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Function of microbial effectors in the establishment of beneficial plant symbioses

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Laura Rehneke

Parts of this work already published or under consideration

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CHAPTER 4

Supplementary Methods

Abbreviations

AMF	-	Arbuscular mycorrhizal fungi
ABA	-	Abscisic acid
AI	-	Artificial Intelligence
AGO	-	Argonaute
Arabidopsis	-	*Arabidopsis thaliana*
ASIL1	-	Arabidopsis 6b-Interacting Protein-Like 1
AUX	-	auxin
CK	-	Cytokinin
Cmu1	-	Chorismate mutase 1
COR	-	Coronatine
CPC	-	CAPRICE
cPEP	-	Complementary peptides
CSN	-	COP9 signalosome
DCL	-	Dicer-like ribonucleases
DNA	-	Deoxyribonucleic acid
DRB	-	DsRNA-binding proteins
ER	-	Endoplasmic reticulum
ET	-	Ethylene
ETI	-	Effector-triggered immunity
FOLD	-	*Fusarium oxysporum f. sp. lycopersici* dual domain
GA	-	Gibberellic acid
gDNA	-	Genomic DNA
GFP	-	Green fluorescent protein
GO	-	Gene ontology
GRF	-	General regulatory factor
HTSP	-	High-temperature seedling-plant
IAA	-	Indol-acetic acid
Isc	-	Isochorismatases
ISR	-	Induced systemic resistance
JA	-	Jasmonic acid

MAMP	-	Microbe-associated-molecular-pattern
MAPK	-	Mitogen-activated protein kinase
miRNA	-	Micro RNA
MiSSP7	-	Mycorrhiza-induced Small Secreted Protein 7
mRNA	-	Messenger RNA
MTF	-	Membrane-associated transcriptions factors
NLR	-	Nucleotide-binding domain leucine-rich repeat
NPR1	-	Nonexpressor of Pathogenesis-related Genes 1
NUE	-	Nitrogen-use efficiency
PAMP	-	Pathogen-associated molecular patterns
PCR	-	Polymerase chain reaction
PRR	-	Pattern-recognition receptor
PTI	-	PAMP-triggered immunity/pattern-triggered immunity
RISC	-	RNA induced-silencing-complex
RNA	-	Ribonucleic acid
RNAi	-	RNA interference
ROS	-	Reactive oxygen species
SA	-	Salicylic acid
SAR	-	Systemic acquired resistance
Si	-	*Serendipita indica*
SIEC	-	*Si* effector candidates
siRNA	-	Small-interfering RNA
sRNA	-	Small RNA
SUC2	-	Yeast invertase, sucrose hydrolysing enzyme
TCP	-	Teosinte branched1/Cincinnata/proliferating cell factor
T-DNA	-	Transfer-DNA
WUE	-	Water-use efficiency
YSST	-	Yeast-signal-sequence-trap
ZFP5	-	Zinc finger protein

Summary

The endophytic root colonising fungus *Serendipita indica* induces growth, as well as biotic and abiotic stress resilience in a broad range of host plants. Similar to plant pathogens, the symbiont needs to overcome host immunity to colonise plants. For this purpose, plant-colonising microbes use, among others, small, secreted proteins termed effectors. Effector proteins are known to alter plant signalling, including hormone pathways, to facilitate host colonisation by pathogens, as well as symbionts. However, *S. indica* is also known to alter hormone signalling toward the increase of host fitness. This project was aimed at analysing whether *S. indica* effectors not only have functions during plant colonisation, but additionally modulate signalling pathways that confer host benefits.

The results confirmed that identified *S. indica* effector candidates (SIECs) are indeed secreted proteins. SIEC-plant protein interaction analyses were carried out to identify the plant proteins targeted by SIECs. Subsequent comparative interactome analyses revealed which targets are common with pathogen effectors or are unique for SIECs. These evaluations already revealed a pronounced SIEC targeting of proteins in host hormone signalling and metabolism. Following functional analyses of SIECs in protoplasts revealed highly specific effector alterations of different hormone pathways. These could be confirmed by phenotyping root traits of plants expressing SIECs. Further, it was shown that the effectors affecting hormone signalling could often increase plant growth. Therefore, effectors are probably part of previously unknown mechanisms for beneficial effects in connection with hormone signalling modulation by symbionts.

To advance our understanding of symbiont increased host fitness under stress, SIECs were analysed for their function in biotic and abiotic stress signalling. Protoplast assays revealed effectors that modulated stress signalling and suggest a connection between stress and hormone signalling function of SIECs. Moreover, protein network analyses uncovered SIEC targeting of plant proteins with known functions in biotic and abiotic stress response. These findings imply that effector function is carried out through specific target interaction. Consistent with this, plants which expressed SIECs were more tolerant to abiotic stresses and increased plant resistance to pathogen infection. The results indicate that effector proteins are part of molecular processes used by symbionts to increase plant fitness.

Combining the SIEC-plant protein interactome and functional protoplast data, the project investigated to what extent SIECs can serve as tools to inform highly interconnected plant protein networks. While effectors frequently target the plant hormone network, the interactome showed connections between hormone modulating SIECs and plant proteins with previously unknown functions. Effector-informed functional analyses of SIEC target mutants proved to be highly successful in uncovering novel plant protein functions and proteins that act as connection points between hormone subnetworks.

Taken together, this project uncovered the function of symbiont effectors in activating host benefits, which at least partially discloses the previously unknown molecular nature of underlying processes. In addition, the symbiont effector data represent a valuable resource for informing plant networks and signalling pathways that are essential for plants to adapt to environmental stresses.

Zusammenfassung

Der endophytische, wurzelkolonisierende Pilz *Serendipita indica* induziert Wachstum, wie auch biotische und abiotische Stressresistenz in einer Vielzahl von Pflanzen. Ähnlich zu Pathogenen, müssen Symbionten die Wirtsimmunität überwinden, um Pflanzen zu kolonisieren. Für diesen Zweck nutzen pflanzenkolonisierende Mikroben unter anderem kleine, sekretierte Proteine, auch Effektoren genannt. Effektorproteine sind dafür bekannt, Pflanzensignale zu verändern, um die Kolonisation von Wirten durch Pathogene wie auch Symbionten zu ermöglichen. *S. indica* modifiziert zudem Hormonsignale, was die Fitness des Wirts verbessert. Dieses Projekt hatte zum Ziel zu analysieren, inwiefern *S. indica* Effektoren nicht nur Funktionen während der Kolonisation von Pflanzen haben, sondern zusätzlich Signalwege modulieren, die nützliche Effekte für den Wirt bewirken.

Die Ergebnisse bestätigten die Sekretion von identifizierten *S. indica* Effektor-Kandidaten (SIECs). SIEC-Pflanzenprotein Interaktionsanalysen wurden durchgeführt, um die Zielproteine der SIECs zu identifizieren. Nachfolgende Analysen umfassten komparative Interaktomstudien und offenbarten gemeinsame Zielproteine von pathogenen als auch symbiontischen Effektoren bzw. Wirtsproteine, die ausschließlich SIEC-Ziele waren. Diese Untersuchungen zeigten bereits eine ausgeprägte SIEC-Interaktion mit Proteinen der Hormonsignalwege und des Hormonmetabolismus des Wirts. Darauffolgende funktionelle Analysen von SIECs in pflanzlichen Protoplasten machten hochspezifische Änderungen von verschiedenen Hormonsignalwegen durch Effektoren deutlich. Diese konnten durch die Phänotypisierung von Wurzelmerkmalen in Effektor-exprimierenden Pflanzen bestätigt werden. Des Weiteren erhöhten Effektoren mit Hormonfunktionen häufig das Pflanzenwachstum. Diese Ergebnisse sind somit ein erster Hinweis, dass Effektoren über bisher unbekannte Mechanismen vorteilhafte Effekte in Wirtspflanzen aktivieren, die in Verbindung mit der Modulation von Hormonsignalwegen stehen.

Um unser Verständnis der erhöhten Fitness von Wirten unter Stressbedingungen durch Symbionten zu verbessern, wurden SIECs auf ihre Funktion in biotischen und abiotischen Stresssignalwegen untersucht. Untersuchungen in Protoplasten zeigten, dass Effektoren Stresssignale verändern und legten die Vermutung einer Verbindung zwischen Hormon- und Stresssignalfunktionen von SIECs nahe. Zudem deckten Netzwerkanalysen die Interaktion zwischen SIECs und Pflanzenproteinen mit bereits bekannter Funktion in biotischen und abiotischen Stressreaktionen auf. Diese Entdeckung deutet darauf hin, dass die Funktionen der Effektoren durch Interaktion mit den Zielproteinen ausgeführt werden. Wie erwartet, waren SIEC-exprimierende Pflanzen toleranter gegenüber abiotischen Stressen und hatten eine erhöhte Resistenz gegenüber Pathogeninfektion. Die Ergebnisse deuten auf Effektorproteine als Teil der molekularen Prozesse hin, die von Symbionten verwendet werden, um die Fitness von Pflanzen zu verbessern.

Durch die Kombination von SIEC-Pflanzenprotein Interaktom und funktionellen Protoplastendaten wurde in diesem Projekt untersucht, inwiefern SIECs als Werkzeug zur Aufschlüsselung hochvernetzter Pflanzenproteinnetzwerke dienen können. Während Effektoren häufig auf das pflanzlichen Hormonnetzwerk zielen, zeigte das Interaktom außerdem Interaktionen zwischen SIECs mit Hormonfunktion und Pflanzenproteinen mit bisher unbekannten Funktionen. Effektor-informierte, funktionelle Analysen von SIEC-Zielproteinmutanten erwies sich als äußerst effizient in der Aufklärung bislang unbekannter Hormonfunktionen von Pflanzenproteinen sowie deren funktionale Rolle in der Verknüpfung und somit *crosstalk* von Hormonsubnetzwerken.

Grundsätzlich deckte dieses Projekt die Funktion symbiontischer Effektoren in der Aktivierung nützlicher Effekte in der Wirtspflanze Arabidopsis auf, was zumindest teilweise die bisher unbekannte molekulare Natur der darunterliegenden Prozesse offenbarte. Zusätzlich zeichnen die Daten symbiontische Effektoren als eine wertvolle Ressource für die Entschlüsselung von Pflanzennetzwerken und Signalwegen aus, welche essenziell für die Adaption von Pflanzen gegenüber Umweltstressen sind.

CHAPTER1 - Introduction

1.1 Increasing food demand and insecurity requires solutions for improved crop yields

In 2021 more than 270 million (20.2%) people in Africa, 425 million (9.1%) in Asia and about 55 million (8.6%) in Latin America and the Caribbean were affected by hunger. While most life in Asia, Africa has the highest prevalence (FAO 2022) and is strongly effected by climate change, because of high drought risks and strongly increasing population size (Ahmadalipour et al. 2019; Schilling et al. 2020). Climate change causes decrease of crop yields, due to increase in extreme weather conditions, which are irregularly distributed with higher impact on developing countries (Mirón et al. 2023). Additionally, the world is challenged with more major factors causing insecurity in food supply. The 2020 pandemic caused economic slowdown, which increased poverty and affected food security especially where supply chains were poorly developed even before COVID-19 (Vos et al. 2022). Starting in the beginning of 2022 the Russia-Ukraine war had various negative socioeconomic impacts. As two major agricultural powers involved, the situation is especially challenging to countries depending on food imports such as the Middle East and North Africa, but also affects global food and fertilizer markets with already high prices (Ben Hassen and El Bilali 2022). Russia and Ukraine supplied 30% and 20% of global wheat and maize, respectively, and Russia being the world leading exporter of nitrogen, potassium and phosphorus fertilizers (FAO 2022). Considering these dire impacts and outlooks on agriculture and food security, options for more stress robust crops and alternative methods for yield increase are urgently needed. One opportunity for improved crop production includes the investigation of plant interactions with beneficial microbes, which are known to improve crop biotic and abiotic stress resilience (Tyagi et al. 2022).

1.2 Climate change endangers crop production by increasing environmental stresses

Environmental challenges on crops are critical in modern agriculture and climate. Increasing temperatures und CO_2 concentrations affect plant and microbe communities globally (Malhi et al. 2021). At the same time climate change is a threat to land, freshwater and oceans due to soil erosion by wind and water (Borrelli et al. 2020). Water deficit because of poor or erratic rainfall and depletion of groundwater is a serious problem in many regions of the world (Hussain et al. 2019). Salinity stress, which can reduce yield, high temperatures, that are threatening plant growth and crop production and impact of climate change on soil fertility are only a few examples of current and future challenges (Pareek et al. 2020). While additionally affected by pests and pathogens, food production must increase to cover the demand of the growing world population (Chakraborty and Newton 2011). Climate change is a main cause for both biotic and abiotic stresses, which drastically endanger agriculture. Species must acclimate, adapt or move, or else die in changing environments. Therefore, plant species may escape inhospitable regions by migration to more suitable areas (Corlett and Westcott 2013). Another effect of climate change involves plant-microbe interactions. While there are many negative environmental impacts on agriculture, effects on microorganisms and interactions with hosts could be positive. Elevation of CO_2, for example, positively influenced mycorrhizal fungi, while effects on beneficial bacteria were variable and comparable results were shown under elevated temperature or drought stress. Generally beneficial microbes are an important factor in influencing plant responses to climate change (Compant et al. 2010). Given that climate change impacts plant yield and the microbiome, we must find mitigation strategies to reduce negative effects on crops (Figure 1.1A-C adapted from Rivero et al. 2022). These strategies could include the plant microbiome and application of beneficial microbes, hormones or chemicals, as well as crop engineering by identification of stress tolerance genes and targeted breeding (Figure 1.1C adapted from Rivero et al. 2022).

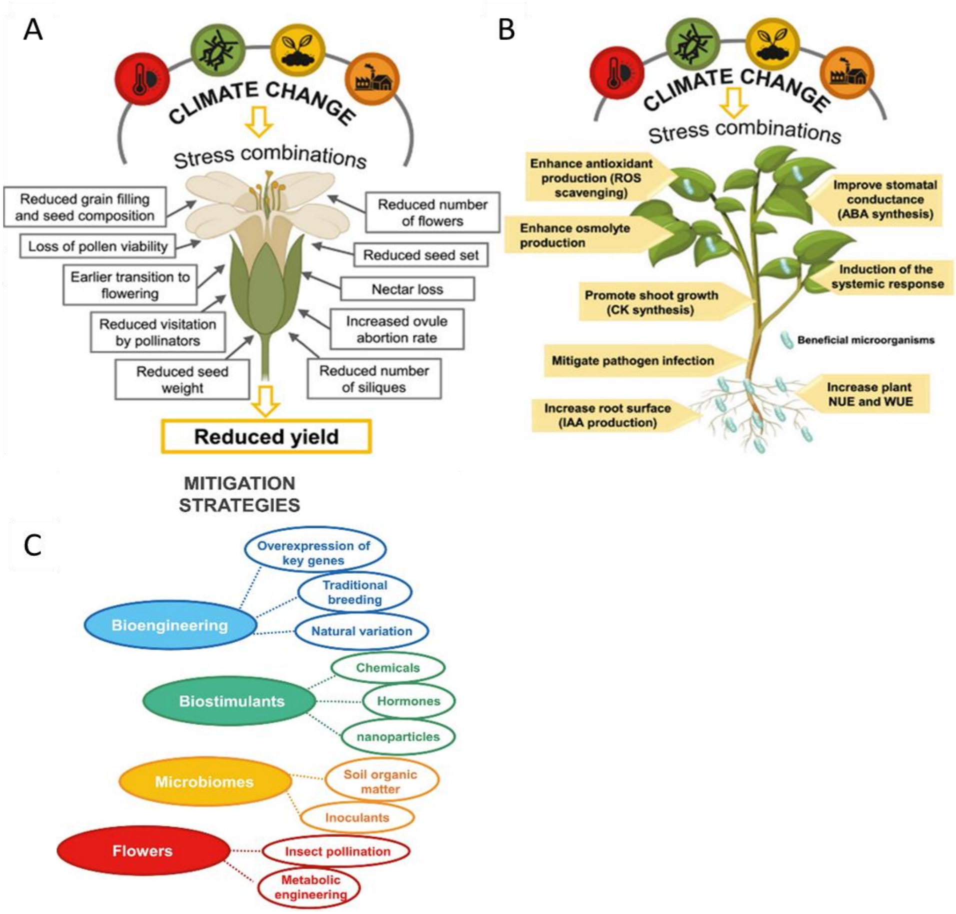

Figure 1.1 Climate change impact on plants and possible mitigation strategies.
A Climate change causes stress combination impacts on plant flowers, reproduction and yield. **B** Possible beneficial effects of the plant microbiome that could mitigate impacts of global warming and climate change on plants. ABA: abscisic acid, CK: cytokinin, IAA: indole-acetic acid, NUE: nitrogen-use efficiency, ROS: reactive oxygen species, WUE: water-use efficiency. **C** Mitigation strategies to reduce the negative impact of climate stress on crops. This figure 1.1 A-C was adapted from Rivero et al. 2022.

1.3 Plant-symbiont interaction driven evolution and land colonisation

During co-evolution with beneficial microbes plants relied on symbioses to withstand environmental challenges (Strullu-Derrien et al. 2018; Kenrick and Crane 1997). Their ability to colonize land more than 400 million years ago fundamentally depended on symbionts transferring nutrients to hosts (Remy et al. 1994; Heckman et al. 2001; Rubinstein et al. 2010).

Although symbiont-host interactions are globally ubiquitous, the reliance of hosts on symbionts shows vast variation depending on transmission mode and symbiont function. While sometimes symbiosis is essential for survival of both partners, others are conditional on specific environments and benefits can diverge (Fisher et al. 2017). Interactions between legumes and nitrogen-fixing rhizo-bacteria, for example, only becomes a symbiosis in certain environments and otherwise maintain facultative relations, while the outcome can also vary (Burdon et al. 1999; Burghardt 2020). Therefore, plant-microbe interactions are influenced by environmental conditions for both pathogenic and symbiont interactions. The critical external circumstances include temperature, moisture and nutrient availability (Cheng et al. 2019). Rising global temperatures have diverse effects on pest infestation, weed flora, crop growth periods, soil microbes and their activity (Malhi et al. 2021). While the microbiome is affected by the climate (Compant et al. 2010), beneficial symbionts might provide a solution for crops to withstand intensified stress conditions (Rivero et al. 2022).

1.4 Plants are constantly confronted with environmental stresses

Plants adapted to and colonized almost every region of the world. Consequently, they are exposed to diverse environmental stresses. These stresses can be categorized into two distinguished groups: biotic and abiotic. Biotic stress includes the interaction with pathogenic organisms such as fungi, bacteria, and oomycetes but also nematodes and herbivores. Together with abiotic stresses like salinity, flooding, drought, high or low temperatures and heavy metals they cause major crop losses worldwide (Bosco de Oliveira 2019).

1.4.1 Abiotic stresses on plants limit crop production

Abiotic stresses are abundant and often multiple stressors appear at the same time (Mittler 2006). Abiotic stress and soil nutrient limitation are reducing plant growth, productivity and quality. As soon as stress is perceived, plants adjust signalling mechanisms to regulate growth and development to guarantee survival and reproduction. These signalling mechanisms include Ca^{2+} signalling, reactive oxygen species (ROS) homeostasis and hormones such as abscisic acid (ABA) (Gong et al. 2020). Heat stress response, for example, affects major physiological processes in plants including photosynthesis, respiration, transpiration, membrane thermostability, and osmotic regulation. In response to heat shock, transcription factors and heat shock protein gene expression is induced (Zhao et al. 2020). Heat stress often occurs together with limitations in water availability and followed by drought. Drought stress is a prominent limiting factor in agriculture. Plants adapt to drought by morphological, physiological and molecular changes to resist water deficit. Drought tolerance related traits include root architecture and morphology, adjustment of water potential and ABA content (Fang and Xiong 2015). Another major abiotic stress is high salinity, which increases intracellular osmotic pressure and can cause toxic levels of sodium accumulation. In response to salinity, plants regulate ion homeostasis, osmotic stress pathways and hormone signalling (Zhao et al. 2021). However, different hormone signalling pathways form elaborate networks ensure flexible and fast responses of plants to diverse biotic and abiotic stress conditions (Verma et al. 2022).

