene Silencing (VIGS) that can modulate expression of key genes in pathogens that are involved in causing plant diseases have been effectively demonstrated (Qi et al., 2019; Koch and Kogel, 2014; Kant and Dasgupta, 2019). Many studies have shown the efficiency of RNAi in controlling plant diseases caused by bacteria, viruses, fungi, insects, nematodes, and parasitic weeds (Saurabh et al., 2014). Even insects feeding on transgenic plants expressing RNAi constructs to silence pest genes have been reported to be severely affected (Huang et al., 2006; Baum et al., 2007; Mao et al., 2007) suggesting the RNAi acts both ways. HIGS technology have been used to control *Fusarium* head blight caused by *Fusarium graminearum*

in barley (Qi et al., 2019). Koch et al. (2013) assembled dsRNA that disrupted the expression of fungal sterol cytochrome P450 lanosterol C-14a-demethylase gene (*CYP51*) in barley, which resulted in the restriction of fungal growth and mitigation of disease symptoms. Also, transgenic rice plants expressing RNA hairpins designed to target *MoAPI* from the rice blast fungus *Magnaporthe oryzae* showed increased resistance to 11 different *M. oryzae* strains (Guo et al., 2019). Sanju et al (2015) further reported that RNAi constructs that targeted a single effector gene (*Avr3a*) from *Phytophthora infestans* (oomycete pathogen that causes late blight in potato) conferred moderate resistance.

6.2 Harnessing *S. indica*, a beneficial microbe with the potential to increase crop production

In nature, plants interact with different kinds of beneficial microbes in the soil (Zolla et al., 2013) and microbial communities play a substantial role in the overall performance of plants by influencing plant physiology and development (Mendes et al., 2013). The outcome of the interaction determines the fate of the plant in terms of growth, yield and fitness. While pathogenic microbes, in many cases, cause different kinds of diseases that interfere with plant growth, development and yield, beneficial microbes support a wide range of vital functions in plant productivity, such as nutrient cycling, mineralization of soil organic matter and enhancing biotic and abiotic stress resistance (Zolla et al., 2013). Khalid et al. (2009) review that significant attention has been given to beneficial microbes in agriculture because of their unique ability to expedite beneficial functions in host plants. One of the beneficial microbes that has been functionally characterized is *Serendipita indica* (formerly known as *Piriformospora indica*). It is a ubiquitous mutualistic fungus that was first discovered in the Indian Thar desert in northwest Rajasthan in 1997 (Deshmukh et al., 2006) and has been extensively studied for its beneficial functions in various plants (Akum et al., 2015). The ability of *S. indica* to increase yield, biotic and abiotic stress resistance in plants has been reported in many studies (Schäfer et al. 2009; Unnikumar et al., 2013; Deshmukh et al., 2006). Currently, this beneficial fungus has been attracting considerable research attention because it has the capacity to colonize roots from potentially all cultivated crops and can be easily cultured on a synthetic media or in bioreactors to be possibly used as biofertilizers for commercial crop production (Singh et al., 2003; Oelmüller et al., 2009; Bagde et al., 2010b; Qiang et al., 2011). In other words, it exhibits no sign of host specificity, a unique feature that makes it ideal for a wide range of applications in biotechnology and crop production (Franken, 2012; Qiang et al.,

2012). Improvement of plant growth and stress resistance by exploiting the intrinsic beneficial capacity of *S. indica* has been considered as a potential means of helping to achieve sustainable and reliable agricultural production (Singh et al., 2011).

6.3 Can effectors from beneficial microbes enhance plant growth and stress resistance?

Effectors were initially considered to simply be virulence-determining small proteins secreted by pathogens (Stergiopoulos and de Wit, 2009; van Esse et al., 2008). Recently, it has been shown that effector proteins are secreted by beneficial microbes and even saprophytes (Rovenich et al., 2014). Similar to other beneficial microbes, *S. indica* has also been proven to have the capacity to secrete and deliver effectors to reprogram host cells (Kloppholz et al., 2011; Plett et al., 2011; Rafiqi et al., 2012). Hassing et al. (2019) however underlined that only a small number of effectors from plant beneficial microbes have been functionally analysed. An effector from a beneficial microbe that has been described is *MiSSP7* from *Laccaria bicolor*, an ectomycorrhizal fungus of poplar roots. Plett et al. (2014) showed that *MiSSP7* is essential for colonization of roots of host trees by modulating the host protein *PtJAZ6* that is a negative regulator of jasmonic acid. Another example is *SP7*, which is secreted by the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (previously known as *Glomus intraradices*) to influence defence signalling by interacting with the pathogenicity-related transcription factor *ERF12* (Kloppholz et al. 2011). They also reported a higher mycorrhization when the effector was constitutively expressed in plant roots. In addition, expression of the effector in the rice blast fungus *Magnaporthe oryzae* diminishes root decay symptoms (Kloppholz et al. 2011). Host-induced gene silencing of the known effector *Strigolactone Induced Secreted Protein 1 (SISI)* secreted by *R. irregularis*, resulted in reduced colonization and the development of stunted arbuscules (Tsuzuki et al., 2016).

Apart from beneficial fungi, some effectors from beneficial bacteria have been functionally characterized. Some of their effector proteins have been reported to be essential for nodulation (Clúa et al., 2018). For instance, type 3 secreted effectors (*T3Es*) of Rhizobia promote nitrogen-fixing nodulation by favouring the activation of symbiosis-related processes but suppressing host immunity (Miwa and Okazaki (2017). *NopJ*, *NopM* and *NopT* effectors secreted by the T3SS of *Rhizobium sp* can reduce nodulation of legumes, while *NopM* and *NopT* have the ability to trigger nodulation in some legumes (Kambara et al., 2009). Kambara et al., (2009)

further revealed that some legumes can identify an effector with a potential pathogenic action as a threat which triggers plant immunity to stop the infection process, whereas others recognize effectors with suppressing and inducing impacts concurrently. The combination of effectors with a variety of impacts on host plants could partly enable symbiosis (Kambara et al., 2009).

Despite the functional characterization of some effectors from beneficial microbes, only a few published studies have attempted to investigate the functions of their effectors in enhancing plant growth, yield and stress resistance. *S. indica* effectors are also poorly characterised. Although hundreds of genes coding for *S. indica* effectors have been recently identified, (Zuccaro et al., 2011; Akum et al., 2015), the only functional study has focused on the effector *PIIN_08944*. Akum et al. (2015) demonstrated that *PIIN_08944* plays a significant function during colonization of *Arabidopsis thaliana* roots by reducing the expression of pattern-triggered immunity (PTI) genes and salicylic acid defence. This lack of functional characterization of *S. indica* effectors despite their interesting roles has hindered their exploitation in boosting crop growth, yield and stress resistance. The aim of this study was to develop tools to analyse the function of *S. indica* effectors and reveal to what extent these effectors mediate the beneficial activities reported for *S. indica*-colonised plants. Investigating the functions of *S. indica* effectors could open up new opportunities to engineer crops with improved yield and resilience against biotic and abiotic stresses as an alternative to the application of the microbe as bio-fertilizers.

6.4 *S. indica* effectors modulate Arabidopsis protoplast responses to biotic and abiotic induced stresses

A large set of 150 effectors from *S. indica* was studied to understand their functions in crop growth and stress resistance in both a single cell and a crop-based system. Although genetic and phenotypic studies using single cell or model plants are scientifically accepted, there is the possibility that the outcome of such analyses is totally different when tested in the intended crops. Investigating the roles of effectors in different systems of varying complexities will give in-depth information on the reproducibility and direct application of research outcomes into different plants/crops. A protoplast-based assay was used to screen 150 *S. indica* effectors in *Arabidopsis thaliana*. Plant protoplasts have been in use for more than 20 years as one of the most useful and versatile analytical tools in plant research (Negrutiu et al., 1992). Transient

expression of effectors in protoplasts has proven to be a rapid and reliable technique for studying the functions of microbial effector proteins (Li et al., 2005; He et al., 2006). It provides the opportunity to test large sets of candidates in a medium to high-throughput manner (Zheng et al., 2014). Such systems have been used to identify a set of *RXLR* effectors from *Phytophthora infestans* (causal agent of potato and tomato blight) to interfere with early stages of flg22-triggered signalling (Zheng et al., 2014).

The expression of *S. indica* effectors (SIEs) were tested for their ability to modulate responses to different stresses. Specifically, using protoplasts SIEs that can affect responses to bacterial flg22 (as PTI elicitors) and abscisic acid (ABA; as abiotic stress mediating hormone) were analysed. Instead of using pathogen inoculation, flg22 was applied to the protoplasts transformed with different SIEs as an immunity elicitor. Exogenous application of ABA has been widely used to stimulate abiotic stress responses in plants. Use of luciferase fused to the promoter of the PTI marker PHI-1 (*pPHI1::LUC*) and ABA marker (*pRD29A::LUC*), allowed measurement of the intensities of luminescence produced in the presence of different effectors under mock, PTI and ABA conditions.