1.4.2 Plant diseases reduce agricultural crop yields

Plant defence is tightly regulated by hormones, with salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) acting as important regulators. Nevertheless, phytohormones are also produced and manipulated by pathogens to suppress plant defence and colonise their hosts (Robert-Seilaniantz et al. 2007). Disease caused by pathogens can lead to immense yield loss. Particularly if abiotic stressors occur simultaneously (Rana et al. 2016). Fungi cause major plant disease and follow diverse colonisation and lifestyle strategies. They are often able to form specialised infection structures to establish colonisation and release virulence factors. These factors can be effector proteins that repress immunity and manipulate plant metabolism but also toxins to kill the host tissue (Doehlemann et al. 2017). Bacteria on the other hand, often surpass plant physical and chemical barriers by motility and chemotaxis pathways to enter the apoplast. In the apoplast phytotoxins and effector proteins are released with various function in plant signalling modulation (Pfeilmeier et al. 2016). Phytotoxins are contributing to bacterial virulence by suppressing plant defence response and inducing chlorosis and necrosis. They can also have similar structural and functional properties as plant hormones to manipulate signalling pathways of host plants (Pfeilmeier et al. 2016). Worldwide more than 200 species of plant-pathogenic bacteria are known and cause substantial crop loss. Among others, bacterial diseases include fire blight of apple and pear, leaf blight of rice, bacterial wilt of tomato, potato, and other crops by *Ralstonia spp*., and many more (Sharma et al. 2022).

The fungal pathogen *Puccinia striiformis* is a prominent wheat disease worldwide. High moisture is essential for its ability to infect plants. The pathogen can be controlled using resistant cultivars, appropriate fungicide or cultivation practices (Chen 2020). The longer dew is present, the higher is the probability of *P. striiformis* infection. Therefore, severe epidemics occur in regions with high moisture during crop growth season. Climate change supports pathogen survival and epidemics are more frequent. Additionally, warmer winters enhance survival and cause more severe epidemics, while cooler and wetter late spring leads to more infection. Because of the changing environmental conditions previously non-favourable areas might become more advantageous to the disease (Chen 2020). Recently severe *P. striiformis* epidemics in Egypt due to new aggressive races were reported (Esmail et al. 2021). In Europe two invasive races have been detected since the beginning of the 21st century, with PstS7 (Warrior) having major impact due to fast spreading (Vidal et al. 2022). Therefore, systems to increase crop resistance to pathogen infections are exceedingly important.

1.5 Plants developed complex immunity mechanisms as defence against microbial infection

During co-evolution with plants, microbes have developed sophisticated strategies for the targeted alteration of plant signalling for colonisation. During the initial interaction between plants and microbes, all microbes are confronted with plant immunity (Nishad et al. 2020). However, the outcome of interactions is vastly different and how this is controlled by plants and microbes is not entirely understood.

The first layer of plant immunity activation includes the perception of microbe-associated molecular patterns (MAMPs), for example flg22 a bacterial flagellin protein (Zipfel et al. 2004), which is referred to as pattern-triggered immunity (PTI). Pattern-recognition receptors (PRR) are involved in perception of bacteria, fungi and oomycetes (Zipfel 2008). MAMPs are found in all kinds of microbes independent of the interaction outcome. Plants are able to detect various different MAMPs to identify interactions and activate defence signalling (Jones and Dangl 2006). PTI, as an initial defence mechanism, stops the majority of potential pathogens (Zipfel et al. 2004) and was described to be activated in leaves as well as in roots (Millet et al. 2010).

To overcome this initial defence mechanisms, pathogens secrete small molecules including proteins, which were defined as effectors. Suppression of plant innate immunity was initially depicted as the principal function of effectors of plant pathogenic bacteria (Hogenhout et al. 2009). However, other pathogenic microbes like fungi and oomycetes have an immense arsenal of effector proteins to guide every phase of plant infection. Effectome studies revealed common pathogen effector target proteins, which helped characterising core immunity network elements (Mukhtar et al. 2011; Weßling et al. 2014). To remain resistant to infections, plants developed a second defence pathway against immunity suppression by effectors termed effector-triggered immunity (ETI). ETI employs nucleotide-binding domain leucine-rich repeat (NLR) receptors to detect effector proteins (Bai et al. 2012). ETI often leads to hypersensitive response, a programmed cell death that stops pathogen proliferation (Dalio et al. 2021) (Figure 1.2 adapted from Dodds and Rathjen 2010).

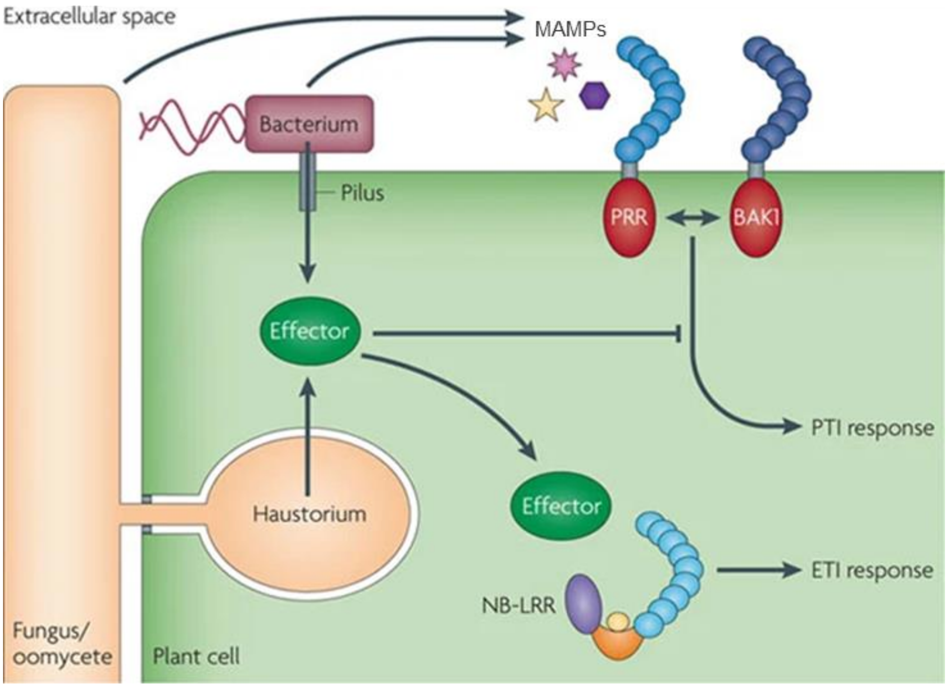

Figure 1.2 Schematic overview of plant PTI and ETI responses to microbe infection.
Microbe interactions with plants trigger PTI responses via MAMP recognition. PTI is repressed by microbes using effector proteins. Effectors are recognised by host NLR-receptors, which activate ETI responses. This figure was adapted from Dodds and Rathjen 2010.

PTI and ETI are regulated by distinct receptors and signalling components. However, recent findings indicate that both can cause similar downstream responses with individual amplitudes and dynamics (Yuan et al. 2021).

1.6 Microbial effectors have functions in plant colonisation and modulation

Typically, effector proteins are described as tools used by pathogens to block MAMP perception and supress PTI (Dangl et al. 2013). However, suppression of immunity for colonisation and the application of effectors is also necessary for successful plant colonisation by beneficial microbes and not a mechanism restricted to pathogen interactions (Yu et al. 2019).

To transfer effectors from the microbe to host cells bacteria such as *Pseudomonas syringae* use type III secretion systems to directly transfer effectors and other molecules into eukaryotic cells (Jones and Dangl 2006; Matteï et al. 2011; Cunnac et al. 2009). Strategies of effector transfer by oomycetes and fungi are more unclear. Some oomycetes develop haustoria in cells and an extrahaustorial membrane to transfer effectors into plant cells (Whisson et al. 2007), several fungi were identified that utilize similar methods (Lo Presti et al. 2015). Many oomycete effectors share an amino terminal signature which have been implicated in effector uptake by hosts (Lo Presti and Kahmann 2017). However, fungal effector uptake into plants might be mediated by endocytosis and could be rather unspecific or have a degree of conservation at protein structure levels (Lo Presti and Kahmann 2017). Applying the Alphafold2 protein structure prediction model (Jumper et al. 2021) could allow the exploration of possible structural similarities between fungal effector proteins more easily and examine their function in the transfer process. Recently a study revealed sequence-divergent but structurally similar effectors shared between symbiotic and pathogenic fungi. Protein structure models of secreted proteins of the AMF *Rhizophagus irregularis* were compared to known pathogen effectors. Proteins with structural similarity to known *Fusarium oxysporum f. sp. lycopersici* dual domain (FOLD) effectors could be identified (Teulet et al. 2023).

Even if the transport and uptake into plants is unclear, all microbes that colonise plants have effectors with manifold functions to establish infections. Effectors themselves can have enzymatic activity. For example, chorismate mutase (Cmu1) from *Ustilago maydis* and isochorismatases (Isc) from the fungus *Verticillium dahlia* were found to reduce the accumulation of the defence hormone SA in plants (He et al. 2020). Additionally, effector proteins can inhibit enzymatic activity of targeted defence-related host proteins (He et al. 2020). Effectors were shown to be able to alter host proteins in their stability, assembling of complexes and localisation to overcome plant defence and establish colonisation (He et al. 2020). *Puccinia striiformis f. sp. tritici* secrets a protein termed PSTG_01766, which targets wheat TaPLCP1 involved in high-temperature seedling-plant (HTSP) resistance. Expression of PSTG_01766 inhibits immunity in tobacco (*Nicotiana benthamiana*) and wheat (*Triticum aestivum*). By subsequent effector-target analyses, TaPLCP1 was identified as a potential central hub of defence responses. Therefore, effector analyses improved our understanding of the molecular mechanism of wheat HTSP resistance, which is especially important as a strategy for controlling stripe rust under the prospect of global warming (Hu et al. 2022).

Furthermore, small RNA (sRNA) effectors were revealed to take part in colonisation and infection processes by intervention of transcription through the RNA interference (RNAi) pathway. They interact with host Argonaute (AGO) proteins and target complementary RNA for degradation using the RNA induced-silencing-complex (RISC) (Ellendorff et al. 2009). New studies proposed this pathway not only to work from pathogen to host, but host plants were found to transfer small-interfering RNA (siRNA) and micro RNA (miRNA) to fungi during interactions (Zanini et al. 2019; Koch et al. 2020).

Interestingly, independent of the interaction outcome, effectors are essential for interactions between plants and microbes. Effectors with function in host colonisation were, for example, identified in the mutualistic fungus *Laccaric bicolor* (MiSSP7) (Plett et al. 2014; Plett et al. 2011), rhizobia (type 3 secreted effectors) (Miwa and Okazaki 2017), and the symbiont *Serendipita indica* (PIIN_08944, Dld1) (Akum et al. 2015; Nostadt et al. 2020). Pathogens as well as beneficial microbes employ effectors that modulate nuclear signalling. While pathogens target nuclear processes to enhance virulence and suppress defence, symbiotic rhizobia effectors seem to have nuclear function for the promotion of nodule development (Tehrani and Mitra 2023). Analysis of effector functions was previously used to investigate plant defence mechanisms and immunity during the establishment of symbiont or pathogen interactions (He et al. 2018; Hogenhout et al. 2009; Mukhtar et al. 2011; Yu et al. 2019)**.** Little is known about the possible molecular function of symbiont effectors not related to colonisation and immunity. It raises the question, whether beneficial microbes employ effectors to manipulate plants to activate beneficial traits**.**

1.7 *Serendipita indica*: a model beneficial microbe with exceptionally large host range

Serendipita indica (*Si*, formerly *Piriformospora indica*) is an endophytic root colonising fungus of the order *Sebacinales*, which was found in association with xerophytes in the Thar Desert in India (Varma et al. 1999). It can establish symbioses with monocotyledonous as well as dicotyledonous species and colonizes a broad spectrum of hosts, including Arabidopsis. The fungus forms inter- and intracellular hyphae in the root cortex, often differentiating into dense hyphal coils and chlamydospores (Pham et al. 2008). In Arabidopsis it shows a biotrophic growth phase in roots followed by a cell death-associated colonisation phase (Jacobs et al. 2011).

Following establishment of the interaction, a symbiosis with *Si* has beneficial effects on the host. For instance increased growth, abiotic stress tolerance and disease resistance (Varma et al. 1999; Waller et al. 2005). The fungus was shown to employ a high affinity phosphate transporter, which is involved in phosphate transfer to the host and possibly part of the symbionts growth promoting effects (Yadav et al. 2010). Yet, *Si* was also found to adjusts host gene expression depending on phosphate restriction and not stimulate phosphate uptake (Bakshi et al. 2017). Therefore, not all beneficial effects can be explained by increased nutrient acquisition. During interaction with *Brachypodium distachyon* (*Pooideae*), for example, a large amount of differently expressed genes and interaction-induced sRNAs was identified (Šečić et al. 2021). Whereas this indicates regulatory processes during the interaction, molecular mechanisms of signalling modulating processes used by symbiotic fungi to confer beneficial traits are largely unknown.

Si colonised hosts gain enhanced biotic and abiotic stress resilience, including resistance to microbial pathogens, insects and drought (Harman 2011). These effects were previously connected to an increase in antioxidants and alteration of gene expression (Baltruschat et al. 2008; Sun et al. 2010). Furthermore, *Si* was shown to protect Arabidopsis from pathogen infection (Sun et al. 2014). It can induce local and systemic resistance to fungal and viral pathogens (Gill et al. 2016; Waller et al. 2005). As a result, *Si* symbioses can enhance plant fitness, including increased crop growth and productivity under changing environmental conditions (Figure 1.3 adapted from Mosaddeghi et al. 2021).

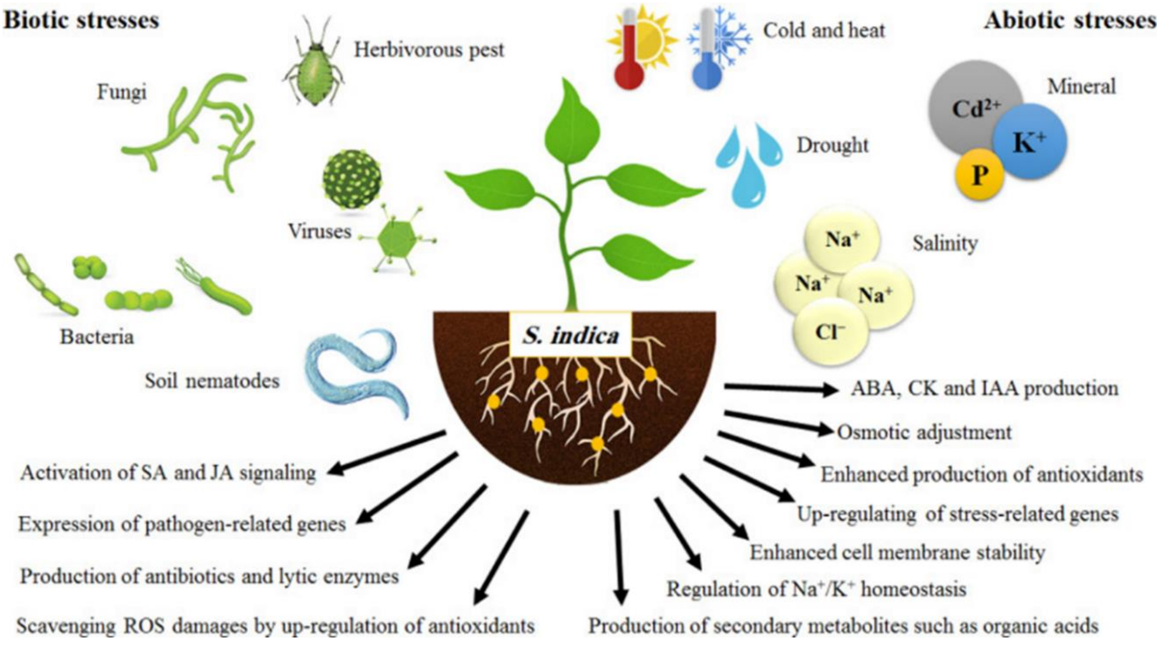

Figure 1.3 Effect of *Serendipita indica* on plant stress
Biotic and abiotic stresses on crops and regulatory mechanisms by *Si* after root colonisation that increase plant stress resilience. The figure was adapted from Mosaddeghi et al. 2021.

While their molecular functions are mostly unknown, *Si* possesses a diverse repertoire of effectors (Lahrmann and Zuccaro 2012; Zuccaro et al. 2011). Beneficial effects mediated by the fungus are at least partially dependent on its ability to alter phytohormone signalling (Xu et al. 2018). To develop a beneficial symbiosis between Arabidopsis and *Si*, a fine balancing of defence and growth response is needed. Plants that are unable to control root colonisation are often over-colonized and as a consequence the benefits for the plant are lost (Nongbri et al. 2012). Environmental conditions may also influence *Si* effects on host, as it was shown that high ambient temperatures strengthened growth promotion in Arabidopsis (Chen et al. 2022). Therefore, fine-tuned signalling between host and fungi are crucial for beneficial interactions. *Si* effectors, which were shown to have hormone signalling modulating function (Akum et al. 2015), might be strongly involved in such processes.

1.8 Phytohormone modulation is essential during beneficial symbioses

Plants use multiple hormones with distinct but highly interconnected signalling pathways (Altmann et al. 2020). A hormone with prominent function in plant development is, for example, cytokinin (CK). CKs regulate many phases of plant growth, including shoot and root meristem formation, development of flowers (Ferreira and Kieber 2005), leaf formation, cell division and senescence (Hwang and Sheen 2001). Thus, they have central functions in cell cycle and plant development (Werner et al. 2001). Auxin regulates cell division, elongation and differentiation of cells in the meristems leading to organogenesis shaping plant shoot and root architecture (Brumos et al. 2018). This is especially important under different environmental conditions. It influences gene expression in plants to adjust plasticity to environmental changes (Gomes and Scortecci 2021). Auxin homeostasis is closely regulated to adjust concentration-dependent growth responses and adaptive responses to both biotic and abiotic stresses (Blakeslee et al. 2019). Together with ABA, auxin is involved in lateral root formation during drought stress (Seo and Park 2009). ABA was originally identified as a regulator of plant stress response. It was shown to modulate numerous aspects of phenotypic and developmental response to disadvantageous abiotic conditions such as drought, low or high temperature and salinity (Hong et al. 2013). In addition, ABA signalling is connected to seed maturation, dormancy and germination (Ali et al. 2022) and affects flowering in response to dry climates (Martignago et al. 2020). In crosstalk with other hormones especially SA and ethylene (ET), ABA was found to be involved in plant response to pathogens and immunity (García-Andrade et al. 2020). SA promotes immunity against biotrophic and semibiotrophic pathogens and has crucial function in basal defence, immune response and systemic acquired resistance (SAR) (Peng et al. 2021). A central regulator of SA-mediated immune response is Nonexpressor of Pathogenesis-related Genes 1 (NPR1), which, activated by SA, induces stress-responsive genes and controls stress protein homoeostasis (Zhou et al. 2023). JA and SA control defence response in an antagonistic regulatory mechanism (Shim et al. 2013). JA is, together with ET, involved in induced systemic resistance (ISR) (Grant and Lamb 2006) and exerts direct control over the production of chemical defence compounds against pathogens and herbivores (Campos et al. 2014). Additionally, studies revealed its function in developmental processes like primary root growth, reproductive development, and leaf senescence (Huang et al. 2017). Further, jasmonates (JA and derivates) are hubs for environmental cues with important functions in moderating salt, drought and freezing stress as well as heavy metal and micronutrient toxicity among others (Ali and Baek 2020).

Si utilizes JA signalling to bypass or supress plant immunity to colonise its hosts. Jacobs et al. (2011) showed, that *Si* is indeed confronted with a root immune system and does not evade detection but supresses PTI. This ability is compromised in *jasmonate insensitive 1-1* and *jasmonate resistant 1-1* mutants, indicating a function of JA signalling during colonisation (Jacobs et al. 2011). Its broad host spectrum indicates a strong capacity to supress plant innate immunity. Therefore, for host colonisation *Si* interferes with plant hormone synthesis and signalling (Xu et al. 2018). Its ability to modulate a large variety of phytohormone signalling pathways has previously been reported (Varma et al. 2012; Gill et al. 2016). This is in line with the observed beneficial effects of the symbiont, as hormone signalling is crucial for balancing growth and biotic stress response after pathogen infection (Verma et al. 2016).

Symbiotic fungi and other beneficial microbes developed mechanisms for targeting hormone signalling involved in defence in a similar way to pathogens but additionally alter growth related phytohormones (Shen et al. 2018). *Laccaric bicolor* uses the effector MiSSP7 to modulate JA signalling in its host (Plett et al. 2014). ET signalling, for example, is involved in balancing host benefits during *Si* symbiosis

(Camehl et al. 2010) and the suppression of host immunity involves ET, JA and gibberellin signalling (Daneshkhah et al. 2018; Schäfer et al. 2009a, 2009b). Yet, root colonisation strategies and phytohormone alteration by *Si* seem to be highly host specific (Liu et al. 2019). However, phytohormone modulation seems to be a strategy for symbionts to enhance host fitness. This is well known for *Si* symbioses (Xu et al. 2018). Still, until now we do not know how effectors contribute to beneficial effects and it is difficult to decipher complex effector functions in highly interconnected plant hormone networks (Altmann et al. 2020).

It is known that modulation of phytohormone metabolism and signalling is an essential part of symbioses. Modulation of different phytohormones was shown to be involved in defence suppression during initial colonisation stages and to confer of beneficial effects by AMF (Bedini et al. 2018). Nonetheless, knowledge is limited on the underlying molecular mechanisms and how symbiotic effectors might alter these hormone processes and thus contribute to the activation of beneficial effects. The analyses of plant hormone functions in fundamental processes such as plant development or stress adaptation is challenged by the vast crosstalk between hormone signalling. The complexity of the hormone network makes it difficult to decipher the contribution of individual proteins or specific interactions to phenotypic traits (Ku et al. 2018; Yang et al. 2019; Khan et al. 2020). Recently, an extensive network-study of protein-protein interactions revealed a highly interconnected network of hormone pathway contacts that represent potential crosstalk points (Altmann et al. 2020)

Even though, effectors have various functions during plant-microbe interaction, not many symbiont effectors are comprehensively functionally analysed. Effectors were previously used to identify proteins that are part of plant immunity and defence signalling. Analyses of *Pseudomonas syringae* effectors revealed multiple interacting host proteins involved in defence, pathogen recognition and innate immune response. Therefore, suppression of immunity was considered as the primary function of these secreted proteins, by affecting protein turnover, RNA homeostasis and phosphorylation and suppression of hypersensitive cell death (Hogenhout et al. 2009). However, little is known about effector functions of beneficial microbes. The analysis of the molecular mechanisms altered by symbiont effectors could advance our understanding of plant stress response and signalling pathways that improve plant fitness. These insights could be highly valuable for future application of symbionts in agriculture or in the generation of crops with advantageous traits, like increased growth under environmental stresses.

1.9 Aims of the study

Based on previous studies effectors might alter plant signalling during symbiont interactions not only to colonise plants, but to additionally increase host fitness and therefore determine interaction outcomes. This study therefore aims at revealing the function and potential beneficial activity of *Si* effectors by focusing on two objectives:

Objective I: Functionally analysing effectors in plant signalling and stress response pathways.

1. Effector modulation of five hormone markers and 4 markers responsive to biotic and abiotic stresses will be evaluated in protoplasts.
2. Effector expressing Arabidopsis plants are going to be analysed for their phenotypical response to hormone and abiotic stress treatment and infection with three pathogens.

Objective II: Identifying effector targets and their role in protein-protein signalling networks.

1. Analyses of host targets based on the interacting effectors.
2. Uncovering of new members and connections of complex plant signalling networks.
3. Disclose the potential of effector-based analysis for the investigation of plant networks.

Based on these objectives, the study aimed at uncovering the molecular nature of symbiont-mediated benefits involved in plant fitness and how *Si* effectors can inform plant protein networks.

CHAPTER 2 – Symbiont effector-guided mapping of proteins in plat networks to improve climate stress resilience

Chapter 2 Introduction

This chapter contains the publication "Symbiont effector-guided mapping of proteins in plant networks to improve crop climate stress resilience" with the subtitle "Symbiont effectors inform highly interconnected plant protein networks and provide an untapped resource for crop climate resilience strategies". The paper was accepted by the journal *BioEssays* on 23rd of January 2024 and published online on 22nd February 2024 with the DOI: 10.1002/bies.202300172.

This chapter elucidates the current knowledge of symbiont effector functions with a focus on their beneficial effects and potential for future crop breeding strategies.

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Symbiont effector-guided mapping of proteins in plant networks to improve crop climate stress resilience

Symbiont effectors inform highly interconnected plant protein networks and provide an untapped resource for crop climate resilience strategies

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Abstract

There is an urgent need for novel protection strategies to sustainably secure crop production under changing climates. Studying microbial effectors, defined as microbe-derived proteins that alter signalling inside plant cells, has advanced our understanding of plant immunity and microbial plant colonisation strategies. Our understanding of effectors in the establishment and beneficial outcome of plant symbioses is less well known. Combining functional and comparative interaction assays uncovered specific symbiont effector targets in highly interconnected plant signalling networks and revealed the potential of effectors in beneficially modulating plant traits. The diverse functionality of symbiont effectors differs from the paradigmatic immuno-suppressive function of pathogen effectors. These effectors provide solutions for improving crop resilience against climate stress by their evolution-driven specification in host protein targeting and modulation. Symbiont effectors represent stringent tools not only to identify genetic targets for crop breeding, but to serve as applicable agents in crop management strategies under changing environments.

KEYWORDS
AlphaFold, climate change stress, effector, hormone signalling, host reprogramming, protein networks, symbiosis

INTRODUCTION

Plants depend on beneficial symbioses for their performance in natural ecosystems and agricultural fields. Nutrient supply is often considered as the canonical driver and outcome of beneficial symbioses where symbionts receive sugars and lipids, while they supply plants with phosphor, nitrogen, irons and mineral nutrients.[1,2] However, it is not clear, how developmental and environmental signals are integrated in this exchange while phytohormones often have important regulatory functions in these interactions.[2] Whereas the molecular mechanisms for increased host stress tolerance and fitness are generally unknown, beneficial effects of microbes often involve changes in plant hormone metabolism and signalling.[3] This is not further surprising as phytohormones are instrumental in every aspect of plant life from germination,[4,5] to vegetative development, reproduction and senescence.[6] In addition, hormones coordinate developmental plant plasticity as an integral part of biotic and abiotic stress

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Abbreviations: ABA, abscisic acid; AMF, arbuscular mycorrhizal fungi; AUX, auxin; cPEP, complementary peptides; ETI, effector-triggered immunity; JA, jasmonic acid; PTI, pattern-triggered immunity; SA, salicylic acid; *Si*, *Serendipita indica*.

adaptation deriving from pathogen attack,[7] nutrient shortage (e.g., nitrate availability),[8,9] drought[10,11], salinity[12] and heat.[13] This multifunctionality of phytohormones originates from their frequently overlapping activities, which adds to the flexibility and robustness of plant adaptions to environmental stress.[14] Effectors, small, secreted proteins from microbes, are known to modulate a vast range of phytohormones during plant microbe interaction.[15]

Climate change is a central cause for biotic and abiotic stress for crops and, thus, severely compromises crop growth and yield.[16] At a global scale, the consequences are manifold and range from the emergence of new diseases, higher pest infestation, shifting of weed flora and reduced crop growth periods to altered soil microbe communities and their activities.[17] These impacts are particularly pronounced in more vulnerable cropping systems in developing countries.[18] As sessile organisms, plants rely on beneficial interactions to cope with changing environments. In addition to promoting plant growth, symbiotic microbes can stimulate phenotypic plasticity and fitness of plants which enhances host tolerances against various stresses.[19,20] Some plant growth promoting bacteria and fungi improve defence against a broad range of pathogens and insect herbivores by eliciting systemic resistance and local resistance.[21–23] This ability of symbionts to enhance plant fitness and resilience is driven by plant-symbiont co-evolution. Plants faced extreme environments, including climate with high CO_2 concentrations and irregular precipitation in the course of land colonisation more than 450 Mio. years ago. To succeed the transition from water to land, plants were highly dependent on beneficial symbioses with fungi.[24–26] Ever since, co-evolution with beneficial microbes is a functional concept of plants to withstand environmental challenges.[27,28] Consistent with this, elevated CO_2 was shown to positively affect beneficial symbionts like mycorrhizal fungi. Such findings indicate symbioses as an important factor in influencing plant robustness and adaptation to changing climates.[29] Plants share common symbiosis signalling components for the establishment of both arbuscular mycorrhizal (AM) and root-nodule symbioses. Early signal transduction upon perception of symbionts is mediated by at least seven shared mechanisms.[30] Despite morphological differences between AM and root nodule symbioses, both genetically control colonisation and lead to transcriptional activation of a common set of symbiosis genes (*SYM* genes).[31] It indicates that plant interactions with mutualistic microbes have co-evolved over time. Additionally, microbe-plant relationships in roots are formed through specific interaction types and a common architecture exists between nodulation and mycorrhizal interactions.[32] Thus, plant-symbiont co-evolution points to promising solutions for enhanced crop fitness under changing climates. It is unclear, however, how symbionts regulate the adjustment of host signalling to confer beneficial effects and to what extent this is linked to the establishment of symbiosis. The establishment of beneficial symbioses are based on a tight molecular communication between both partners. Recent studies, for instance, demonstrated the significance of lipid exchange between AM fungi (AMF) and most ancient plant lineages. This lipid exchange essentially supports the formation of arbuscules, a fungal interface in plant cells that facilitates cross-species communication and nutrient exchange.[33] It was revealed that lipids in arbuscle-containing cells are transferred to AMF, following upregulation of lipid synthesis and metabolism pathways for example by *WRINKLED* genes in the host cells. Without these lipid synthesis-regulating genes symbiosis with AMF was strongly reduced.[33]

Interestingly, many conserved signalling and recognition mechanisms as well as transcriptional and cellular responses highly overlap between beneficial and pathogenic microbes.[34] This is not further surprising as the lifestyle of fungi is ranging from mutualism (beneficial for both partners) to commensalism (beneficial for the microbe but no effect for the host) to parasitism (host is harmed on the benefit of the fungus). Among others, factors such as partner combination, developmental stage, physiology, habitat conditions and stress situations determine if a mutualistic interaction can shift to commensalism or even parasitism.[35] Most critical for the symbiotic outcome are external circumstances such as temperature, moisture and nutrient availability.[36] Overall, it suggests that the establishment of symbioses (in terms of colonisation) is not necessarily dependent on the regulation of beneficial effects. It raises the question about the molecular basis of mutualism in plant-microbe symbioses and if it can uncover solutions to enhance crop fitness under climate stress. The intimate interaction of beneficial symbionts with plants relies on the reprogramming of cellular processes in the host.[37,38] Similar to pathogens, symbionts use secreted proteins (effectors) to modulate hosts.[39] Functional analyses of pathogen effectors have greatly advanced our knowledge about the organisation and regulation of the plant immune system.[40] Considering the importance of symbionts for environmental adaptation of plants,[28] symbiont effectors can be instrumental in the identification of plant proteins and pathways with key functions in climate stress resilience. In this article, we will give an outlook on our current knowledge about beneficial effector functions and possible applications of symbiont effectors to sustain crop production.

Microbial effectors in plant colonisation

During co-evolution with plants, microbes have developed sophisticated strategies for the targeted alteration of plant signalling to prepare host cells for colonisation. At the initial interaction stages, all microbes are confronted with plant immunity.[41] Plants perceive microbe-associated molecular patterns (MAMPs), for example flg22, a 22-amino acid conserved peptide in bacterial flagellin,[42] to activate highly effective layers of plant immunity in leaves and roots,[43,44] which is referred to as pattern-triggered immunity (PTI). Plants use pattern recognition receptors (PRRs) for the specific recognition of a large number of different MAMPs from diverse microbes such as bacteria, fungi and oomycetes.[45,46] The activated cellular immune signalling aims at establishing an antimicrobial micro-environment and includes intracellular reactive oxygen and calcium bursts, kinase cascade activation and transcriptional reprogramming.[47]

To overcome PTI, pathogens secrete proteins that were later defined as effectors. Suppression of plant innate immunity was first

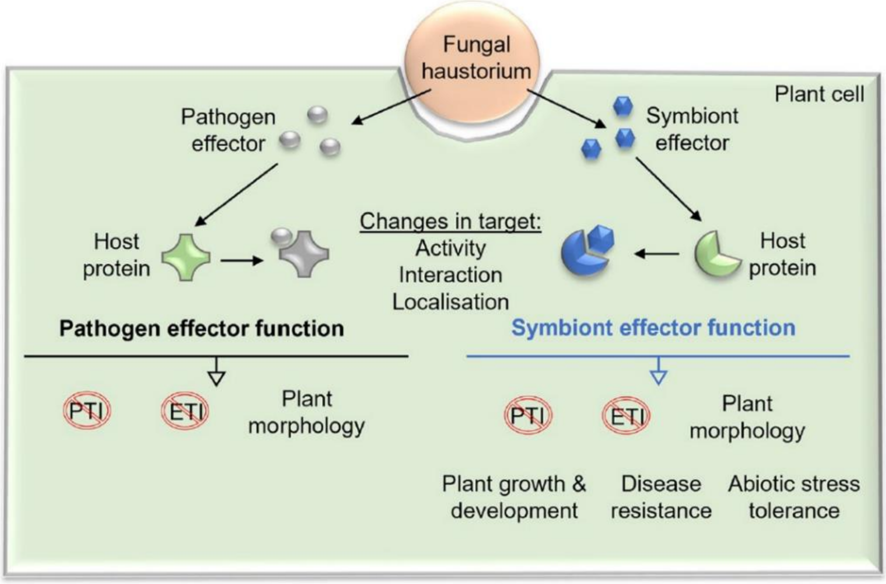

FIGURE 1 Effector proteins from pathogenic and beneficial fungi are often translocated into plant cells, where they modulate target localisation, interaction and activity.[40] While microbes utilise effectors to suppress host PTI and ETI and affect plant morphology,[48] symbiont effectors additionally have possible functions that increase plant growth, biotic and abiotic stress resilience.[85]

described as principal effector function of plant pathogenic bacteria by affecting post-translational modification of host proteins (e.g., ubiquitination, phosphorylation) and RNA homeostasis.[48] Similarly, pathogenic fungi and oomycetes have an arsenal of several hundred effector proteins to channel every step of plant infection.[40] Global effectome studies revealed that effectors from pathogens of different kingdoms and different life-cycle strategies manipulate common host target proteins. This convergent targeting of host protein networks helped to identify core immunity network components.[49,50] In response plants developed an additional immunity layer called effector-triggered immunity (ETI), which utilizes nucleotide-binding domain leucine-rich repeat (NLR) receptors to detect effectors.[51] Even though, PTI and ETI require different receptors they share some signalling components, and ultimately unite in similar downstream responses with distinct amplitudes and dynamics.[52] Recent studies even indicate components previously thought to be related to ETI to function in PTI responses and suggest convergence points of defence signalling cascades.[53] Current concepts consider ETI to be essential in the restoration and potentiation of PTI to launch a more robust overall plant immune response.[54]

Pathogen effectors from model pathogens (e.g., *Pseudomonas syringae*) have been instrumental for our understanding of the regulation and interconnection of PTI and ETI.[48] In addition to the immunity-interfering activity, pathogen effectors were later recognised for altering plant behaviour and morphology during pathogen infection.[48] This functional versatility seems to be even more elaborated for symbiont-derived effectors (Figure 1).

Effectors are required in beneficial symbiosis

Pathogens and beneficial symbionts follow, in general, highly similar colonisation and cellular interaction strategies.[55] The suppression of PTI is therefore equally important to them. The outcome is, however, detrimentally different. In contrast to diseases caused by pathogens, symbionts enhance host fitness.

Therefore, fungal symbioses are of great value for agriculture, by promoting plant health, growth, and development. Fungal effectors play an important role in complex plant-symbiont crosstalk and were previously defined as proteins that modulate plant physiology to facilitate fungal colonisation and growth within the host.[56] The relevance of common symbioses genes[31] suggests that nodulating rhizobacteria and arbuscular mycorrhiza employ effectors with overlapping host targets.

Si (*Serendipita indica* formerly *Piriformospora indica*) is an endophytic root colonising fungus that establishes symbioses with a broad spectrum of monocotyledonous as well as dicotyledonous plants. The fungus forms inter- and intracellular hyphae in root cells that eventually differentiate into chlamydospores.[57] The biotrophic growth phase is occasionally followed by a cell death-associated colonisation phase; possibly to turn cells into vessels for spore deposition.[43,58] *Si* is confronted with a root immune system and does not necessarily evade detection but supresses PTI to colonise host roots.[43] As a result of this intimate colonisation, *Si* transfers various beneficial effects to monocots and dicots. In addition to improved growth and development, colonised plants show increased abiotic stress tolerance as well as local and systemic disease resistance.[59,60] Growth and developmental effects might be partly attributed to improved nutrient acquisition. The fungus was shown to employ a high affinity phosphate transporter, which is involved in P transfer to the host.[61] A more recent study revealed that *Si* adapts host gene expression depending on phosphate limitation but does not stimulate phosphate uptake.[62] Additionally, a high-affinity sulphur transporter of *Si* was reported to improve maize growth by compensating for low sulphur availability and *Si* colonisation allowed maize to allocate resources away from sulphur assimilation.[63] A fungal high-affinity iron transporter has a functional impact on improved performance of rice. Rice interacting with *Si* with functional Fe transporter under iron limiting conditions was larger, contained more chlorophyll and had higher biomass.[64] These findings indicate that promotion of growth and developmental effects of symbionts have great agricultural potential and cannot be solely explained by an improved nutrient supply but involve an alteration of plant signalling and physiological processes.

Limitation of nutrients is one of many abiotic stresses plants have to cope with in nature. Other abiotic stress conditions include drought, heat, salinity and cold, often occurring in different combinations. Plants activate signalling pathways that for example involve a variety of molecules ranging from reactive oxygen species (ROS) to hormones.[65] Independent of biotic (e.g., pathogens, herbivores) or abiotic stress, stimuli often result in a fast Ca^{2+} influx into the cytoplasm recognised by Ca^{2+} sensors which transfer signals through hormone pathways.[66] Hormone signalling in response to stresses causes various adaptational changes in all plant cell regulating processes, like translational modifications, transcription, activation and deactivation of signalling pathways.[12,67,68] Especially, abscisic acid (ABA) and jasmonic acid (JA) signalling are coordinated by Ca^{2+} signalling and were shown as convergent points between biotic and abiotic stress regulation.[69] The symbiont *Si* is known to affect host signalling pathways related to stress responses including Ca^{2+} signalling and ROS production.[70,71]

Correspondingly, *Si* mediated abiotic stress tolerance and disease resistance to microbial pathogens and insects[72] is associated with host signalling and subsequent transcriptional reprogramming. Improved salt-tolerance observed in barley was connected to an increase of the antioxidative capacity of host plants, while increasing barley resistance to fungal diseases. Elevated antioxidative capacity resulted in an overall increase in grain yield.[59,71] Consistently, *Si* stimulated antioxidant enzymes and expression of drought-related genes to confer drought tolerance in Chinese cabbage leaves and promoted root and shoot growth and lateral root formation.[73] The fungus was reported to regulate transcription of genes to mediate cold tolerance under freezing stress (members of freezing responsive *WRKY*, *ERF*, *bHLH*, *HSF*, *MYB* and *NAC* transcription factor families),[74] drought stress (e.g., *DREB2a*, *CBL1*, *ANAC072* and *RD29a*)[73,75] and salt stress response (*HKT1*, *KAT1*, *KAT2* functional in regulation of Na^+ and K^+ homeostasis).[76] In addition, local and systemic resistance to fungal and viral pathogens is based on *Si*-mediated adaptation of genetic and physiological host programs.[59,77,78] As a result, *Si* symbioses lead to a better plant performance, including root proliferation, crop growth and productivity under changing environmental conditions with great potential for improvement of agriculture.[77,79] Even though, effector proteins of symbionts are known to promote host interactions,[80,81] how exactly symbiont effectors are involved in these stress regulations is mostly unknown. The studies indicate, however, a diversity of *Si*-mediated benefits that have an obvious dependency on host reprogramming in common. It suggests a direct involvement of symbiont effectors in launching beneficial effects (Figure 1). Beneficial microbes rely on effectors with highly variable functions for host colonisation.[82] Irrespective of beneficial or pathogenic lifestyles, previous effector analyses centred around their function in immunity suppression and host colonisation[48,50,82,83] by altering host protein stability, disturbing protein complexes and relocalization of their targets.[39] The mutualistic fungus *Laccaric bicolor* was among the first to reveal its dependency on an effector (MiSSP7) for colonisation.[80,84] Likewise, effector targeting of the PTI backbone mitogen-activated kinases was identified for rhizobia to suppress host defence.[81]

Despite various indications, a potential function of symbiont effectors in the activation of beneficial effects is still underinvestigated. Rhizobia, for instance, use effectors not only to promote nodule formation but to stimulate other symbiosis-related activities.[81] To receive a more comprehensive overview of effector functions in plant benefits we analysed effector proteins of *Si* and their targets in comparative and functional effectome studies.[85] *Si* possesses a diverse repertoire of effectors[86,87] and *Si* effector Dld1 (PIIN_05872) was identified to affect metal ion homeostasis and oxidative stress[88]; traits of known relevance for beneficial plant effects.[77,89] Our comparative effectome analysis identified *Si* effectors that shared targets with pathogen effectors and were most likely involved in PTI modification at initial cell colonisation. The majority of symbiotic effectors, however, had exclusive targets[85] which may indicate *Si* effector-host protein interactions involved in the activation of beneficial pathways. A subsequent annotation analyses revealed an abundant function of *Si* effectors in interacting with hormone pathways.[85] The data suggested a function of effectors on plant hormones to regulate beneficial host traits. It will be interesting to find out in future, to what extent effectors of symbionts with a broad host range have conserved targets and/or functions (e.g., in hormone pathways) across their different host plants. This knowledge will be critical for exploiting effector-based mechanisms in future biotechnological application strategies.