In this study, only the top 10 SIEs that significantly induced or suppressed flg22 or ABA responses, as identified in the protoplast assay (in collaboration with Dr Silke Lehmann) were further analysed. The ability of some of the SIEs to alter protoplast response under mock, biotic and abiotic treated conditions was in accordance with previous reports that showed that beneficial microbes use their effectors to reprogram host cells (Rovenich et al., 2014; Klopffholz et al., 2011; Plett et al., 2011; Rafiqi et al., 2012; Clúa et al., 2018). Among the SIEs tested, only two effectors (*SIE10* and *SIE87*) were identified to modify both flg22 and ABA responses. *SIE10* strongly induced PTI and ABA responses in protoplast under mock conditions and when treated with either flg22 or ABA. *SIE87* repressed the PTI and *RD29A* responses in protoplast in both mock and stressed conditions. This postulates that, as characterised for pathogen effectors (Toruño et al., 2016; Snelders et al., 2018; Wu et al., 2018), mutualistic effector proteins could alter specific pathways in host plants. As observed in this study, some effectors might affect only biotic stress or abiotic stress signalling in plants.

6.5 Functional analyses of *S. indica* effectors in plant growth and biotic stress resistance in a model plant

In order to understand effector functions in a more complex system than in a single cell protoplast-based system, the SIE candidates tested in protoplasts were stably transformed and tested in the model plant *Arabidopsis thaliana*. *A. thaliana* is an important and commonly used model organism for studies of plant stress and growth physiology (Meyerowitz and Somerville, 1994; Meinke et al., 1998; Dean, 1993; Pyke, 1994). Apart from those top SIE candidates identified to affect flg22 responses in protoplasts, SIE candidates that influenced protoplasts responses to other hormone or abiotic treatments (i.e. *SIE126* and *SIE69* in ABA, *SIE88* and *SIE64* in auxin, *SIE109* in cytokinin, *SIE76* in salicylic acid, and *SIE91* in salt stress assays) were included. In total, 32 effectors were investigated in *A. thaliana*. For each effector, a pool consisting of three independent lines was used and primary root growth was measured under both mock and ABA conditions. The growth analysis of all lines (individual and pooled lines) expressing SIEs showed that some effectors significantly promoted while other repressed root growth under mock and flg22-treated conditions. Kambara et al. (2009) revealed that the beneficial bacterium *Rhizobium sp.* NGR234 uses a combination of effectors with suppressing and inducing activities to colonize and trigger nodulations in legumes. In addition to growth phenotyping, selected SIEs were assessed for their ability to alter root sensitivity to flg22. While 5 SIEs significantly increased flg22 sensitivity, 4 SIEs significantly reduced sensitivity to flg22. Individual lines of 4 SIEs were further examined for root sensitivity to flg22 for reproducibility, but only *SIE67* and *SIE76* had reproducible results. Positional effects or insertion of multiple gene copies might contribute to the phenotypic variation among the individual lines and emphasises the relevance of using individual lines rather than pooled lines for functional analyses of SIEs *in planta*. For further studies, it will be interesting to challenge the individual lines expressing *SIE67* and *SIE76* with pathogens such as *Pseudomonas Syringae*, *Fusarium oxysporum* as to assess ability of these effectors to enhance disease resistance in plants.

Effectors often lack conserved domains, which is often explained by high selective pressure against known domains and, hence, to avoid host recognition (Dodds et al., 2009; de Jonge et al., 2010; Rafiqi et al., 2010). Out of 6 SIEs that mediated an altered flg22 sensitivity (*SIE23*, *SIE67*, *SIE76*, *SIE99*, *SIE106*, and *SIE146*), *SIE67* was found to carry a known protein domain, defined as DJ-1_PfpI with no known protein domains for the others. The DJ-1/ThiJ/PfpI

superfamily embodies a diverse group of proteins found in nearly all organisms (Wei et al., 2007) including humans (Zhu et al., 2010; Clements et al., 2006). DJ-1/PfpI domains were found to be ~165 amino acids in soybean (Islam and Ghosh, 2018), 140 in rice, 150 in Arabidopsis and 170 in *Escherichia coli* (Ghosh et al., 2016). Few members of DJ-1/ThiJ/PfpI family proteins that have been functionally described (Zhan et al., 2014) appear to be involved in increasing stress resistance. The proteins may have distinct functions in different organisms (Smith and Wilson, 2017). For instance, in human, *DJ-1* protein has been found to be associated with cancer and Parkinson's disease (Wilson, 2011). However, they have also been reported to function in the scavenging of reactive oxygen species (ROS) produced under oxidative stress in animal systems (Lee et al., 2012) as well as in plants (Ghosh., et al 2016). Expression of *DJ-1* transcripts in rice (*OsDJ-1*) was increased in response to different abiotic stresses such as salinity, drought, cold, heat and oxidative stresses (Ghosh., et al 2016). Likewise, the transcript level of *AtDJ-1A* in Arabidopsis was induced in response to different external stresses including strong light, CuSO₄, H₂O₂ and methyl viologen (Xu, et al., 2010). Overexpression of the yeast *DJ-1* homolog *Hsp31* in the model plant *Nicotiana tabacum* enhanced abiotic and biotic stress resistance (Melvin et al., 2017). Islam and Ghosh (2018) also reported that expression of the *GmDJ-1D1* transcript in soybean was highly induced when plants were treated with various abiotic stresses such as salinity, dehydration, oxidative stress, dicarbonyl stress and after ABA treatment.

To further understand the significance of *SIE67* and three other SIEs (*SIE76*, *SIE99* and *SIE146* as candidates altering flg22 responsiveness) in stress resistance in plants, the expression of three PTI-induced marker gene (*FRK1*, *PHI-1* and *NHL10*) was analysed upon flg22 treatment of lines overexpressing these effectors. *SIE67* did not alter PTI response in Arabidopsis in consistent pattern in all the replicates. More studies on *SIE67* are needed to understand its roles in plant stress resistance. The reduced root sensitivity to flg22 in *A. thaliana* (compared to the control line) obtained might be due to the presence of the DJ-1 protein domain identified in *SIE67*. It will be interesting to investigate if the effect is caused by the identified domain through. This could be done by mutation of the active domain of the protein of *SIE67* using site-directed mutagenesis or CRISPR/Cas9 technology before expressing the effector *in-planta* for genetic functional analysis.

6.6 Functional analyses of *S. indica* effectors in abiotic stress tolerance in *Arabidopsis*

In order to understand if some of the SIEs could also play important roles in abiotic stress response in *Arabidopsis thaliana*, 20 SIE candidates identified in the ABA-protoplasts assay were stably transformed in *A. thaliana*. ABA was used to stimulate abiotic stress response in the model plant and, as for flg22 assays with whole SIE-expressing lines, the selected effectors were tested to see if they promoted or repressed root growth under ABA conditions. It was found that not all these effectors expedited beneficial effects in plants. For instance, lines expressing *SIE44*, *SIE88* and *SIE120* significantly promoted growth while those expressing *SIE10* repressed growth. In addition, *SIE10* showed reduced root sensitivity to ABA whereas *SIE44*, *SIE88* and *SIE120* increased it, suggesting a role for *SIE10* in integrating development under stress and perhaps in modulating growth-immunity antagonism. In this respect it is important to highlight the ability of *S. indica* to uncouple growth-immunity trade-offs (Jacobs et al. 2011). In interaction with host plants, *S. indica* is likely to use some effectors to suppress plant immunity in the process of host colonization. In turn, once the interaction with the host is established, another set of SIEs could be released to participate in conferring beneficial effects to the host plants. Similarly, Kambara et al. (2009) revealed that the beneficial bacterium *Rhizobium sp.* NGR234 uses a combination of effectors with both suppressing and inducing activities to colonize and initiate nodulation in legumes.

Of 6 SIEs altering ABA responsiveness, only *SIE10* and *SIE120* had known protein domains. Two domains; *inhibitor_I9* (proteinase propeptide inhibitor) and *peptidase_S8* were found in *SIE10*. The I9 family of protease inhibitors have been found in fungi (Hohl et al., 2017). They specifically inhibit the S8 family of serine peptidases, which comprise subtilisins (SBTs) in bacteria and plants, kexin in yeast and related mammalian proprotein convertases (Schaller et al., 2012). The *peptidase_S8* family (also called the subtilisin or subtilase family) is the second largest family of serine proteases (Rawlings et al., 2010). SBTs are among the large group of degrading enzymes found in almost all organisms (Muszewska et al., 2011). They are very important for fungal adaptation to new environments and to colonise different hosts. (Prasad et al., 2015). Fungi use them to penetrate and colonize their host (Varshney et al., 2016). For instance, Hypocrealean fungi, which can be saprophytic, entomopathogenic and mycoparasitic (Rossman et al. 1999) use them for host invasion and pathogenesis, for breaking down of insect cuticles or protein-rich components of egg shells (Lai et al., 2014; Donatti et al., 2008). It has

also been reported that *Purpureocillium lilacinum* recruit them to degrade protein constituents of nematode and insect eggs (Varshney et al., 2016). The proteins are also employed by nematode-trapping fungi against their hosts (Li et al. 2010). *Beauveria bassiana* and *Cordyceps militaris* use them to degrade insect cuticles (Xiao et al., 2012; Zheng et al., 2011).