Symbiont effectors identify beneficial genetic traits in highly interconnected plant networks

Plant hormones are principal regulators in all aspects of plant development and integrate environmental stress and developmental processes.[90] Phytohormone signalling and regulation of plant stress response and growth is highly dependent on vast crosstalk and pathways regulating each other.[8,10,69,91] In plant immunity salicylic acid (SA) and JA are key hormones in regulation of defence signalling against pathogens.[92] Additionally, hormones primarily known for abiotic stress signalling (e.g., ABA) or developmental function (auxin, cytokinin) also affect immunity. Signalling pathways are interconnected in complex networks for rapid adaptation to the biotic environment.[22] ABA interacts with the SA and JA signalling pathways to mitigate effects of abiotic stress versus pathogen defence. Apart from initial Ca^{2+} signalling, transduction of these pathways are often carried out via GTP-binding proteins. They comprise a large group of proteins with a high variation of structure and function.[69] Thus, hormones are attractive points of attack for both pathogens and symbionts to modulate plants for colonisation, disease, or beneficial effects.[15,93–95]

Symbiotic fungi and other beneficial microbes developed mechanisms for targeting defence- and growth-related hormones.[15,40] *L. bicolor*, for instance, promotes mutualism though blocking JA action via the interaction of the effector MiSSP7 and the Populus host protein JAZ6.[84] In addition to modulation of hormones for defence suppression during initial colonisation, hormones were found to be involved in conferring beneficial effects in symbioses as reported for arbuscular mycorrhizal fungi.[96] Consistent with this, cytokinin (CK) signalling is an important factor for rhizobia nodulation[97] and auxin (AUX) production by root-associated microbes is necessary for regulating plant growth.[98]

Si interferes with the synthesis and signalling of various plant hormones.[77,94,99] *Si* colonisation success involves ethylene (ET), JA and gibberellin (GA) signalling.[43,100–102] Earlier studies revealed *Si* effector PIIN_08944 to play an important role in its ability to colonise Arabidopsis by interfering with SA signalling.[103] Nevertheless, plant hormone modulation is not only recruited to establish colonisation, but to confer benefits and balancing *Si* benefits as reported for example for ET.[94,104] AUX signalling modulation and production by *Si* is important for altering root development and involved in growth promoting effects.[105] Besides changing hormone signalling, *Si* increases hormone levels/hormone production in hosts like AUX, ABA, SA and GA in addition to betaine, glycine, and proline. Increased levels of theses hormones and molecules were shown to improve plant resistance to environmental stresses, like salinity.[79] Proline and glycine betaine are osmolytes involved osmotic balance during stress.[106]

The vast crosstalk of the hormone network makes it generally difficult to decipher the contribution of individual proteins or specific interactions to phenotypic traits.[69,91,107]. In an effort to disentangle hormone networks and to identify principal regulators, a recent extensive network-study based on protein-protein interactions revealed hormone pathway contacts that represent potential crosstalk points.[108] In our comparative effectome analysis, we employed the same protein-protein interaction platform as well as effector target networks of plant pathogens in Arabidopsis.[85] More than 80% of *Si* effectors either directly interacted with proteins connected to hormone signalling pathways, or targeted proteins interacting with proteins involved in particular hormone pathways.[85] This pronounced hormone targeting effect was further evaluated in comparison to pathogens. Symbiont and pathogens both significantly interact with hormone related proteins, as the frequency of effector interactions with hormone network proteins was higher than the simulation-based random expectation. However, *Si* targeted these pathways more frequently and more extensively. It indicated that modulation of hormones is not only involved in colonisation strategies but supports the hypothesis that *Si* effectors recruit specific proteins of the hormone network to confer beneficial traits.[85]

Functional assays confirmed that in addition to targeting hormone related proteins symbiotic effectors alter hormone signalling pathways in planta. Hormone marker assays in protoplasts identified extensive and highly specific hormone signalling modulation by *Si* effectors. Interestingly, hormone effects translated into individual increases in primary root growth and/or hypocotyl length in *Si* effector-expressing Arabidopsis plants.[85] These assays uncovered previously unknown, highly specific function of Si effectors in hormone signalling to confer host benefits independent of improved nutrient transfer. It further indicates the suitability of effectors as tools to disclose molecular mechanisms and genetic determinants operating in the establishment of symbiont-mediated plant benefits. Considering that they are the result of plant symbiont co-evolution, symbiont effectors have a high potential to identify regulatory principles of plant stress resilience and, thus plant fitness under environmental challenges.

Strategies to identify and use the genetic potential of symbiont effectors

Uncovering the underlying symbioses-associated genetic resources requires a deeper understanding of symbiont effector targets and the functional context of these targets within interconnected protein networks. Network mapping based on systematic protein-protein interaction analyses has given a profound insight into the organisation of the highly entangled plant hormone signalling network.[108] Applying such a systems level approach we generated a symbiont-host interactome map and discovered symbiont effectors that target specific host proteins often associated with the hormone network.[85] Such effector-guided approach can be very effective in assigning previously unknown functions to plant proteins and help in the identification of pathway contacts and pathway connecting points conferring host benefits (Figure 2). More importantly, it can reveal effectors with beneficial effects such as growth promotion.[85] These effector targets represent possibly new host protein candidates that improve plant fitness under environmental stress.[85]

In addition to experimentally demanding protein-protein or comprehensive interactome studies, recent advances in in silico prediction of protein structures have opened-up exciting opportunities for hypothesis-driven functional analyses. AlphaFold2 and RoseTTaFold are currently the most advanced software tools to predict protein structures based on artificial intelligence trained on multiple sequence alignments.[109,110] Alphafold-Multimer is an extension of this system that predicts structures of protein complexes developed by DeepMind[111] and was used, for instance, to screen ~1800 pathogen small, secreted proteins for interaction with six defence related hydrolases.[112] Utilising the AlphaFold-Multimer prediction model allows to screen previously identified effector candidates[85] for interaction partners in plants. While these tools cannot substitute wet bench experiments, they are highly suitable to screen larger sets of effectors from different organisms for putative interactors in

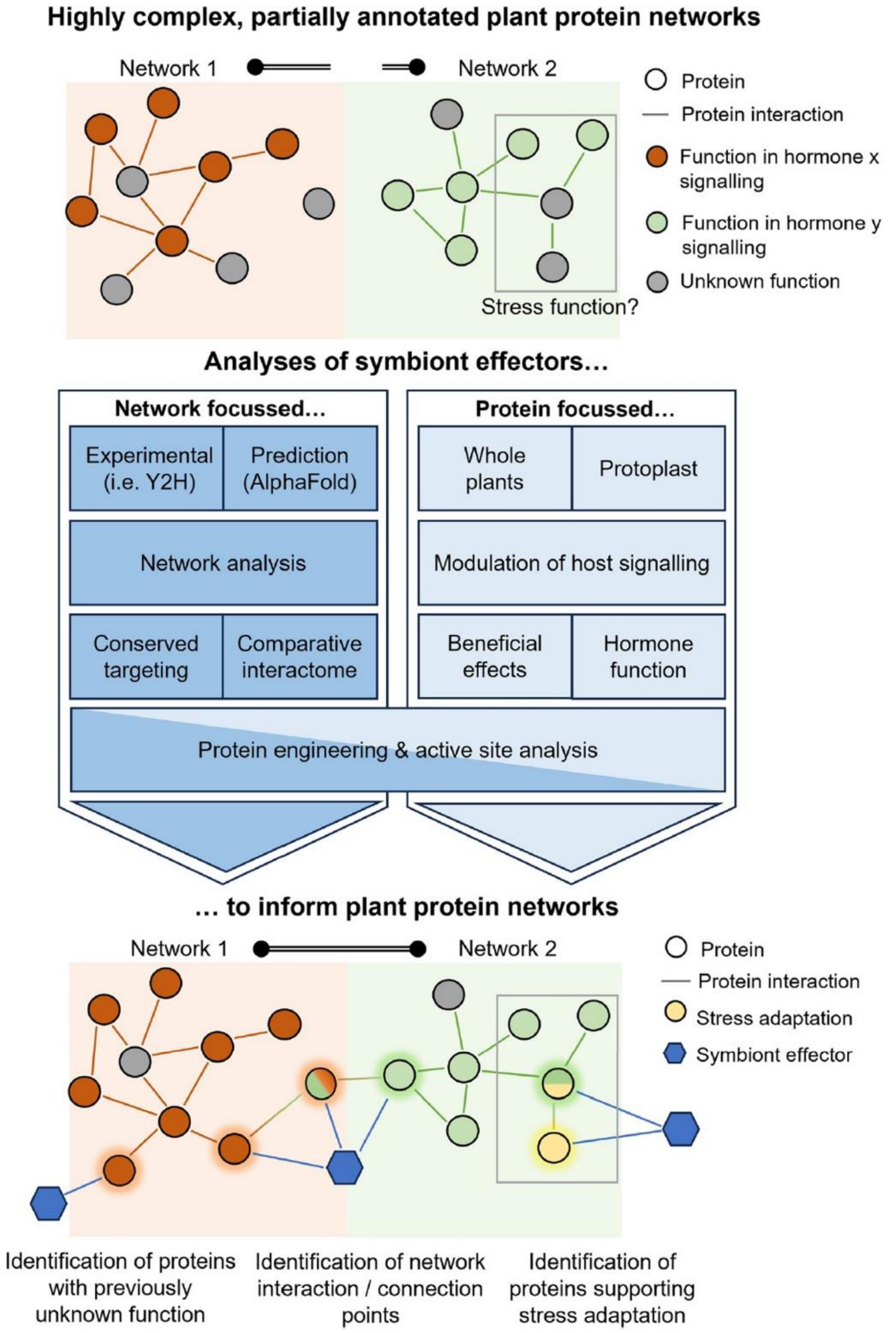

FIGURE 2 Symbiont effector analysis informs highly complex plant hormone networks and assigns previously unknown functions to host proteins.[85] Protein networks involved in hormone and stress signalling can be studied with a diversity of effector analysis approaches focusing on interactions, networks and protein functions. Combination of methods allows protein engineering and active site analyses resulting in informed plant networks regarding protein function, network connection points and stress signalling.

different plants. This is especially relevant for systems where respective analytic platforms (e.g., comprehensive yeast-two-hybrid libraries) are not available and it can assist in the identification of proteins involved in improving plant stress resilience and development under changing climates. These tools can elucidate conserved targets of beneficial microbes that are particularly valuable for future application of symbionts in agriculture or generation of crops with advantageous traits.

An important aspect of Alphafold-Multimer is the ability to predict active sites for protein interaction.[112] In addition to expand our

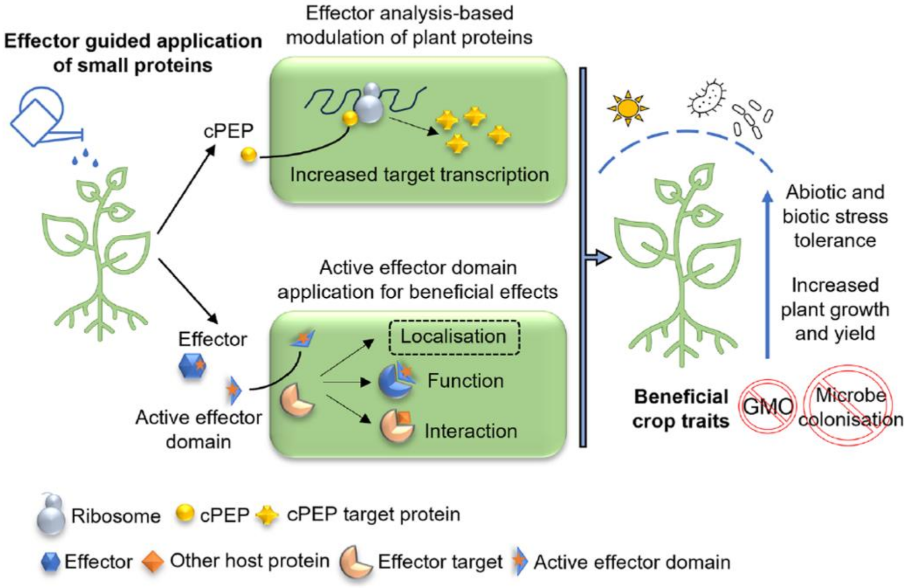

FIGURE 3 Application of small proteins introduces a system for symbiont effector analysis-informed crop stress adaption and overall fitness improvement. Complementary protein (cPEP) application increases, for example, target transcription and improves crop fitness,[113] which can be informed based on symbiont effector targets with beneficial stress tolerance or growth function. The application of effectors represents a potential strategy to improve plant fitness and effectiveness of fertilizers to address environmental challenges and protect crops. These approaches are GMO free and do not require microbial colonisation of crops.

understanding of effector targeting and target modulation it can help to develop active peptide versions of effectors or effector targets. In this respect, a recent technical study suggested simple spraying or watering of plants with complementary peptides (cPEP) from any gene to increase target protein abundance resulting for example in improved growth and heat stress tolerance without genetic modification.[113] The cPEP method is based on findings, that short peptides produced in planta interact with their nascent RNA sequences.[113,114] Ormanecy et al. (2023) showed that peptides with 5–40 amino acids in size increased target activity with strong specificity. cPEPs, when externally applied, were able to modulate mRNA translation by increasing ribosomal recruitment at translation initiation sites. For this function, RPL19 (AT4G17560) was necessary, which is assumed to increase stability of inter-ribosomal subunit bridges.[113] The cPEP approach could become highly valuable in protecting crops more flexibly and immediate against rapidly changing climates and new emerging diseases and pests. Apart from the identification of possible cPEP targets for crop improvement it may also be possible to apply effectors directly to induce beneficial signalling pathway modulation and favourable plant phenotypes (Figure 3). Fungal effectors are small, secreted proteins and plant uptake is possibly unspecific or depending on protein structure.[115] In the latter case AlphaFold2[110] may help to define structures that would facilitate plant uptake. Our recently identified effector proteins as well as fungal effectors in general are very variable in size, sequence, and function.[85,116] Functional domains of effector peptides could be transferred into plants by spray-application. While plant uptake of cPEPs is based on a currently unknown mechanism, cPEPs must have some hydrophilic properties to facilitate solubilization and between 5 to 40 amino acids in size. Functional cPEP might be applied by drainage or spraying leaves or seedlings.[113] Therefore, small functional domains of effector proteins could be designed, that are possibly taken up and confer beneficial effects in plants similar to their native function during *S. indica* interaction. We showed previously that expression of single effectors in Arabidopsis was sufficient to improve seedling growth.[85] The application of single or a combination of effectors could represent a strategy to improve the effectiveness of biological fertilizers or to flexibly address multiple environmental challenges to protect crops (Figure 3). The feasibility of this approach in large-scale agricultural settings will certainly depend on an understanding of effective protein treatment concentrations, the efficiency of uptake mechanisms and economic factors such as peptide production costs.

CONCLUSION

Effector analyses were shown as useful tool to identify plant immunity and defence pathways and the mechanistic processes of pathogen microbe colonisation.[48] Additionally, these studies aided in the development of plant disease protection products.[117] Symbiont effector analyses regarding their function in conferring beneficial effects to plants was previously neglected, even though numerous studies identified signalling modulation by symbionts as necessary for increased plant stress tolerance.[77,94] However, the investigation of plant signalling networks can be exceptionally challenging because of vast pathway crosstalks and overlapping functions.[10,91]

In this review and our previously published study[85] we show not only, that symbiont effector analyses reveal molecular mechanisms regulating the establishment and beneficial outcome of plant-symbioses, but also several potential applications of symbiont effectors. Studies of effectors derived from beneficial microbes are able to inform highly complex and interconnected signalling networks.[85] Effector network analysis is capable of identifying signalling network connection points and assign new, beneficial functions to host proteins.[85]

Based on this identification of previously unknown functions of plant proteins in hormone signalling and plant stress resilience, new application points for crop improvement are available. Using in silico methods like AlphaFold-Multimer[111] extensive studies of beneficial effectors are promising to investigate signalling pathways for increased plant stress tolerance, growth and yield. Modulation of the identified symbiont effector targets as well as direct application of effectors in previously demonstrated GMO-free approaches like cPEP[113] may be important tools for improved crop stress tolerance in the future. These techniques are highly valuable in drastically changing environments for improved, greatly flexible crop production and protection products to ensure foot security for a growing world population.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

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Chapter 2 Discussion

This chapter provides a profound review of current knowledge about microbial effector proteins, focusing on their function in signalling pathways and involvement in beneficial effects conferred by symbionts. It further elucidates their potential in uncovering complex plant networks by interaction and functional analyses. The paper discusses novel, promising methods based on symbiont effector analyses, which could improve agriculture practices and crop breeding under changing environments.

CHAPTER 3 – Symbiont-host interactome mapping reveals effector-targeted modulation of hormone networks and activation of growth promotion

Chapter 3 Introduction

Chapter 3 of this thesis was published in the journal *Nature Communications* with the title " Symbiont-host interactome mapping reveals effector-targeted modulation of hormone networks and activation of growth promotion". The paper was accepted on 27th June 2023 and published online on 10th July 2023 with the DOI: 10.1038/s41467-023-39885-5.

While microbe effector proteins are known to be involved in host signalling modulation for immunity suppression and colonisation (Hogenhout et al. 2009; Lo Presti et al. 2015), little is known about symbiont effectors and their potential role in regulating host benefits. This chapter aims to analyse *S. indica* effector functions and interactomes especially in the context of plant hormone networks to elucidate previously unknown mechanisms of symbiont-mediated host fitness. Additionally, effector-informed target analyses will be explored as tool for uncovering complex plant signalling networks.

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Symbiont-host interactome mapping reveals effector-targeted modulation of hormone networks and activation of growth promotion

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Plants have benefited from interactions with symbionts for coping with challenging environments since the colonisation of land. The mechanisms of symbiont-mediated beneficial effects and similarities and differences to pathogen strategies are mostly unknown. Here, we use 106 (effector-) proteins, secreted by the symbiont *Serendipita indica* (*Si*) to modulate host physiology, to map interactions with *Arabidopsis thaliana* host proteins. Using integrative network analysis, we show significant convergence on target-proteins shared with pathogens and exclusive targeting of Arabidopsis proteins in the phytohormone signalling network. Functional *in planta* screening and phenotyping of *Si* effectors and interacting proteins reveals previously unknown hormone functions of Arabidopsis proteins and direct beneficial activities mediated by effectors in Arabidopsis. Thus, symbionts and pathogens target a shared molecular microbe-host interface. At the same time *Si* effectors specifically target the plant hormone network and constitute a powerful resource for elucidating the signalling network function and boosting plant productivity.