SBTs are largely conserved in different species, but their function can differ among organisms; for instance, genetic and functional differences of SBTs exist among different fungal genomes (Li et al., 2010). Evolutionarily, SBTs are conserved in all three domains of life (Archaea, Bacteria and Eukarya), but many more SBTs have been recently found in plants than in other organisms (Schaller, et al., 2012). The expansion of the SBT family in plants was thought to be possible because of its importance in plant diversification and survival during evolution (Schaller, et al., 2012). They play important roles in protein turnover and organ development in plants (Schaller et al. 2012; Siezen and Leunissen 1997). They are also vital in plant developmental processes which include development of lateral roots, cuticles and differentiation of epidermis and xylem (Neuteboom et al. 1999; Tanaka et al. 2001; Zhao et al. 2000). They have been identified to be involved in plant growth, seed and fruit development, and in plant resistance to biotic (such as in programmed cell death), and abiotic stresses (Schaller, et al., 2012). Similarly, they have been proven to be essential in different kinds of symbiotic (including arbuscular mycorrhization and nodulation) and pathogenic interactions (Taylor and Qiu, 2017) in plants. Plant SBTs share many similarities with their bacterial and mammalian homologs, but their exclusive functions especially in plant physiology along with their distinctive biochemical and structural characteristics differentiate them from SBTs present in other organisms (Schaller, et al., 2012).

Based on the comprehensive information that microbes use SBTs for host colonization and invasion, this suggests *SIE10* that has a SBT domain could be among the effectors that *S. indica* use to colonize its host. Some effectors have already been identified to support host colonisation by beneficial microbes. Akum et al. (2015) demonstrated that *S. indica* candidate effector *PIIN_08944* plays a significant function during colonization of *Arabidopsis thaliana* roots by reducing expression of PTI genes. Plett et al. (2014) showed that *Laccaria bicolor* (an ectomycorrhizal fungus) effector *MiSSP7* is essential for colonization of roots of poplar trees by modulating the host protein *PtJAZ6*, which is a negative regulator of jasmonic acid. Furthermore, *SP7* effector from the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (formerly known as *Glomus intraradices*) modulates defence gene signalling by interacting

with the pathogenicity-related transcription factor *ERF12* to enable colonization (Kloppholz et al., 2011).

SIE10 was among the few effectors that repressed root growth in Arabidopsis, which could be as a result of modulating impact of the effector in the plant. This modulation does not result in obvious necrotic cell death as associated with pathogenic effectors. Voß et al. (2018) recently reported that Crinkler (*CRN*) effectors in pathogens have been shown to be associated with cell death in plants while *CRN* from *Rhizophagus irregularis* did not cause cell death symptoms when expressed in *Nicotiana benthamiana* leaves or in *Medicago truncatula* roots. In addition, the reduced expression of the three ABA markers (*RAB18*, *RD29A* and *RD29B*) observed in *SIE10*-expressing lines under mock condition further showed its modulating capacity on abiotic stress signalling. This inhibitory effect on ABA responsive markers could result in the insufficient endogenous ABA being generated for proper root growth and development. ABA has originally been viewed as a growth inhibitor, but it also has growth promoting roles in plants (Arteca, 1996; Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992). Young tissues contain high levels of ABA; mutant plants deficient in ABA are stunted and show growth inhibition (Finkelstein, 2013). Xiong and Zhu (2003) further emphasized that ABA is essential for normal plant growth as ABA-deficient Arabidopsis mutants showed reduced cell vigour and plant growth (Xiong and Zhu, 2003). The reduced growth is partly because ABA-deficient plants have decreased transpiration and compromised turgor, and also because of over-production of ethylene (Sharp, 2002). Hormonal profiling of the lines expressing *SIE10* will be important to understand the modulating impact of this effector especially on endogenous ABA signalling in plants.

The *Methyltransf_11* domain was also identified in effector *SIE120*. Lines expressing *SIE120* showed consistently and significantly improved growth under mock and ABA conditions. The growth inducing ability of *SIE120* was also demonstrated even when the lines were tested under flg22 vs mock treated conditions. Methyltransferases are enzymes involved in the transfer of a methyl group from S-adenosyl-l-methionine to different substrates such as DNA, RNA, proteins, and small-molecule metabolites and catalyzes DNA methylation (Cai, 2016, Adhikari and Curtis, 2016). DNA methylation is essential for normal growth and development in plants and animals (Huang et al., 2016). Reduction in DNA methylation results in floral abnormalities in *Arabidopsis thaliana* (Finnegan et al., 2000). Some bacteria can use certain DNA methyltransferases that lack restriction endonucleases and called orphan methyltransferases to

control some vital cellular processes such as DNA replication, DNA repair and gene regulation (Lu et al. 1994; von Freiesleben, Rasmussen and Schaechter 1994; Woude, Braaten and Low 1996; Wion and Casadesús 2006). It has also been shown that bacterial DNA modified by those enzymes can be involved in microbial self-defence (Naito et al., 1995). The importance of methyltransferases, especially in DNA methylation, could be the reason *SIE120*-expressing lines showed a significant better root growth than control lines. The expression of ABA induced markers was analysed in lines expressing *SIE120* and *SIE44* that promoted root growth. Reduced induction of *RD29A* was found under ABA treatment compared to the control line. This could explain the increased root sensitivity of both lines to ABA. These effectors, particularly *SIE120*, could target and modify host processes important for higher yield in crops.

6.7 Optimization of a barley scutella transformation system for rapid functional studies of effectors

This work established an improved barley transformation system that can facilitate functional studies of *S. indica* effectors in a monocot crop in the future. Barley is an important global cereal and model crop (Bartlett et al., 2008; Kumlehn et al., 2006) which was chosen to further test the functions of SIEs that showed interesting activities in *A. thaliana*. Conventional barley transformation has been used to develop transgenic barley varieties with different economic important attributes that improved traits such as yield, seed quality and stress resistance. Transformation via *Agrobacterium tumefaciens*-mediated alone has produced over 10,000 transgenic barley plants over the past 10 years (Marthe et al., 2014). One of the most important advantages of an *Agrobacterium*-based transformation systems is the relatively high transformation efficiency (Nadolska-Orczk et al. (2000). Despite this advantage over other transformation methods (such as particle bombardment, electroporation), transformation efficiency is still one of the limiting factors in the production of large numbers of genetically engineered barley plants (Bartlett et al., 2014). Transformation efficiencies of about 25% or more have only been reported in barley (Bartlett et al., 2014, Hensel et al., 2008; Harwood, 2014). Using this transformation system in functional analyses of a large number of genes for instance, testing thousands of genes would be prohibitively costly and a high number of barley scutella would need to be transformed to compensate for the non-transformed scutella (~75%). Moreover, it requires at least 1 year to generate homozygous lines expressing genes of interest if the transformation process is successful. The absence of more rapid, high-throughput

transformation systems in cereal crops that can keep pace with ‘omics’-derived knowledge from transcriptome, proteome, or metabolome studies is a major drawback that limits our abilities to functionally examine and develop novel traits in crops (Imani et al., 2011; Harwood, 2012).

Imani et al. (2011) established a new approach for barley called stable root transformation system (STARTS) which aimed to reduce the period of time usually required before homozygous lines expressing the genes of interest can be phenotyped. STARTS thus allowed functional genetic analyses at a higher throughput. This system is highly time efficient as it takes about 6 weeks to generate plant material (roots) in comparison to the conventional method that requires at least 1 year to generate homozygous lines. STARTS follows the same process as in the conventional method but transformed calli were used to generate roots instead of whole plants. This new system would not entirely replace the conventional transformation method but to complement it. Only genes or sequences that have been confirmed to have an important function will be forwarded to the generation of homozygous plants, while ‘ineffective’/inconclusive genes will be discarded at an early stage. STARTS could be a powerful and rapid tool for larger scale genetic studies in barley. However, in the course of optimizing the system, it was observed that the calli produced did not have a uniform transformation pattern. The transformation intensity (the area fully transformed relative to the entire plant area intended to be transformed) was low. Although the transformed scutella showed high transformation intensity, calli generated were consistently composed of transformed and untransformed fragments that were randomly interlaced. When the calli were advanced to produce roots, there was a mix of transformed and untransformed roots. These heterogenous calli or roots will display qualitative and quantitative differences in transgene expression levels at different points on the same callus/root. This anomalous variations in transformation intensities have to be resolved for STARTS to be efficiently suitable for larger scale gene functional studies. High transformation efficiency combined with uniform transgene expression patterns is essential for functional studies of genes to be utilized in molecular breeding (Li et al., 2017).

In an attempt to resolve the problem of the non-uniform transformation pattern, the first step taken was to understand the cause of the problem. The source of the problem was traced to the transformed scutella. When scutella are transformed only the periphery of scutella where the embryonic axis was removed will be initially transformed, resulting in the observed chimeric

transgenic state of calli, even on higher selective antibiotic- containing media. As observed in this study, culturing scutella on media containing high (but optimal) antibiotics improved the transformation efficiency (approximately 100% was possible), but low transformation intensity (variation) was still an issue. This showed that it is impossible to transform all scutella parts. The only option was to manipulate the calli formed to facilitate homogeneous transgene expression patterns. By culturing the growing calli (after one month of scutella transformation) for additional 1-2 weeks, the calli crumble into fragments. The uniformly transformed parts were carefully separated from the untransformed parts (without damaging calli tissue) and regenerated on higher selective antibiotics (more than the concentration used for selecting transformed scutella) containing media to reach the size of the mother calli (within ~3 weeks). Using higher concentrations of selective antibiotics reduces the chance of growth of any untransformed tissue and the regenerated calli were found to be uniformly transformed. Accordingly, the calli obtained by this procedure resulted in regeneration of ~100% stably transformed roots per callus. The results here explain why transformation efficiency of about 25% have only been reported in barley (Bartlett et al., 2014, Hensel et al., 2008; Harwood, 2014). If technical modifications described above are incorporated into the conventional transformation systems of barley and other cereal crops to generate uniformly transformed calli rather than using calli with chimeric transgene expression, the transformation efficiency of the transgenic plants will likely rise above 70%. With these important improvements, STARTS will facilitate the use of the system to complement the conventional transformation system for more large-scale and rapid functional genetic studies as demanded in our era of post-genomic studies.