Plants continuously interact with a plethora of prokaryotic and eukaryotic microbes. While understanding the molecular details of pathogen-host interactions has commanded most attention by researchers, it is increasingly recognised that many microbes benefit their host by improving nutrient acquisition, accelerating growth and boosting pathogen resistance and abiotic stress tolerance[1–5]. In fact, by enhancing plant fitness, symbionts enabled colonisation of the hostile land environment by plants more than 400 million years ago[6]. Ever

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since, plants have co-evolved with and relied on beneficial microbes to withstand challenging environments[7–9]. However, while individual mechanisms underlying health-promoting effects have been worked out in some cases, a systems-mechanistic-level understanding of how symbionts interact with plant hosts is lacking. Moreover, beneficial microbes and evolutionary close pathogenic relatives share many host-interacting mechanisms and it remains a fundamental open question how these interactions turn into a pathogenic or beneficial outcome[10].

Irrespective of their different colonisation strategies, the ability of plant pathogenic bacteria, fungi, and oomycetes to establish diseases is based on the secretion of an arsenal of effector proteins for the targeted manipulation of host pathways. Global interactome studies revealed that effectors from the fungal leaf pathogen *Golovinomyces orontii* (*Gor*), the oomycete leaf pathogen *Hyaloperonospora arabidopsidis* (*Hpa*), the bacterial leaf pathogen *Pseudomonas syringae* (*Psy*), and bacterial root pathogens *Ralstonia pseudosolanacearum* (*Rps*) and *Xanthomonas campestris* pv. *campestris* (*Xcc*) interact with *Arabidopsis thaliana* (hereafter Arabidopsis) host proteins with high specificity, while also sharing common target proteins involved in the regulation of plant immunity. This significant interspecies convergence on targeted host protein networks by pathogens of different kingdoms and trophic colonisation strategies suggested the existence of a common molecular pathogen-host interface that is targeted by diverse microbes[11]. In the absence of systematic interactome data for any beneficial microbes, however, it is unclear whether this common interface is indeed specific to pathogens, or whether it might be a universal microbe host interface. We aimed to address this fundamental question.

In addition, the beneficial (mutualistic) fungal root endophyte *Serendipita indica* (*Si*, formerly *Piriformospora indica*) induces a broad spectrum of beneficial effects (e.g. growth promotion) in its host plants[4,12–20]. Thus, *Si* constitutes an important genetic resource for improving crop productivity under changing environments, which remains untapped due to our lack of understanding of many of the underlying mechanisms. Here, we identify 106 candidate effectors from *Si* and mapped the protein contact points of these in their Arabidopsis protein host. Combining this symbiont-host interactome with that of pathogens revealed, in addition to a common host-microbe protein-interaction interface, an over-representation of *Si-specific* targets within the host hormone network. By implementing an *in planta* phenotyping platform, we show that over 80% of *Si* effectors modulate hormone signalling, and that overexpression of hormone-modulating effectors promotes growth in Arabidopsis. Finally, by integrating our interactome and phenotyping data with an updated hormone protein network, we successfully confirm effector-informed hormone functions for hitherto uncategorised Arabidopsis proteins. Our study thus indicates the translational potential of effectors from the beneficial fungal endophyte *S. indica* in assigning proteins to the highly interconnected plant hormone network and in advancing our understanding of the molecular nature of beneficial plant effects.

Results

Interactome mapping of *S. indica* effector-host targets

As effectors from beneficial microbes likely mediate plant symbioses and beneficial host effects[21–24], we aimed to identify *Si* effector candidates (SIECs). We performed RNA-seq of Arabidopsis roots colonised by *Si* at early, biotrophic[25] (3 days after inoculation, dai) and late, cell death inducing[25] (10 dai) colonisation stages (Supplementary Fig. 1a, Supplementary Data 1, 8). Employing approved effector identification pipelines[26–28] we found 106 SIECs that met the stringent selection criteria, such as high expression during colonisation, presence of a signal peptide, and absence of transmembrane domains (Supplementary Data 1). Using the heterologous yeast signal sequence trap (YSST) system[29], we experimentally confirmed the functional integrity of the secretion signals for 11 randomly selected SIECs. The YSST yeast strain is not able to grow in sucrose medium due to a deletion of the *SUC2* gene encoding a secreted invertase. N-terminal fusion of a protein with a functional signal peptide restored SUC2 secretion and yeast growth in sucrose media, as did *SUC2*-deficient cells complemented with SUC2 (lacking endogenous signal peptide) fused to any of the 11 *SIECs* tested (Fig. 1a, Supplementary Fig. 1b). Expression of *SIEC*s did not affect yeast growth under control conditions on glucose media (Supplementary Fig. 1c). Confirming that our search criteria revealed secreted effector candidates, the 106 SIECs were forwarded to systematic large-scale SIEC-Arabidopsis protein interaction (interactome) mapping.

We generated an SIEC-host protein interactome network map using a high-quality pipeline that we previously employed to generate plant pathogen networks[11,30], the Arabidopsis Interactome map AI-1[31], and a systematic map of the phytohormone signalling network[32]. SIECs were screened as DNA-binding protein fusions (DB-SIECs) against a library of 12,000 Arabidopsis proteins (12k_space) and 500 additional proteins associated with hormone signalling[32]. After removal of autoactivating DB-SIECs, we uncovered and verified 207 protein-protein interactions (PPI) between 156 Arabidopsis proteins and 33 SIECs (Fig. 1b and Supplementary Data 2). 14 SIECs interacted with only one host protein, while 19 interacted with two or more host proteins (Fig. 1b, c). 115 host proteins were targeted by only one SIEC, while 41 were targeted by more than one (Fig. 1d). This partition pattern of SIEC targeting is consistent with previously observed pathogen effector-host protein interactions[11,30]. To confirm the quality of our dataset experimentally, we analysed six randomly selected interactions between SIECs and host proteins in independent co-immunoprecipitation assays *in planta* (Fig. 1e). After individual optimisation, all interactions could be confirmed, thus further suggesting a high biophysical quality of the data. Subsequent gene ontology (GO) enrichment analysis indicated that SIEC-targeted Arabidopsis proteins function in processes that are known to regulate symbiotic *Si*-Arabidopsis interactions, including defence response (GO:0031347), regulation of metabolism (GO:0009893), regulation of developmental process (GO:0050793) and cellular response to hormone (GO:0032870) (Supplementary Data 1, 3)[25]. Further supporting the validity of the SIEC interactome map (Fig. 1b), 19 GO terms were common between SIEC targets and differentially expressed Arabidopsis genes (DEGs) in our RNA-seq analyses (Supplementary Data 1, 3), including programmed cell death (GO:0012501), response to radical oxygen (GO:1901700), plant organ development (GO:0099402), indolalkylamine biosynthesis (GO:0046219) and response to ethylene (GO:0009723). In both the DEGs and the interactome, enrichments for over 150 biological processes were over-represented ($p < 0.05$) illustrating in both datasets the broad extent to which *Si* modulates host systems. Terms associated with more specific secondary metabolite biosynthesis such as glucosinolate were observed only for the DEGs, although this may reflect the increased depth of RNA sequencing vs. the less exhaustive Y2H screen. Overall, 20% (34/174) and 32% (59/183) of terms were linked to host defences or hormone signalling in the *Si* interactome and DEGs, respectively. This immune targeting by symbiotic *Si* is consistent with previous reports[25,33,34] and supports in addition to the robustness, the biological validity of the dataset.

Comparative symbiont-host pathogen-host interactome analyses

Next, we aimed to explore the relationship of symbiotic *Si* host targets to those of pathogen effectors. We previously described the convergence of pathogen effector proteins on few functionally important host proteins, which we coined *intraspecies convergence*, i.e. the targeting of host proteins by multiple effectors from one pathogen[11]. We also described *interspecies convergence*, for the targeting of common host proteins by different and evolutionary distant pathogens (*Pst*, *Hpa*, *Gor*[11,30]), which was later extended to *Ralstonia pseudosolanacearum* (*Rps*) and *Xanthomonas campestris* (*Xcc*)[35]. Notably, we showed that topological convergence corresponds to biological importance: in infection assays the level of convergence correlated with the frequency of enhanced resistance and enhanced susceptibility phenotypes[11]. Beyond being important for the successful colonisation of diverse

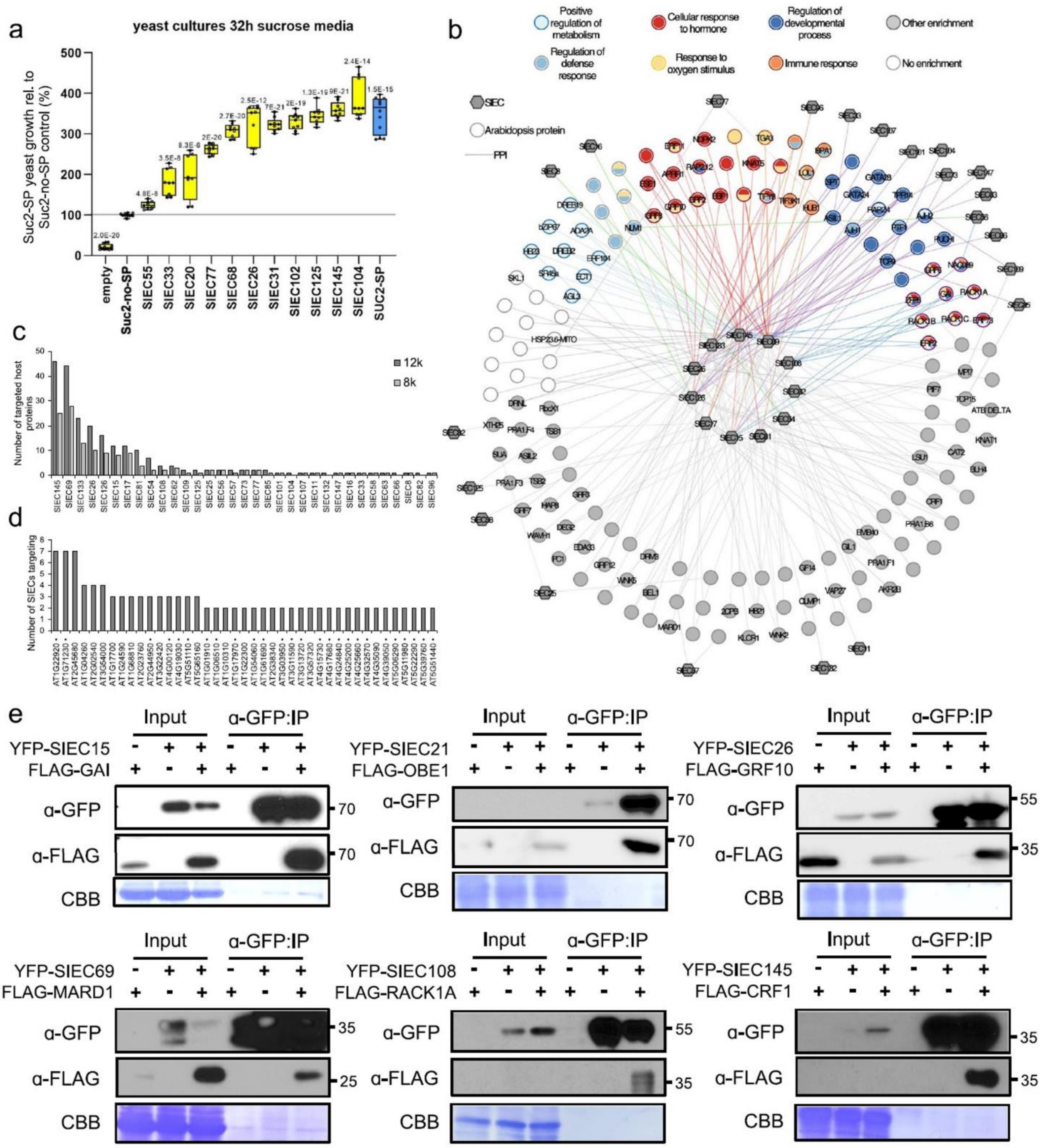


Fig. 1 | *Serendipita indica* - Arabidopsis interactome map. a The expression of signal peptide (SP)-containing SIECs fused to yeast SUC2 allows for growth of yeast cells on secretion selection media when compared to expression of SUC2 without SP (no-SP). Empty = untransformed yeast Y02321, SUC2-SP = SUC2 with endogenous SP (blue; positive control). Error bars represent min to max from $n = 3$ biological replicates. Yellow colour indicates significantly growing yeast cultures according to two-tailed, unpaired t-test: numbers above plots indicate p-values for significantly different comparisons. See also Supplementary Fig. 1. All box plots indicate minimum to maximum values, the 25th to 75th percentile with lines indicating the median of the data. **b** Interaction network displaying Arabidopsis proteins (circles) targeted by *Si* effector candidates (SIECs, hexagons), and the gene ontology (GO) term enrichment for these proteins (see Supplementary Data 3 for details). **c** Degree distribution of SIECs interacting with Arabidopsis proteins in the 12k_ (dark grey) and 8k_spaces (light grey). **d** Degree distribution of Arabidopsis proteins that are targeted by at least two SIECs. Dots indicate 8k space plant proteins. **e** Co-immunoprecipitation assays confirm interactions of SIECs with their predicted Arabidopsis target proteins *in planta*. Numbers indicate size (kDa).

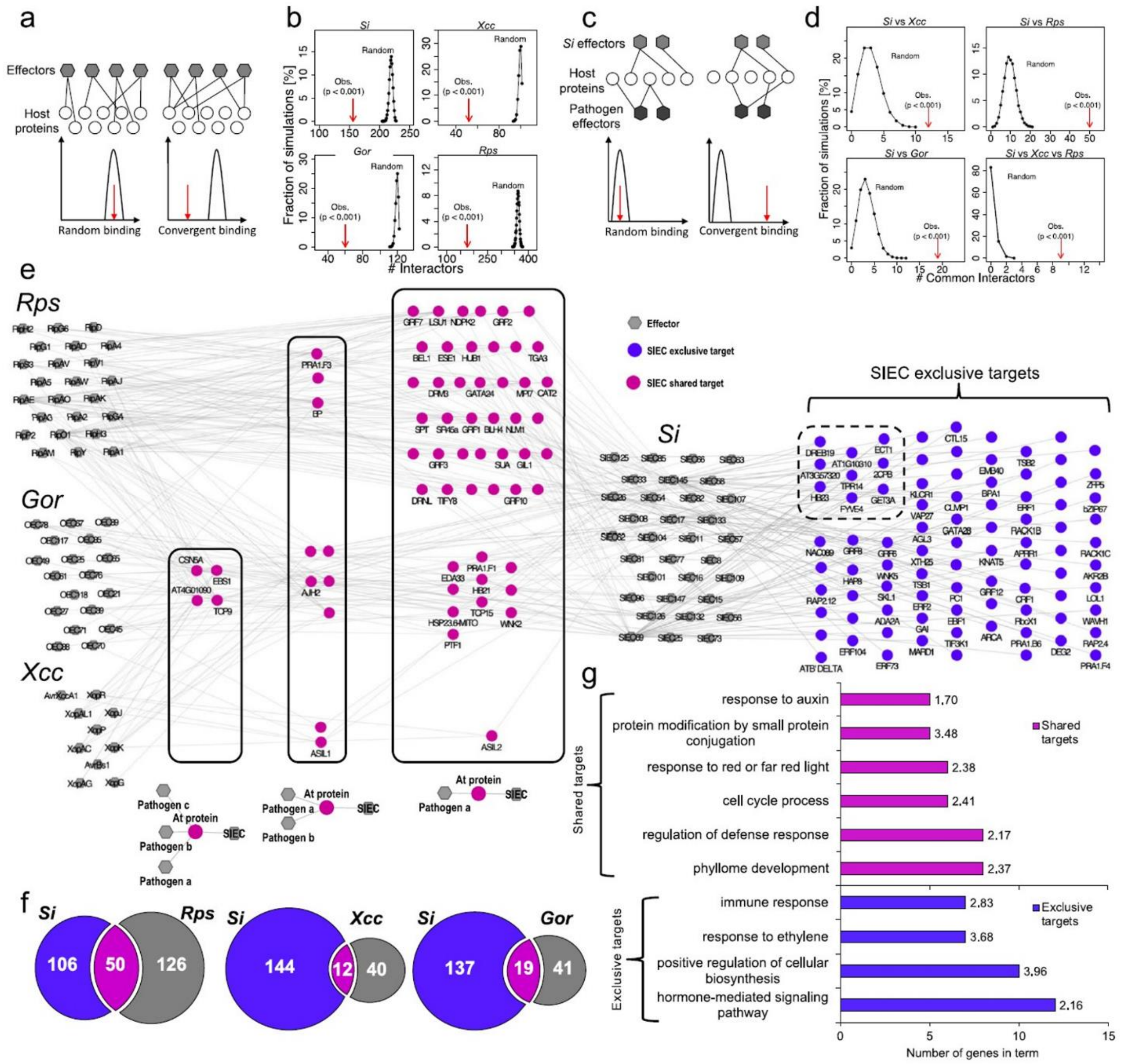


Fig. 2 | Comparative interactomics (12k_space). a Network rewiring between effectors (hexagons) and host proteins (circles) to determine the likelihood of random and convergent binding between effectors and host proteins. **b** Distribution of the number of simulated interactors between effectors from *Si*, *Xcc, Gor and Rps*, and *Arabidopsis* proteins vs. the observed number (red arrows). **c** Network rewiring of interactions from effectors of multiple organisms targeting host proteins to determine the likelihood of interspecies convergence. **d** Distribution of the number of random simulated common interactors between SIECs and effectors of *Xcc*, *Rps*, *Gor* vs. the observed number of common interactors (red arrows) in two- and three-way convergence analyses. **e** Classification of SIEC target proteins as either exclusive to *Si* (blue) or shared with at least one pathogen effector (magenta) from *Rps*, *Xcc* or *Gor*. Shared nodes are hierarchically displayed according to the number of microbes with interacting effectors. Pathogen exclusive interactions not shown. Dashed inset: convergent targets exclusive to *Si*. **f** Overlap between SIEC and pathogen effector interactions from *Rps*, *Gor* and *Xcc*. **g** Comparative GO term enrichment analysis showing number of genes represented by terms which were present in exclusive (blue) or shared (magenta, *Rps*, *Xcc*, *Gor*) SIEC targets. Numbers at the end of bars represent the -$\log_{10}$ of the adjusted p-value for each term.

pathogens, the convergence targets are linked to population genomic signatures[11] and even conserved as pathogen targets between plant host species[36]. Because of the intimate co-evolutionary link between plants and microbes, we wondered if convergence targets are perhaps not solely important for pathogens, but instead might be universal molecular microbe-host contact points. We therefore examined if SIECs also exhibited intraspecies convergence, and if some of the convergence targets are shared with pathogens[11,35]. Our analysis clearly revealed intraspecies convergence for SIECs (Fig. 2a, b), where the simulated number of unique interactors is higher than the number observed, indicating that redundant targeting is not exclusive to pathogens but may be a common feature of plant-microbe interactions. For analysing interspecies convergence, we integrated our data with pathogen-host interactions for *Rps, Xcc, Gor, Hpa*, *Psy* effectors[11,30,35] taking the search space of each experiment into account. The effectors of *Hpa* and *Psy* were tested in an 8k_space (8,000 host proteins)[30], which is a fully contained subset of the 12k_space[11,35] subsequently used for screening *Gor, Rps* and *Xcc*[11,35]. In both search spaces degree-preserving network rewiring revealed clear evidence for interspecies convergence involving SIECs (Fig. 2c, d and

Supplementary Fig. 2a–c) in all possible comparisons, which cannot be explained by random effects. In line with the GO analyses (Fig. 1b and Supplementary Data 3) this indicates that *Si* and pathogens employ effectors interacting with some of the same host proteins to manipulate common processes in Arabidopsis irrespective of evolutionary origin (prokaryote, eukaryote) and the lifestyle (beneficial, pathogenic) of the microbes. For example, 13 SIECs shared four Arabidopsis protein targets with all three pathogens in the 12k search space: TCP9, EBS1, CSN5A, and AT4G01090 (Fig. 2e), all of which have been implicated in plant defence. This analysis also revealed several convergent host proteins which were not targets of pathogen effectors, such as HOMEOBOX PROTEIN 23 (HB23) and DEHYDRATION RESPONSE ELEMENT-BINDING 19 (DREB19), which may be important to *Si* colonisation specifically. Overall, *Si* shared most targets with *Rps* effectors (12k_space) and *Hpa* effectors (8k_space) (Fig. 2e, f and Supplementary Fig. 2e), respectively, which likely reflects the common target tissue (roots) and colonisation strategy (biotrophy with a subsequent cell death phase) of the microbes.