6.8 Investigating the functions of *S. indica* effectors in *Fusarium graminearum* resistance in barley

The next step was to use the improved barley scutella transformation to investigate the roles of SIEs in biotic and abiotic stress resistance in barley as a crop-based system. *Fusarium graminearum* as causal agent of root, crown, floral and seed rot, and Fusarium head blight (FHB) diseases in barley and wheat (Machado et al., 2018, Taheri, 2018) was used. In the same vein, salt stress, a major threat to global food security (Oyiga et al., 2016) that severely impairs plant development and yield (Hasegawa et al., 2000) was selected as abiotic stress in plants. For the *F. graminearum* assay, the challenge was to establish how to effectively inoculate the roots generated from the calli and to identify a reproducible method to quantify any stress

resistance launched by SIEs in the transgenic roots. In comparison to dipping roots from barley seedlings in the spore suspension that is normally used for *Fusarium* root rot studies (Machado et al., 2018, Taheri, 2018), establishment of rot disease in roots from barley calli was not effective using the same inoculation method. This might be attributed to the poor root hair development (in terms of maturity and number) from calli, meaning there was no effective point of attachment for the fungal spores. Bishop and Cooper (1983) also stated that after fungal spore germination, hyphae require adherence to the host root surface before fungal infection begins. Instead, inoculating the surface of media on plates with spores either by use of glass beads or spreaders before placing calli with roots was effective to result in visible root rot diseases within 3 days after inoculation. In accordance with studies of Imani et al. (2011), phenotypic and genetic analyses of roots from barley calli inoculated with *F. graminearum* or *S. indica* inoculation were similar to the previous findings reported using roots from the crop seedlings. The major challenge in establishing the assay was to determine the right quantity of fungal inoculum (e.g. number of spores) in combination with an optimal sugar concentration to provide reproducible conditions for fungal proliferation and infection without inducing media-related stress to calli or roots. Using media with insufficient sugar to support fungal growth would lead to fungal starvation and would alter the ability of the fungus to produce secondary metabolites that are required for colonization. There is high possibility that excess of sugar may instead encourage the fungus to grow only on the media and avoid the costly processes involved in colonization and invasion of the host. This could lead to ineffective inoculation and disease establishment. Each parameter (e.g. inoculation method, media composition) needs to be defined for each microbe and may differ between the present system with roots regenerated from calli compared to barley seedlings.

Having established the inoculation procedures, the next step was to develop a method for semi-automated quantification of disease levels in transgenic roots. Assessing fluorescence intensity in *GFP*-expressing roots was not sensitive enough to determine any change in *GFP* intensity irrespective of treatment. There might have been a reduction in the *GFP* expression but the signal variation was not adequate for quantification. Visualizing roots and root rot symptoms (browning) using bright field images was efficient for quantification as outlined by Beccari et al. (2011) and Percy et al. (2012). Alternatively, more precise methods based on the quantification of fungal DNA by qPCR would be available although the throughput would be lower. Imani et al. (2011) demonstrated that qPCR can be applied to quantify fungal DNA in roots from calli in a similar fashion as reported for roots from whole plants. In this study, roots

expressing SIE effectors could not be screened for altered resistance against *F. graminearum* because of time constraint. However, the protocol for the fungal assay has been successfully established for future analyses. The effectors expressing lines that altered PTI pathway in *A. thaliana* are recommended to be screened first.

6.9 Understanding the functions of *S. indica* effectors in salt stress tolerance in barley

Finally, selected *S. indica* effectors were screened for their ability to alter salt tolerance in barley. For this, uniformly transformed calli were used for the screening as they were easier and faster to generate than roots. To determine reproducible conditions for fast screening against salt stress different parameters were tested. Callus weight and intensity of *GFP* signal intensity in calli were found to correlate under salt stress as compared to unstressed conditions. Different studies have confirmed that *GFP* expression can be useful as a quantitative reporter of expression of candidate genes *in vivo* (Soboleski et al., 2005, Scholz et al., 2000). In this study, salt stress screens in barley paralleled assays in Arabidopsis. In total, 8 SIEs and *GFP* (as a control) were successfully transformed and transformed lines screened for salt stress levels. *SIE39* and *SIE88* appeared to increase callus weight under both mock and salt stress conditions. *SIE39* showed reduced sensitivity to ABA when expressed in Arabidopsis and *SIE88* significantly promoted growth in Arabidopsis. Conversely, *SIE129* and *SIE120* reduced callus weight. It was unexpected that *SIE120*, which significantly promoted root growth in Arabidopsis on both mock and stress conditions repressed callus weight in barley. Although further confirmation is needed, there is possibility that some effectors may alter different pathways when tested in the intended crops. The future work plan will be to screen all the candidate effectors that altered ABA pathway in *A. thaliana*, in this case both roots and calli parts can be used in parallel. For the fact that *S. indica* is a root mutualist, expressing the effectors in barley roots might be more appropriate for the stress resistance assay.

7 General Conclusion

Beneficial microbes have attracted considerable attention in research as they are considered to be potential bio-agents to improve crop production under changing climates and nutrient availabilities (Bhattacharyya et al., 2016; Singh et al., 2011; Khalid et al., 2009). Most studies focus on exploiting microbes as bio-fertilizers and remediators. However, beneficial microbes also release metabolic and protein effectors involved in activating beneficial effects in plants. Knowledge of functions of pathogenic effector proteins in host plants has accelerated our understanding of plant immunity and the development of novel molecular techniques for controlling plant diseases (Win et al., 2012; Stam et al., 2014). These techniques include Host-Induced Gene Silencing (HIGS) and Virus Induced Gene Silencing (VIGS) (Qi et al., 2019; Koch and Kogel, 2014; Kant and Dasgupta, 2019). In addition, gene editing may be applied to manipulate the host proteins that pathogenic effectors target; this could result in enhanced disease resistance in the host plants. It was discovered that beneficial microbes such as *S. indica* can secrete effectors to reprogram their host plants similar to plant pathogens. It is postulated that beneficial microbes could use their effectors to expedite numerous positive benefits that have reported in their colonized plants. However, substantial research has not been carried out to reveal the function of their effectors in plant growth and stress resistance. The dearth of information on their functions has hindered harnessing them as part of sustainable strategies to elevate crop yield and fitness against stresses. The aim of this project was to investigate the roles of a selected set of *S. indica* effectors in single cell, whole plant and crop-based systems. The study represents a new holistic approach in which the functions of a whole set of effectors from a beneficial microbe were analysed in diverse plant systems with different complexities. In *Arabidopsis* protoplasts, its effectors were identified to modulate responses to biotic and abiotic stresses. Moreover, *Arabidopsis* lines and barley calli stably expressing effectors identified in the protoplast assay showed that some effectors have potential to modulate plant pathways involved in crop growth and stress resistance. It suggests *S. indica* effectors are valuable resource for identifying crop traits determining crop performance and yield in the field.

Effectors *SIE44*, *SIE76*, *SIE106* and *SIE120* could be important in enhancing crop growth and yield. For instance, based on the analyses presented here (e.g. qPCR and root stress sensitivity assays) and the information obtained from the domain protein predictions, *SIE67* could be useful in improving stress resistance in plants. Another interesting candidate identified was

SIE10. This effector showed the highest PT1 or ABA response in protoplasts together with lowest reduced root growth in Arabidopsis. The presence of the *peptidase_S8* (subtilisins) domain on this effector suggests it could be important in *S. indica* colonization of host plants. In addition, the reduced expression of the ABA markers observed in *SIE10*- expressing lines suggested its modulating capacity. This effector might be recruited by *S. indica* to suppress immunity as part of the colonisation of host plants. In nature, beneficial microbes likely use a combination of effectors, with activity similar to pathogenic effectors and beneficial ones. These could act in a complementary fashion to interact and confer positive benefits to host plants. It will be interesting in future to further functionally analyse these effectors as a means to better understand how beneficial microbe use effectors to colonise host plants and coordinate the modulation of various plant processes including suppression of immunity, activation of systemic resistance, growth promotion and abiotic stress tolerance.

For further functional validation of *S. indica* effectors in a crop-based system, some steps were improved in a previously established barley transformation system. The major improvement was in the generation of plant materials such as calli and roots with uniformity in transformation. The technical adjustments introduced here to optimize barley scutella-based transformation can be applied to other cereal crops and can help in fast-tracking rapid and larger scale genetic screening systems as demanded in our era of post-genomic studies. With the target of exploiting effectors for crop improvement, the conditions for investigating the roles of effectors in protecting barley against fungal pathogen such as *Fusarium graminearum* and salt stress were established. Because of time constraints, functional analyses of a set of selected effectors only focused on their ability to confer salt stress tolerance in barley. Effector *SIE88* appeared to promote in Arabidopsis and calli growth in barley. *SIE39*, that showed reduced sensitivity to ABA in the model plant seemed to promote calli growth even in salt stress conditions. While further studies are needed, this research showed that effectors from beneficial microbes could be important in developing sustainable crop productivity under changing climates. Investigating combinations of effectors rather than individually could increase the possibility of identifying subsets of effectors with synergistic effects for crop production.

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