S. indica effectors target the host hormone network

While the convergence analyses revealed shared targeting of SIECs and pathogen effectors, we next explored the extent of SIEC exclusive targeting and the function of these targets. In the 12k_space, *Si* had 87 exclusive targets; in the universally interrogated 8k_space, 76 Arabidopsis proteins exclusively interacted with SIECs (Supplementary Fig. 2d, e). To identify any discriminate functional trends between shared and exclusive SIEC targets we performed a second enrichment analysis with each set of proteins restricted to a reference set of Y2H positive genes (see Methods). As most enriched terms were associated with broad regulatory processes, we focused on annotations that were restricted to <10% of the total number of genes in the reference set (280), and subsequently removed terms for which <5% of the query set were enriched. Positive regulation of cell biosynthesis (GO:0031328), pertaining to increases in cellular anabolism, was the most significant term in the exclusive set, whilst protein modification by small protein conjugation (GO:0070647) was most highly enriched in the shared targets. Hence these distinctions indicate the diverse function of *Si* effectors, among which the exclusive targets might regulate growth via anabolic processes, whilst others suppress host immunity, e.g. through the ubiquitin proteasome system, a central hub already implicated in many plant-pathogen interactions[37–40]. Terms associated with innate immune response (GO:0045087) and regulation of plant-type hypersensitive response (GO:0010363) were enriched in SIEC exclusive targets, but not in the shared target set. Conversely, regulation of defence response (GO:0031347) was enriched in shared targets only. These differences in the enrichment of immunity-related GO terms may reflect incomplete saturation of the pathogen-host screens[41], but could also point to *Si-specific* mechanisms of immune modulation. Intriguingly, *Si*-exclusive host targets were distinctly enriched for GO terms associated with hormone signalling and response to ethylene, whilst shared host targets were enriched for response to auxin only (Fig. 2g and Supplementary Data 3, 4). While the significance of hormones in the establishment of *Si* symbioses is well known[17,25,42], only recently have we begun to understand the relevance of hormone function in *Si*-mediated beneficial effects[19,43–45]. The strong enrichment of hormone-related GO terms for exclusive *Si* host targets suggests an important role for SIECs in operating beneficial host activities. To gain a more comprehensive insight into the interconnection of *Si* and the host hormone network, we refined the SIEC interactome to uncover SIEC-hormone interaction points (SHIPs). We extended this concept of SHIPs from our previous definition of pathway contact points to identify crosstalk in the phytohormone network[32]. After systematically mapping the Arabidopsis phytohormone interactome network, we reported an abundance and functional significance of physical contacts between proteins associated to different phytohormone signalling pathways, and validated that such contacts point to signalling cross-talk and functional pleiotropy of the involved proteins[32]. In fact, interactions between differently annotated Arabidopsis proteins reliably pointed to unknown functions for at least one of them, often both[32]. To identify SHIPs in this study, we first integrated the SIEC interactome with the systematic portion of the Arabidopsis Interactome 1 (AI-1_{MAIN}), and annotated hormone functions in this network using the Arabidopsis hormone database 2 (AHD2)[46,47] and hormone-related GO terms (Fig. 3a). This revealed interactions of SIECs with Arabidopsis hormone proteins that we defined as direct SHIPs (1st degree) and 2nd degree SHIPs, i.e. SIEC-hormone protein interactions via hormone-un-annotated mediators[31] (Fig. 3a–c). Of the 33 SIECs in our SIEC interactome (Figs. 1b and 2e), 20 formed 1st degree SHIPs with 50 hormone-annotated targets, and 7 SIECs formed 2nd degree SHIPs with 62 hormone proteins. 6 SIECs had no 1st or 2nd degree hormone-interaction and their targets remained un-annotated (Fig. 3a, b). The observed frequency of SIEC interactions with hormone proteins was higher than simulation-based random expectation, and more significant when compared to pathogen-effector interactions with the hormone network (Fig. 3d, Supplementary Fig. 3). Given that we were unable to find interactors for 73 of the 106 SIECs, and that 66 SIEC targets remained un-annotated (Fig. 3a), we hypothesised that the interconnection between SIECs and the hormone network was considerably deeper than we had observed. Our findings, the known completeness limitations of large-scale interactome maps[31,48] and the potential for beneficial activities of hormone-targeting SIECs, encouraged us to systematically explore the function of all 106 SIECs in hormone signalling in complementary, functional plant screens.

Host hormone regulatory functions of *S. indica* effectors

To study functions of the 106 SIECs in hormone signalling we employed our previously developed promoter-based hormone reporters (hereafter *pHORMONE*) for Arabidopsis protoplast assays. These *pHORMONE* reporters are highly specific for each of five hormones (ABA, AUX, CK, JA, SA)[49] which cover a wide range of plant signalling processes, including growth, development, biotic and abiotic stress responses. We were unable to find specific reporters for the remaining hormones. For the functional protoplast screen, we expressed a *LUCIFERASE* (*LUC*) coding sequence using the promoters of respective AUX-, CK-, ABA-, SA- or JA-responsive genes (*pHORMONE::LUC*) together with individual SIECs under the control of the cauliflower mosaic virus *35S* promoter (*35S::SIECs*); *UBIQUITIN10* promoter driven expression of *GLUCURONIDASE* (*pUBQ10::GUS*) was used for normalisation (Fig. 4a). We first conducted a landmark screen followed by a validation screen. In the landmark screen, each of the 106 SIECs x 5 hormone marker combinations were tested in stimulated (respective hormone treatment) and unstimulated (mock) conditions and in two replicates, resulting in 1060 protoplast assays (Fig. 4a–c). Following data normalisation, changes to LUC signals were considered significant if the SIEC altered a reporter more than 2-fold (stronger/weaker) versus empty vector controls. For each SIEC/reporter combination we obtained one “mock ratio” capturing suppressing or inducing SIEC effects on the *pHORMONE::LUC* reporter in the absence of hormone treatment, and a “treatment ratio” to capture suppressing or inducing SIEC effects on *pHORMONE::LUC* activity in the presence of hormone treatment. The product of these ratios was used to quantify the overall effect of each SIEC on the tested hormone pathway. This processing step highlighted SIECs which function synergistically (marker induced or repressed) in both basal and hormone-treated conditions, adding stringency and simplicity to the analysis by revealing robust and continuous effects in the tested conditions (Fig. 4b). Of 530 tested SIEC/hormone marker combinations we detected significant changes in 166 (31%) and of these, 43% (72) were reporter inductions, while 57% (94) were suppressions (Fig. 4b).

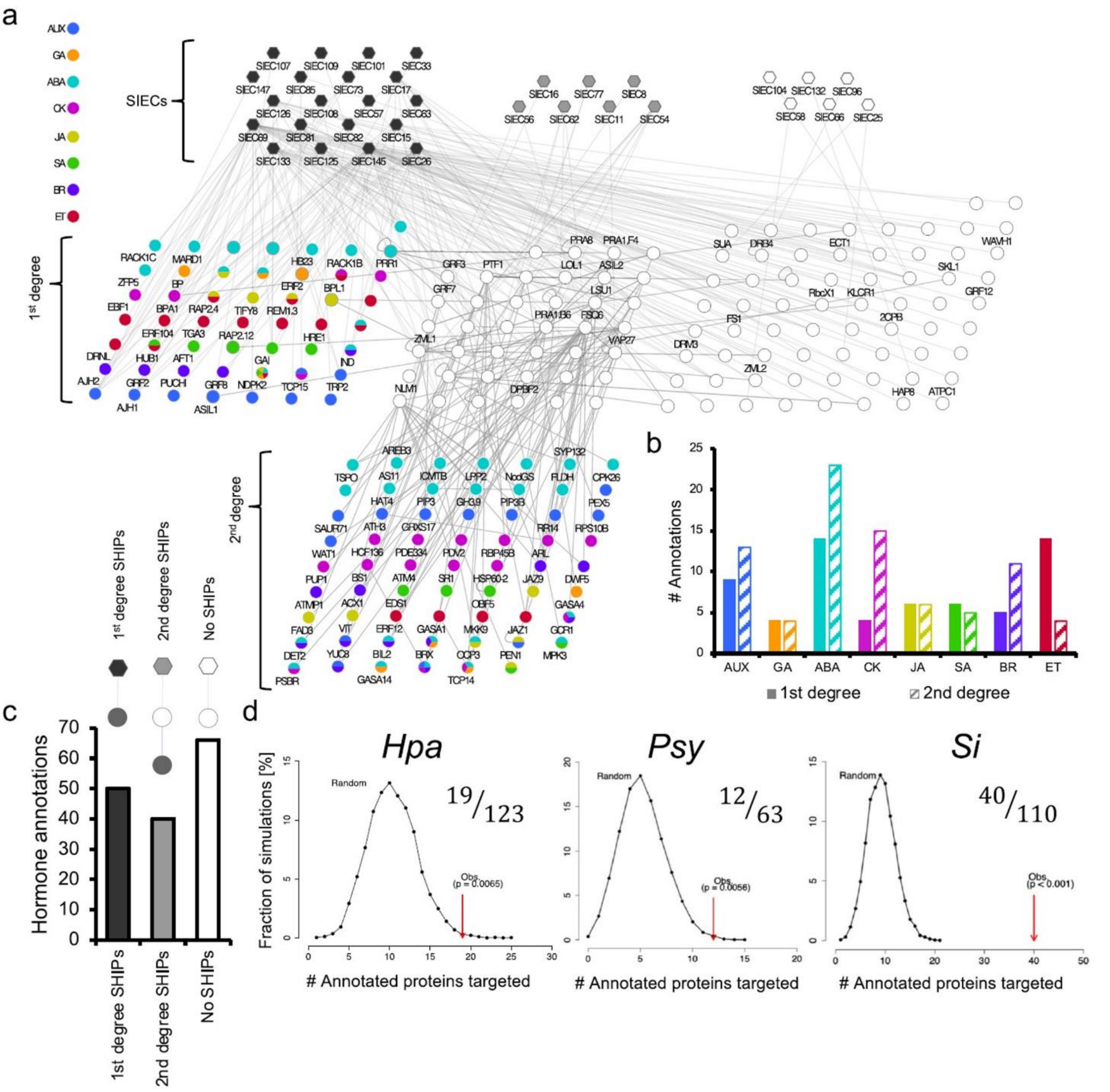


Fig. 3 | SIECs target hormone pathways in Arabidopsis. **a** Combined phytohormone annotations from the Arabidopsis Hormone Database v2 and Gene Ontologies mapped onto SIEC target proteins. 1st degree SIEC hormone interacting points (SHIPs) interact directly with respective SIECs (dark grey). 2nd degree SHIPs are not themselves annotated but form secondary interactions with other annotated proteins within AI-MAIN1. **b** Summary of hormone annotations in 1st and 2nd degree SHIPs. For bar colours see legend in **a**. **c** Number of SHIPs in the SIEC interaction network. Legend: Grey and white nodes represent annotated and unannotated proteins respectively. **d** Frequency distribution of the number of annotated proteins targeted by effectors (8k space) in random networks vs the observed number for *Hpa*, *Psy* and *Si*. Inset numbers represent the # of annotations (top) vs the total # of unique effector targets (bottom).

The highest number of SIEC-dependent reporter changes was observed for the AUX and SA reporters (37 SIECs each), followed by ABA (36), JA (35), and CK (21) (Fig. 4b). Except for the SA reporter, suppressions were more prevalent than inductions. Overall, 86 of the 106 SIECs (80%) changed at least one of the 5 hormone pathways in the landmark screen. An independent validation screen (Fig. 4d) confirmed all SIECs for JA, 90% for AUX, 70% of CK and 60% of ABA effects. Only 10% of SIEC effects on the SA reporter were validated (Fig. 4d) likely due to the overall low, albeit highly specific, LUC signal of the SA reporter. Consistent with the observed target-specificity of SIECs in our SIEC interactome map (Fig. 1b, c), we detected a high SIEC-pathway specificity in our protoplast screen, as 39 of the 86 hormone-modulating SIECs caused significant changes in only one of the five tested hormonal pathways (Fig. 4c). In addition to these very specific SIEC effects, we also identified six broad range hormone signalling modulating SIECs that changed at least four of the five tested markers (Supplementary Data 5). Given the complexity of the host hormone network, and that small perturbations in the plant metabolism (signalling/biosynthesis/perception) can alter hormone regulated gene expression, particularly via crosstalk, it is important to consider whether measured changes in marker expression represent direct activities, or reflect the response by host cells to upstream activities of each

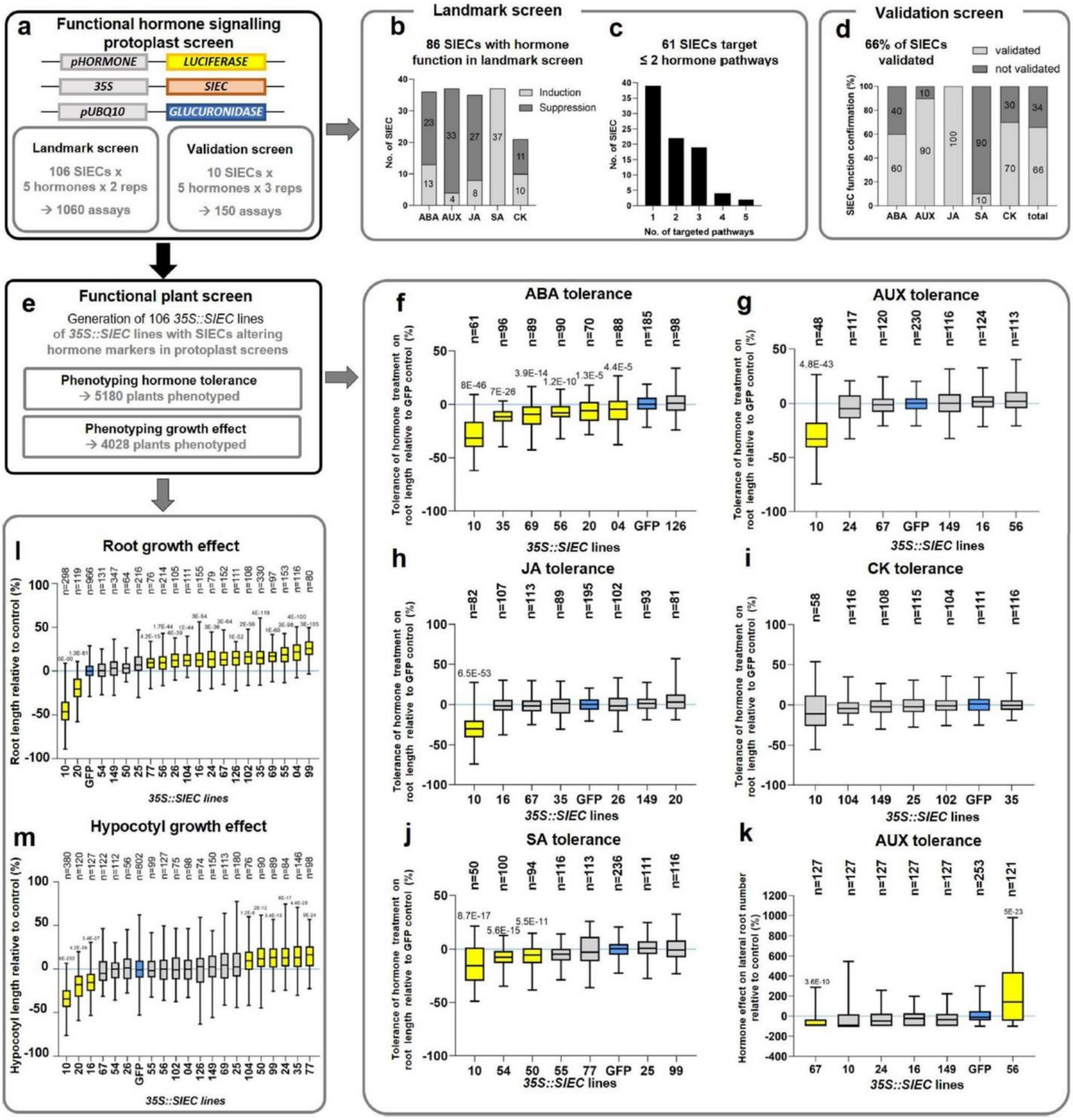


Fig. 4 | Phenotyping of hormone-related effects of SIECs in protoplasts and whole plants. a Workflow for annotating hormone functions of SIECs in functional Arabidopsis protoplasts landmark and validation screens. Protoplasts were transformed with an SIEC overexpression construct (*35S::SIEC*), hormone markers consisting of promoters of hormone-specific genes (see Materials & Methods for details) fused to LUCIFERASE (*pHORMONE::LUCIFERASE*) and a *pUBQ10::GLUCURONIDASE* construct for data normalisation. **b** Number of SIECs that induce or suppress tested hormone pathways in the landmark screen with all 106 SIECs tested against five hormones (1060 assays in total). **c** Specificity of SIECs in modulating hormone pathways. **d** Validation screen for the top ten SIECs (based on 106 SIEC from the landmark screen) on hormone marker regulation (150 assays in total). **e** Outline for functional plant screen to phenotype the effect of SIECs on altering hormone tolerance or growth in whole plants. Hormone tolerance of *35S::SIEC* lines compared to *35S::GFP* control plants (blue) for ABA (**f**), AUX (**g**, **k**), JA (**h**), CK (**i**), and SA (**j**) (5180 plants in total). Error bars represent min to max from 3 biological replicates. Yellow colour indicates significant growth differences compared to *35S::GFP* plants. Statistical significant difference was calculated using two-tailed, unpaired t-test: numbers above plots indicate *p*-value for significantly different comparisons. All box plots indicate minimum to maximum values, the 25th to 75th percentile with lines indicating the median of the data. **l**, **m** *35S::SIEC* lines with altered primary root or hypocotyl length when compared to *35S::GFP* control plants (blue). Error bars represent min to max from at least 2 biological replicates (4028 plants in total). Yellow colour indicates significant differences compared to *35S::GFP* plants. Statistical significant difference was determined by two-tailed, unpaired t-test: numbers above plots indicate *p*-value for significantly different comparisons. All box plots indicate minimum to maximum values, the 25th to 75th percentile with lines indicating the median of the data.

effector. In either case this analysis gives strong evidence toward which pathways are modulated in the presence of each SIEC, particularly as all *pHORMONE::LUC* constructs tested were highly specific toward their respective hormones[49], and many effector candidates only affected one marker.

To validate the protoplast-detected SIEC signalling functions in whole plants, we stably expressed all 106 SIECs in Arabidopsis seedlings (Fig. 4e) and confirmed *SIEC* expression by quantitative real time-PCR (qRT-PCR) (Supplementary Fig. 4). We phenotyped primary root length reduction (relative to *35S::GFP* plants) upon hormone treatment as a proxy for hormone-tolerance of the lines (Fig. 4f–j, Supplementary Data 6 and Supplementary Fig. 5). Analysing 5180 *35S::SIEC* plants (~860 plants/hormone treatment), we observed phenotypes for JA, SA, ABA and AUX, but not CK (Fig. 4f–j). Interestingly, almost all observed phenotypes indicated SIECs reduced tolerance to respective hormones. For example, 6 of the 7 *35S::SIEC* lines showed diminished ABA tolerance as indicated by increased root growth attenuation (Fig. 4f). We further analysed lateral root number (LRN) for AUX and CK treatment as a second phenotype regulated by both hormones, and anthocyanin production under constant light for CK tolerance (Fig. 4k and Supplementary Fig. 7g, h). While SIEC effects were not detected for CK, reduced and enhanced lateral root formation were observed for the AUX-treated lines *35S::SIEC67* and *35S::SIEC56* (Fig. 4k) respectively. *35S::SIEC10* plants showed reduced root growth on control media which might affect its performance after hormone treatment (Fig. 4l). SIEC10 strongly induced expression of all 5 hormone markers in protoplast screens (Supplementary Data 5), suggesting this effector might influence multiple fundamental processes in plants. We were able to confirm an *in planta* function for 4 out of 5 hormones. Whilst as expected, the direction/amplitude by which an effector modulated each hormone marker in protoplasts did not clearly correlate with the direction of hormone responsiveness at the root level (Supplementary Fig. 6), the confirmation of SIEC effects in whole plants indicated the specificity and suitability of the protoplast assay in analysing and detecting hormone functions of SIECs. This is particularly relevant for hormones and their involvement in a multitude of processes and response reactions of plants. In our study we focused on two root phenotypes and additionally anthocyanin content for cytokinin treatment. Thus, we did not examine all possible traits altered by SIEC expression and hormone treatment. Still, we were able to confirm 39% of the SIECs with effects on hormone signalling, functionally validating the dramatic impact of SIECs on the phytohormone signalling network.

S. indica effectors promote growth in Arabidopsis

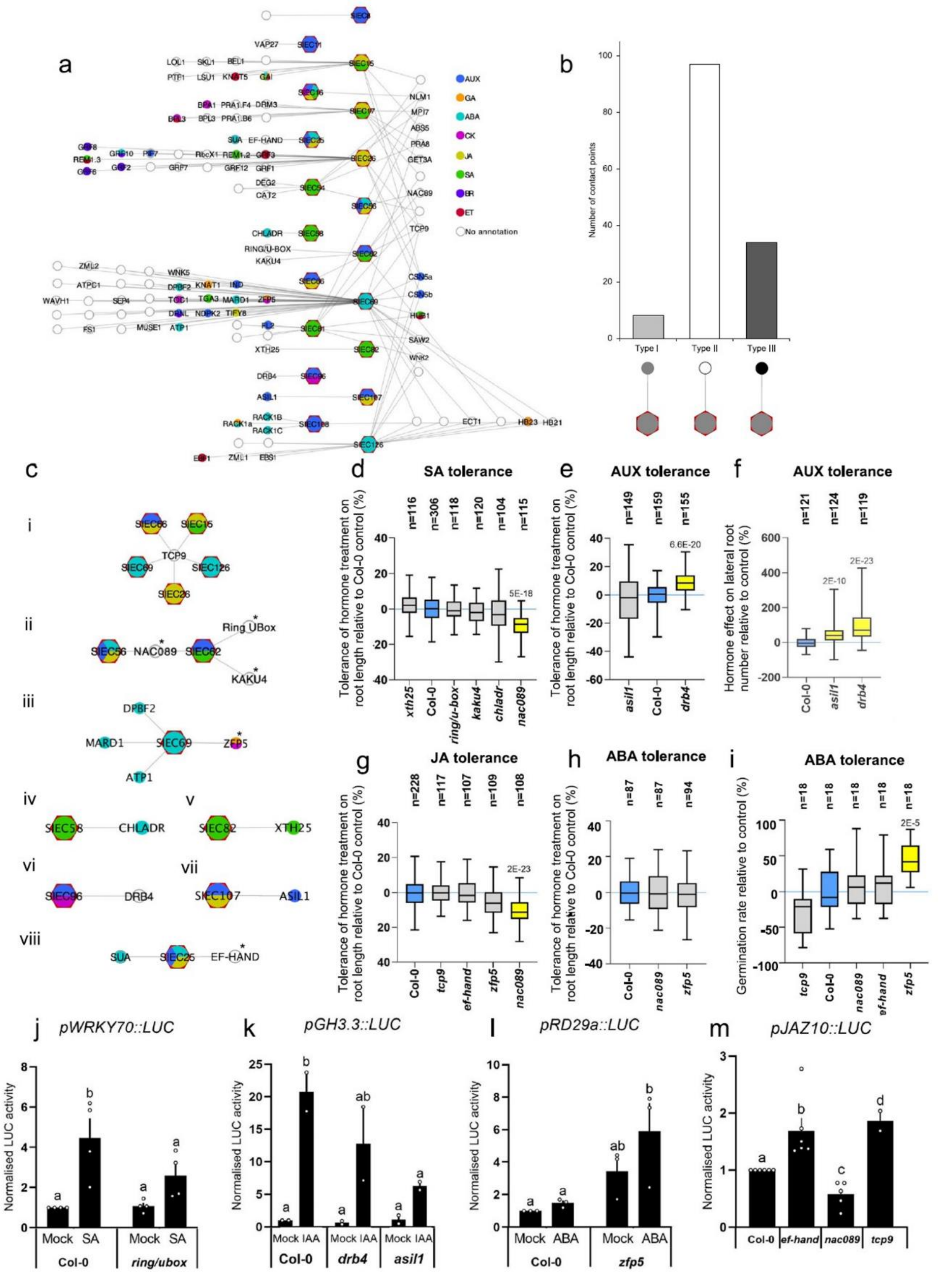
Given that *Si*-mediated benefits involve hormone signalling[19,43–45], we wondered if individual SIECs supported *Si* mediated phenotypes, such as growth promotion in Arabidopsis. We phenotyped 20 *35S::SIEC* lines that previously showed altered hormone activity in protoplasts, using primary root and hypocotyl length as a proxy for growth promotion in below- and above-ground tissue. These included 3 SIECs which modulate the response to the growth hormones AUX and CK, 3 SIECs affecting responses to the abiotic stress hormone ABA, 6 SIECs altering the responses to JA and SA, and 8 SIECs with overlapping effects on multiple hormones. Overall, 16 of the 20 (80%) tested SIECs impacted growth control of Arabidopsis roots, and 9 altered hypocotyl length (Fig. 4l, m). A majority of 14 SIECs promoted root growth, whilst only two SIECs impaired it; 6 promoted hypocotyl length, whilst 3 suppressed it. Intriguingly, not all lines with enhanced root length produced longer hypocotyls, and vice versa, suggesting in some cases highly specific physiological activity of SIECs. Thus, even individual SIECs can confer well-known benefits of *Si* symbiosis on host plants. Considering the tight connection of SIECs to the hormone-signalling network it can be anticipated that many other phenotypes are mediated by individual effectors.

S. indica effector-based informing of the host hormone network

The complex and integrated nature of plant hormone signalling networks challenges mapping of protein function to specific hormone pathways[32]. We have previously demonstrated that physical interactions among differently annotated signalling proteins can reliably inform on novel functions of one or even both partners in the respective other pathway[32]. Based on the SIEC effects on hormone pathways, we thus wondered if SHIPs could be used similarly to identify unknown hormone functions of their Arabidopsis targets. To this end, we combined our SIEC-phytohormone protein network (Fig. 3a) with the SIEC protoplast phytohormone assay data (Fig. 4a–d) to assign functions to Arabidopsis proteins according to their interactions with hormone-modulating SIECs. 99 Arabidopsis proteins and their 19 SIEC interactors fit these criteria and were used to generate the Functionally Informed Symbiont-Plant Interaction Network (FI-SPIN, Fig. 5a). We applied the concept of SHIPs to define the different SIEC-hormone protein interactions. 5.6% and 24.4% of edges within FI-SPIN were classified as SHIPs, where SIEC-inferred hormone assignments of Arabidopsis targets match published Arabidopsis protein functions (type I) or do not match (type III), respectively (Fig. 5b). A majority of 70% of SIEC-Arabidopsis target combinations involved un-annotated Arabidopsis proteins, which we refer to as type II SHIPs (Fig. 5b). To elucidate the accuracy of these SHIP-based hypotheses regarding the Arabidopsis hormone network, we chose ten SIEC-interacting Arabidopsis proteins covering type I-III SHIPs (Fig. 5c, Supplementary Fig. 7). Our selection included ASIL1 and XTH25 for type I SHIPs; EF-HAND, DRB4, KAKU4, NAC089, AT3G29270 /RING/U-BOX, and TCP9 for type II SHIPs; and CHLADR and ZFP5 for type III SHIPs (Fig. 5c). We subsequently phenotyped T-DNA mutants lacking respective SIEC-interacting proteins (Supplementary Fig. 7a–j) for altered hormone tolerance by assessing primary root length and LRN (Supplementary Data 6) (~530 mutant plants/treatment, 2643 plants in total), as well as by quantifying respective *pHORMONE::LUC* activities in mutant protoplast assays (Fig. 5d–m).

Among the type II mutants, *ef-hand* and *tcp9* mutants exhibited wild type phenotypes in JA and ABA assays (Fig. 5g, i and Supplementary Fig. 8a). However, in accordance to altered JA tolerance of EF-HAND and TCP9-interacting SIECs in protoplasts assays (Fig. 5c), protoplasts of both mutants revealed higher JA marker expression (*pJAZ10::LUC*) in the absence of JA treatment, suggesting an elevation of basal JA signalling in both mutants (Fig. 5m). Consistent with the altered AUX responsiveness of protoplasts expressing ASIL1-interacting SIEC107, the AUX marker (*pGH3.3::LUC*) was repressed in *asil1* mutant protoplasts and LRN was enhanced in *asil1* mutants in the presence, but reduced in the absence of AUX (Fig. 5f, k and Supplementary Fig. 8c). In line with the AUX induction by DRB4-interacting SIEC96 (Fig. 5c), *drb4* mutants produced longer roots and more lateral roots under AUX treatment (Fig. 5e, f and Supplementary Fig. 8a, c). This suggests a previously unknown AUX function of DRB4. As detected for interacting SIEC69, *zfp5* mutant protoplasts altered ABA marker expression (*pRD29A::LUC*) upon ABA treatment. In addition, *zfp5* mutant seeds exhibited a higher germination rate in the presence, but not absence of ABA, indicating a previously unknown ABA function of ZFP5 (Fig. 5h, i, l and Supplementary Fig. 8b). *nac089* mutant protoplasts were reduced in JA marker expression, and, consistent with its interactors SIEC56 and SIEC62, mutant plants showed a reduced JA and SA responsiveness (Fig. 5d, g).

Thus, of the ten candidate proteins, mutants for seven (70%) showed altered hormone responsiveness in the root and/or protoplast assays as predicted by our FI-SPIN (Fig. 5a, c). This indicates that indeed SIECs are helpful to identify hitherto unknown functions of Arabidopsis proteins in hormone signalling, and to disentangle highly interconnected pathways. More importantly, these experiments demonstrate the reliability and integrated power of FI-SPIN. Our SIEC ORFeome and the highly validated FI-SPIN network will be a powerful



resource to advance the understanding of specific effectors and of the molecular basis of symbiont-host interactions in general.

Discussion

In co-evolving with plants, microbes have developed highly specific ways of manipulating their hosts for colonisation, nutrient acquisition and even reproduction with a wide range of outcomes for the host. Effectors were first identified in pathogens as secreted proteins that counter plant immune mechanisms. The diversity of effector functions was instrumental both for understanding plant immunity at the molecular level[50,51] and for the development of crops with novel resistance traits[52,53]. However, the understanding of plant interactions with

Fig. 5 | Function-based informing of the *Si*-Arabidopsis interactome. **a** Functionally informed symbiont-protein interaction network (FI-SPIN) showing SIECs which changed at least one hormone pathway in Arabidopsis protoplast (landmark) screen (Fig. 4a–d). Host targets were annotated using AHDv2.0 with additional information from GO terms. Node colours indicate assignment to hormone pathways (top right). **b** Quantification of SIEC-hormone interacting points (SHIPs) in FI-SPIN with type I SHIPs (matching hormone annotation of SIEC and targeted Arabidopsis protein), type II SHIPs (no annotation of SIEC-targeted protein) or type III SHIPs (mismatching hormone annotation of SIEC and targeted Arabidopsis protein). **c** Subnetworks (i-viii) extracted from FI-SPIN (**a**), indicating SIEC-targeted Arabidopsis proteins. T-DNA mutants of these genes were evaluated for phytohormone phenotypes based on their interacting SIEC annotation. Where subnetworks contained multiple Arabidopsis proteins. * Indicates the selected target. Node colours indicate assignment to hormone pathways (top right in **a**). **d–i** Phenotyping of Arabidopsis T-DNA insertion mutants lacking SHIP targets (see **c**) for altered responses to ABA, AUX, JA and SA). Error bars represent min to max from 3 biological replicates. Yellow colour indicates significant differences in hormone sensitivity compared to Col-0 plants (in blue). Statistical significant difference was calculated using two-tailed, unpaired t-test: numbers above plots indicate *p*-value for significantly different comparisons. All box plots indicate minimum to maximum values, the 25th to 75th percentile with lines indicating the median of the data. **j–m** Normalised *pHORMONE::LUC* data in protoplasts of selected Arabidopsis T-DNA mutants relative to Col-0 for *pWRKY70::LUC* (SA), *pGH3.3::LUC* (AUX), *pRD29A::LUC* (ABA) and *pJAZ10::LUC* (JA) with and without hormone treatment. Error bars represent standard errors from at least $n = 2$ biological replicates. Letters indicate significant differences as calculated by ANOVA and Tukey test after normalisation between biological replicates. Data are presented as the mean +/− the SEM.

beneficials and symbionts lags behind that of pathogens, and it is still unclear what exactly determines the outcome of an interaction, e.g. can plants categorically discriminate between pathogenic and beneficial microbes. While some plants possess specific plasmamembrane-localised receptors (such as Nod Factor Receptor 1 (LjNFR1) and LjNFR5 in *Lotus japonicus*) to recognise symbionts and facilitate their accommodation, it is unclear how widespread such specific recognition is among plant species[54] given the diversity of host-beneficial plant-microbe combinations. Moreover, these receptors and the defined set of "common symbiosis genes" required for symbioses in legumes with arbuscular mycorrhizas and N-fixing rhizobia are not required for *S. indica* colonisation[55]. At the same time, all microbial symbionts display microbe-associated molecular patterns that are perceived by pattern-triggered immunity (PTI) receptors. To overcome the effective root immune system, many beneficials, including *S. indica*, employ effector-based strategies analogous to pathogens. In addition to manipulating host immunity at initial interaction stages however, mutualists like *Si* might also employ effectors to elicit effects that are beneficial for their host plants[6,25,56]. The extent to which mutualist and pathogen effectors share host targets and possible functions on one hand, and how symbiont effectors contribute to beneficial effects on the other, are fundamental questions we set out to address.

Si possesses a diverse repertoire of effectors[33,57–59], which we used for systematic interactome network mapping. As the same platform was previously used to map plant targets of pathogen effectors[11,30], this allowed us to analyse shared and specific targets of pathogen and symbiont effectors without distortion by differences due to the search space and screening pipeline. Notably, we found that *Si* effectors converge on some of the same host targets that we previously identified as targets for evolutionary distant pathogens, which have important functions in the infection process. The observation that *Si* effectors converge on some of these shared targets suggests that these host proteins are important, independent of the specific outcome of the microbe-host interaction, and constitute key mediators of plant-microbe interactions. Furthermore, our analysis revealed several convergent *Si* effector targets which did not interact with pathogen effectors. Given that we previously reported a correlation between the likelihood of a colonisation phenotype and the number of intra- and cross-species effectors Arabidopsis proteins interacted with[30], these targets will be highly valuable in elucidating the balance between enhanced host fitness and colonisation of *Si* in the future. In addition to *Si* exclusive targets, we found substantial overlap in targets with different pathogens. This observation was most pronounced with the bacterial root pathogen *Rps* and hemi-biotrophic oomycete *Hpa*. Similar targeting thus appears to be driven by similarities in lifestyle and tropism rather than evolutionary proximity; *Si* and *Rps* share a broad host range and target tissue[60,61]. The relatedness of colonisation strategy (biotrophic followed by cell-death associated growth phase for *Si*), in turn, might determine effector target overlaps with hemi-biotrophic *Hpa*.

Beneficial effects mediated by fungi or rhizobacteria often involve changes in plant hormone signalling[62] and previous studies have linked the *Si*-Arabidopsis symbiosis and beneficial effects in growth and defence to modulated hormone signalling[18,24,58,61,63]. It was therefore noteworthy that *Si* effector-targeted proteins are enriched for unique hormone signalling proteins, considerably more when compared to pathogen effector targets, suggesting a broad and deep modulation of the pleiotropic plant hormone signalling network by the symbiont. Our functional screens confirmed this tight connection as 86 out of 106 tested SIECs changed growth and defence hormone marker activities in protoplast assays, many of which were confirmed *in planta*. Whether these changes to hormone marker expression are linked directly to effector function, or are the result of pleiotropy in the plant hormone network, this clearly supports previous observations that irrespective of lifestyle, microbes utilise effectors to target hormone pathways, including those associated with growth and development (BR, CK, AUX), as well as defence (SA, JA, ET)[64–66]. Our analysis suggests this might be particularly important for the balancing of symbiosis in host-microbe interactions, where in addition to the suppression of host defences the activation of beneficial host effects requires manipulation of the hormone network. Given the diversity of SIEC-targeted pathways and the multifunctionality of phytohormone signalling, this intense targeting may be exploitable for biotechnological applications.

However, we also noticed that in many cases the experimentally observed hormone signalling changes could not be directly linked to the annotated hormone functions of SIEC-targeted proteins. We recently demonstrated that such mismatches often result from the pleiotropy of the hormone-signalling network and incompletely characterised protein functions, and that physical interactions reliably identify new protein functions in this network[32]. Thus, to identify indirect links and facilitate hypothesis development about previously unrecognised hormone-related functions of Arabidopsis proteins, we integrated the systematic $\text{AI-1}_{\text{MAIN}}$ host-interactome with our SIEC-interactome and the protoplast data. The analyses demonstrate the involvement of ER stress-associated NAC089[67] and its interactors SIEC56 and SIEC62 in JA and SA signalling, and an AUX-signalling function of SIEC96 and its target DRB4, a protein so far only known for its role in antiviral defence responses via RNA silencing[68]. Overall, a previously undescribed function in specific hormone signalling pathways could be shown for 7 of the 10 tested SIEC-target mutants, which not only supports our interpretation, but further demonstrates the immense potential of our resource to serve as a reliable basis for advanced hypotheses.

Our study thus provides a deeper insight into *Si* connection to the hormone network, helps assign new hormone-related activities to Arabidopsis proteins, and indicates the usefulness of effectors in decoding highly interactive protein networks such as the hormone system. It further advanced our understanding of the molecular mechanism of *Si*-mediated plant fitness. Many symbiotic plant microbes take part in nutrient exchange at the fungal-plant interface by providing the host with additional nutrients such as phosphate in exchange for carbon[69–71]. Although some evidence suggests that *Si* behaves in a similar fashion[72–74], systemic benefits associated with the fungus could not be explained simply by an improved nutrient availability. Our findings that SIECs can promote root length demonstrate the potency of SIECs as keys to uncover fundamental processes in plants. Just as pathogen effectors have helped in deepening our understanding of defence signalling in plants, effectors from beneficial organisms, such as *Si*, can help us in identifying beneficial plant traits, and in applying them in the next generation of crops toward an improved fitness under changing environments.

Methods

Experimental model and subject details

All Arabidopsis mutants and transgenics are in the Col-0 accession (wild type, WT). *A. thaliana* genetic materials including *xth25*, *asil1-1*, *nac089* and *kaku4-2* were described previously[75–79], except all *35S::SIEC* lines, which were generated in this study, and the *chladr*, *drb4*, *tcp9*, *AT3G29270* (*Ring/U-Box protein*), *zfp5* and *ef-hand* mutants, which were described in this study. Growth conditions for specific experiments are given below in the Arabidopsis growth and transgenic lines section.

Method details

Arabidopsis growth and generation of *35S::SIEC* lines. Plants were grown in vertical squared petri dishes on sterile ½ strength Murashige & Skoog (MS) medium with 0.7% agar or on *Arabidopsis thaliana* salts (ATS) media[80] [5 mM KNO_3, 2.5 mM KH_2PO_4 buffered with 2.5 mM K_2HPO_4 to pH 5.5, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 70 µM H_3BO_4, 50 µM FeEDTA, 14 µM $MnCl_2$, 10 µM NaCl, 1 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.2 µM $NaMoO_4$, 0.01 µM $CoCl_2$ and 0.45% (w:v) Gelrite® (Duchefa Biochemie, Netherlands)], in short day conditions with an 10–12 h light (60–120 µmol m^{-2} s^{-1}), at 22 °C in a growth cabinet 8 h Before genotyping, plants were grown in ökohum® Anzuchterde mixed with Celaflor Careo® granules (1.5 g in 1 l soil) in a 10 h light (100 µmol m^{-2} s^{-1}), 14 h dark cycle at 22 °C. T-DNA insertion mutant lines for putative *Si* effector target genes were acquired from the Nottingham Arabidopsis Stock Centre (NASC): *chladr* (AT3G04000, SALK_068469C), *xth25* (AT5G57550, SALK_204573C), *drb4* (AT3G62800, SAIL813H11), *asil1-1* (AT1G54060, SALK_124095C), *tcp9* (AT2G45680, SALK_201398C), *nac089* (AT5G22290, SALK_201394C), *ring/u-box* (AT3G29270, SALK_082480), *kaku4* (AT4G31430, SALK_076754C), *zfp5* (AT1G10480, SALK_113106C) and *ef-hand* (AT5G28900, SAIL_891_B10). Homozygous integrations of T-DNAs and reduced target gene expression were confirmed by genotyping PCR and qRT-PCR, respectively (see below).

35S::SIEC lines were generated by *Agrobacterium tumefaciens*-mediated transformation of expression constructs into Arabidopsis Col-0 wild type plants. SIEC ORFs (see below) were cloned into the Gateway compatible pEarleyGate201 vector[81] and transformed into *A. tumefaciens* strain GV3101. After Arabidopsis transformation, T1 (in soil) and T2 (on agar plates) plants were selected by 150 and 10 µg ml^{-1} Basta treatment, respectively, and genotyped using PCR. Overexpression of SIECs was confirmed by quantitative real-time PCR (qRT-PCR, see below). Three independent lines were generated for each of the 106 SIEC.

Serendipita indica (*Si*) cultivation and treatment

Si wild-type strain DSM11827 (Leibniz Institute, Braunschweig, Germany) was grown on CM agar [per litre; 20 g glucose, 2.4 g $NaNO_3$, 2 g peptone, 1 g casamino acids, 1 g yeast extract, 600 mg KH_2PO_4, 200 mg $MgSO_4 \cdot 7H_2O$, 200 mg KCl, 6 mg $MnCl_2 \cdot 4H_2O$, 2.65 mg $ZnSO4 \cdot H2O$, 1.5 mg H_3BO_3, 0.75 mg KI, 0.13 mg $CuSO_4 \cdot 5H_2O$, 2.4 ng $Na_2MO_4 \cdot 2H_2O$, 1.5% (w:v) agar] for at least 6–8 weeks in the dark at 25 °C. *Si* spore suspension was prepared by adding H_2O 0.02% (v:v) Tween-20 to mature *Si* plates, and scraping off spores and mycelium using a sterile cell scraper. The material was sonicated for 5 min at 4 °C and filtered through two layers of miracloth. Spores were then centrifuged at 2,200 g and 4 °C for 7 mins, and washed in H_2O 0.02% (v:v) Tween-20 three times. Spores were counted using a Fuchs-Rosenthal haemacytometer and made up to 500,000 spores ml^{-1}. Arabidopsis seedlings were grown in squared petri dishes on ATS media for 9 days, and then treated with 1 ml per plate *Si* spore suspension. Control plants were treated with 1 ml per plate H_2O 0.02% (v:v) Tween-20 (mock). Roots were harvested 3 and 10 days after inoculation and flash frozen in liquid N_2.

In silico identification of SIECs and cloning

Flash frozen mock and *Si* treated Arabidopsis roots were ground in liquid N_2 and total RNA was extracted from two biological replicates using TRIzol (Invitrogen)/chloroform. RNA was purified using the RNeasy Plant Mini Kit (Qiagen), including on column Dnase digestion. Library preparation was performed using the TruSeq RNA sample prep v2 kit (Illumina) and raw reads were generated using Illumina HiSeq (50 million reads per sample).

Raw reads were filtered using FastQC[82] and aligned to the Arabidopsis genome (TAIR10) using Bowtie2[83]. SIECs were identified by aligning reads, which did not align to TAIR10, to the *Si* genome[59]. The 852 SIECs identified were then filtered based on completeness of sequence, upregulation during colonisation[84], presence of a signal peptide (SignalP[26]), lack of transmembrane domains (TMHMM[27]) and presence of functional domains (Pfam, ScanPROSITE). The 106 SIECs that remained after filtering were synthesised without signal peptide in the Gateway compatible vector pENTR221 (Life Technologies). For Arabidopsis differential gene expression analysis, mapped read counting was performed using Htseq-count[85]. Differentially expressed genes (DEGs) were identified using DESeq2 after normalisation using default parameters with a log2 fold change cut off of +/−0.6 and an adjusted *p*-value of <0.05 after Benjamini-Hochberg correction.

GO term enrichment analysis

Gene ontology enrichment analysis was conducted in R studio using the TopGO package[86]. For analysis of proteins identified in protein interaction networks, the reference library of genes is described as Y2H-positive, or genes which showed interaction in AI1-Main[31], PPIN-1[30], PPIN-2[11] and the *Si, Rps and Xcc* interaction networks. For comparative GO enrichment, GO filtering was implemented to increase specificity and reduce redundancy. GOs were removed if >10% of the total number of genes in the Y2H-positive set (280) were annotated, and if the total number of genes in each term represented <5% of the query set. To calculate GO enrichment of DEGs after *Si* inoculation of Arabidopsis roots, all expressed genes in the Arabidopsis reference genome (TAIR10) were used as a reference library. We used the function runTest to calculate both Fisher and Kolmogorov-Smirnov statistics for each term. A *p*-Value cut-off of <0.05 was used to evaluate significant GO term enrichment.

Yeast two-hybrid (Y2H) and DPNR

SIEC-Arabidopsis protein interactions were identified by yeast two-hybrid (Y2H) analysis and mapped as described in Altmann et al., 2020[32], Mukhtar et al., 2011[30] and Wessling et al., 2014[11]. Full details can be found in the supplementary information of these articles. All 106 SIECs were screened against the Arabidopsis ORF library reported in Wessling et al., 2014[11], as well as an additional 500 plant hormone proteins[32]. Network analysis was performed using Cytoscape (v3.9.1) and R studio (v3.1). All network graphs were created using tools in the base Cytoscape program. For intra- and interspecies convergence analysis, degree preserved network rewiring (DPNR) was used. For intraspecies convergence, *N* genes were sampled randomly with replacement from the AI1-MAIN network[31], where *N* was the number of total PPIs between microbial effectors and host proteins observed by Y2H. The total number of host proteins sampled from one simulation were stored and this process was repeated 10,000 times. Significance was calculated by dividing the number of simulations where the calculated number of targets was fewer than the number of targets observed by Y2H. If the simulated number was never lower than the observed number by the simulations, the *p*-Value was set to <0.001. For interspecies convergence, performed between *Si* and the five pathogens, *N* genes were sampled randomly with replacement from the AI1-MAIN network, where *N* was the total number of unique targets to each set of effectors. The number of common interactors between samplings was stored and this simulation was repeated 10,000 times. Significance was calculated by dividing the number of simulations where the calculated number of common interactions was higher than the number observed by Y2H. If the calculated number was never higher than the observed number, the *p*-Value was set to <0.001.

Yeast signal sequence trap (YSST)

To confirm secretion of identified SIECs in vivo, we used the yeast signal sequence trap (YSST) system as described by Krijger et al., 2008[29]. For yeast transformations we used the pSMASH vector, and generated fusions of full-length SIECs containing their native signal peptide with the yeast invertase SUC2. Growth of yeast cultures on media with only sucrose as carbon source indicates SUC2 secretion due to a functional effector signal peptide.

Co-immunoprecipitation

For co-immunoprecipitation assays, SIECs were subcloned into pEarleyGate104[81] and thus fused N-terminally with YFP. Putative Arabidopsis targets of SIECs were N-terminally tagged with a FLAG-tag by subcloning them into pEarleyGate202[81]. Leaves of 4-week-old *Nicotiana benthamiana* plants were co-infiltrated with *A. tumefaciens* (GV3101) containing respective constructs for SIECs and Arabidopsis targets, as well as the p19 silencing repressor[87]. After 3 days, leaf tissues were ground and frozen. Proteins were extracted in extraction buffer [150 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 5% (v:v) glycerol, 0.2% (w:v) polyvinylpyrrolidone, 1% (v:v) IGEPAL® CA630, 10 mM dithiothreitol (DTT), 1% (v:v) Plant Protease Inhibitor Cocktail (Sigma), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and centrifuged at 30,000 g, 4 °C for 25 min. Samples were incubated with GFP-Trap® affinity matrix (gta-10, Chromotek, Alpaca, nanobody) for 3 h. Affinity matrix was washed 5 times using wash buffer [150 mM Tris-HCl pH7.5, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 5% (v:v) glycerol, 10 mM DTT, 0.5% (v:v) Plant Protease Inhibitor Cocktail (Sigma), 0.5 mM PMSF]. Samples were separated using SDS-PAGE and subsequently analysed by Western blot. SIECs were detected using an α-GFP-HRP antibody (sc-9996, Santa Cruz Biotechnology, mouse, monoclonal, 1:10,000), Arabidopsis proteins were detected using α-FLAG ((F3165, Merck, mouse, monoclonal, 1:2000) and α-mouse-HRP (71045, Merck, Goat, polyclonal, 1:10,000) antibodies.

Arabidopsis genotyping

For genotyping of Arabidopsis mutants, one leaf per individual plant was flash frozen in liquid N_2 and ground to a fine powder with metal beads using a TissueLyser (Qiagen). Leaf material was incubated under constant mixing for 10 min in 500 µl of DNA extraction buffer [200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% (v/v) SDS], and then centrifuged for 10 min at 13,000 g. The supernatant was transferred into 500 µl chloroform and mixed for 5 min. After centrifugation for 10 min at 13,000 g, the supernatant was transferred into 500 µl isopropanol and let to precipitate for at least 2 h at −20 °C. After centrifugation for 10 min at 13,000 g, the supernatant was removed, and the pellet washed with 70% ethanol. After centrifugation, the supernatant was removed, the pellet dried completely and dissolved in H_2O. 100 ng of genomic DNA served as template in PCR reactions with a standard *Taq* polymerase in a thermo cycler using a standard PCR program. Primers are listed in Supplementary Data 7.

Gene expression analysis by quantitative real time-PCR

Whole seedlings were flash frozen and ground to a fine powder. Total RNA was extracted using TRIzol® reagent (Invitrogen). 2 µg RNA were digested with DNAse I (Thermo Fisher Scientific) in the presence of RiboLock Rnase Inhibitor (Thermo Fisher Scientific) to remove genomic DNA. cDNA synthesis was performed using the qScript™ cDNA Synthesis Kit (Quantabio) according to the manufacturer's instructions. qRT-PCR was performed using the SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma) following a standard protocol. The $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods[88] were used to determine absolute and relative gene expression, respectively. Primers are listed in Supplementary Data 7.

Protoplast screening

Protoplast screening was conducted as described in Lehmann et al., 2020[49]. Arabidopsis mesophyll protoplasts were generated from the leaves of 4-5-week-old Col-0 plants. 3-4 leaves from 24 plants were sliced into 1 mm strips and incubated in 3 ×6 ml enzyme solution [20 mM MES (ph 5.7), 400 mM mannitol, 20 mM KCl, 1.5% (w:v) cellulase R10 (Melford Laboratories Ltd., C8001), 0.4% (w:v) macerozyme R10 (Melford Laboratories Ltd., M8002), 10 mM $CaCl_2$, 0.1% BSA] for 2.5–3 h at 25 °C with gentle shaking. Protoplast suspensions were filtered through a 70 µm nylon cell strainer and spun for 2 min at 100 g at 4 °C. Protoplasts were resuspended in W5 buffer [2 mM MES (pH 5.7), 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl] and spun again under the same conditions. Pellets were resuspended in MMG buffer [4 mM MES (pH 5.7), 400 mM mannitol, 15 mM $MgCl_2$]. Protoplasts were transformed in 96 well microtiter plates or manually in tubes using 3 µg of DNA per 10,000 protoplasts; 1 µg *35S::SIEC*, 1 µg *pHORMONE::LUC* (ABA marker: *pRD29a::LUC*, AUX marker: *pGH3.3::LUC*, JA marker: *pJAZ10::LUC*, SA marker: *pWRKY70::LUC*, CK marker: *pARR6::LUC*) and 1 µg *pUB-Q10::GUS* as internal control for transformation efficiency. Plates were incubated in a growth chamber overnight (22°C, 12 h light period). Following overnight incubation, 100 µl of supernatant were removed from each well before cells were treated with mock (0.05% EtOH, 0.05% DMSO or water), 50 µM MeJA, 30 µM SA, 500 nM NAA (AUX), 10 µM ABA or 20 µM t-zea (CK). Plates were mixed by gentle shaking for 1 min and incubated for 3–5 h in a growth chamber. For quantification of LUC activity, 20 µl of luciferase substrate [1 mM beetle luciferin, 3 mM ATP, 15 mM $MgSO_4$, 30 mM HEPES (pH 7.8)] were added to white 96-well plates (NUNC U96, Greiner) before treated protoplasts were transferred using cut tips. Plates were incubated in the dark for 15–30 min and then imaged using a Photek camera system or Tecan plate reader Infinite® M Plex. Photon integration was performed for 15 min − 1 h with the Photek camera or 1000 ms measured over a period of 30 min with the Tecan plate reader. After quantification of LUC activity, 90 µl of supernatant were removed from each well. 100 µl of lysis solution [25 mM Tris/H_3PO_4 (pH 7.8), 2 mM DACTAA, 2 mM DTT, 10% (v:v) glycerol, 1% (v:v) Triton X-100] were added at room temperature. Plates were shaken at 1,000 rpm for 5 min and spun at 1,000 g for 2 min. 10 µl cell lysate was mixed with 100 µl GUS substrate [10 mM Tris-HCl (pH 8), 1 mM 4-methylumbelliferyl-beta-D-glucuronide (MUG), 2 mM $MgCl_2$] in a black flat bottom 96 well plate and incubated at 37 °C for 1–1.5 h. GUS activity was measured by fluorescence excitation at 360 nm and detection at 465 nm. For analysis, LUC values were normalised using the GUS activity fluorescence value as a control for transformation efficiency. Wells with a GUS activity <50% of the highest 10% GUS fluorescence value on the same plate were omitted. Two technical replicates for each SIEC-marker combination were used to evaluate mock and basal LUC activity, respectively. Integration images were quantified using Image32 (Photek). To select effector candidates that trigger robust changes in phytohormone signalling, we calculated a ranking factor by multiplying the effector/empty vector ratios for mock and hormone-treated samples. This preprocessing prioritises effectors which have a strong effect on the marker, particularly those candidates that change both basal and induced expression of the marker in the same manner (either induction or suppression). Following a log2 conversion, the absolute values were ranked from largest to smallest and the Top 10 effectors in each hormone pathway were used for repetitions of protoplast transfections to confirm the observed changes on the respective markers. For evaluation of *pHORMONE::LUC* activity in T-DNA insertion mutants, GUS-normalised LUC values were normalised to the Col-0 mock value to reduce variation between biological replicates. Data were analysed by two-way ANOVA followed by a Tukey test.

Hormone tolerance assays

Arabidopsis *35S::SIEC* lines were phenotyped for growth promotion and hormone tolerance by comparing their mock vs treated % root reduction to that of control plants (*35S::GFP*). T-DNA insertion lines of SIEC targets were phenotyped by comparing them to Col-0. Seeds were surface sterilised and plants were grown for seven days on ½ MS media after stratification in the dark at 4 °C for 48 h. SIEC-expressing seedlings were selected with 10 μg ml^{-1} BASTA. Seedlings were then transferred to mock plates (½ MS without hormone) or ½ MS plates supplemented with a respective hormone (10 μM ABA, 40 nM IAA, 200 nM BA, 10 μM SA, or 0.5 μM MeJA). After seven days, photos of plates were taken and root lengths and lateral root numbers were measured using ImageJ. Hormone tolerance was quantified by calculating the relative change in root length/LRN between *35S::SIEC* lines grown on treatment vs mock plates. These values were then compared to the relative change in *35S::GFP* treated in the same way, by dividing the *35S::SIEC* % change by the mean relative % response of *35S::GFP* control lines, to calculate the percentage difference in responsiveness. Tolerance was then calculated by subtracting 100, where negative values indicate reduced tolerance, and positive values indicate increased tolerance. For anthocyanin quantification[89], seeds were surface sterilised, stratified for 48 h, and SIEC-expressing seedlings selected on BASTA containing ½ MS media after 7 days in short day conditions. 14 GFP and SIEC expressing plants per plate were then transferred to media containing 0 nm, 200 nM, 500 nM, 1 μM and 10 μM CK and kept in continuous light. After 7 days, seedlings were harvested in liquid N_2. After grinding, 5 volumes of anthocyanin extraction buffer (45% methanol, 5% acetic acid) per mg fresh weight were added. Samples were centrifuged at 12,000 g for 5 min and the supernatant was transferred to a new tube. This step was repeated, and 200 μl of the final sample transferred into transparent V-bottom 96-well plates. Absorbance was measured at 530 and 637 nm using a Tecan Infinite® M Plex plate reader. Relative anthocyanin content was calculated by [Abs530 − (0.25 × Abs657)] × 5. The experiment was repeated 3 times using 2 technical repeats each time.

ABA tolerance was determined by evaluating seed germination following ABA treatment. Seeds were surface sterilised and plated on ½ MS media (mock) and ½ MS media containing 0.1 μM ABA in 6 rows with about 20 seeds per row. After stratification for 48 h at 4 °C in the dark, the plates were transferred into short day conditions for 2 days. Germinated seeds per row were counted, and germination rates determined. The experiment was repeated 3 times.

Quantification and statistical analysis

All experiments were completed at least three times except where indicated in the figure legend. In these instances, the experiment would be based on at least two biological replicates. Statistical analyses of the RNAseq data and networks, including the integrated 12k_space cross-kingdom interactome map and FI-SPIN are detailed above. For comparative analyses, *Si* interactions identified in the 500 hormone gene search space were omitted to reduce bias. Luciferase activity data from protoplast assays was processed and analysed as described above. Due to large variation of all samples between biological replicates, luciferase activity values were normalised to Col-0 mock. The normalised values from at least two biological replicates were then used for one- or two-way analysis of variance (ANOVA) with Tukey's multiple comparison test, considering genotype, treatment and interaction, where applicable. For phenotyping assays, significance was determined using a two-tailed, unpaired *t*-test on either the absolute root length/lateral root number, or for hormone assays the percentage difference to the mock treated samples. For YSST assays two-tailed, unpaired *t*-test was used on either absolute $OD_{600\ nm}$ or the percentage difference to samples containing *pSMASH* without signal peptide. We used the function runTest to calculate both Fisher and Kolmogorov–Smirnov statistics for each term

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All functional, transcriptional, genetic and interaction data is available in Supplementary Data 1–8. The transcriptional analysis of *Si* colonised Arabidopsis roots and SIEC identification, including DEGs in host tissues at 3 and 10 dai are available in Supplementary Data 1. The interactions of SIECs and Arabidopsis proteins identified through Y2H, as well as published pathogen effector interaction data and the search spaces used here are presented in Supplementary Data 2. All GO enrichment results for the SIEC interactome in the 8k and 12k spaces, exclusive and shared SIEC targets, and DEGs at 10dai are presented in Supplementary Data 3. Significantly enriched GO terms were calculated by Fisher and Kolmogorov-Smirnov tests with, a p value cut-off of 0.05. The classification of SIEC target proteins as exclusive to *Si* or shared with a pathogen effector in the 8k and 12k are presented in Supplementary Data 4. The initial and confirmation screens of SIEC function in protoplasts against *pHORMONE::LUC* constructs are presented in Supplementary Data 5. All physiological data for TDNA and *35 S::SIEC* line performance in phenotyping assays and outcomes of two-sided, unpaired t-tests are presented in Supplementary Data 6. All primers used in this study are listed in Supplementary Data 7. RNA sequencing data is deposited with GEO under accession code GSE222356. Source data for all figures are provided with this research article. Read counts mapped to TAIR10 are available in Supplementary Data 8. Source data are provided with this paper.

Code availability

Scripts for performing DPNR in R are available at https://doi.org/10.5281/zenodo.7749043[90].

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Author contributions

Research concept and design: S.L., P.F.-B. and P.S. SIEC-At interactome mapping, data integration and convergence statistics: R.O., M.A., S.A. and P.F.-B. Immunoprecipitation and yeast secretion assays: R.O. and L.R. Si-At RNAseq analyses: R.E. and C.R.-G. Protoplast assays: L.R., E.K. A.D.-F. and S.L. Generation of SIEC lines and phenotyping: R.O., L.R., S.L., E.K., J.R., Y.Z., E.O., C.S. and W.S. Mutant genotyping and phenotyping: L.R. and E.K. Manuscript writing: R.O., L.R., S.L., P.F.-B. and P.S. Critical manuscript reading and editing: R.E., V.N. and W.S.

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The authors declare no competing interests.

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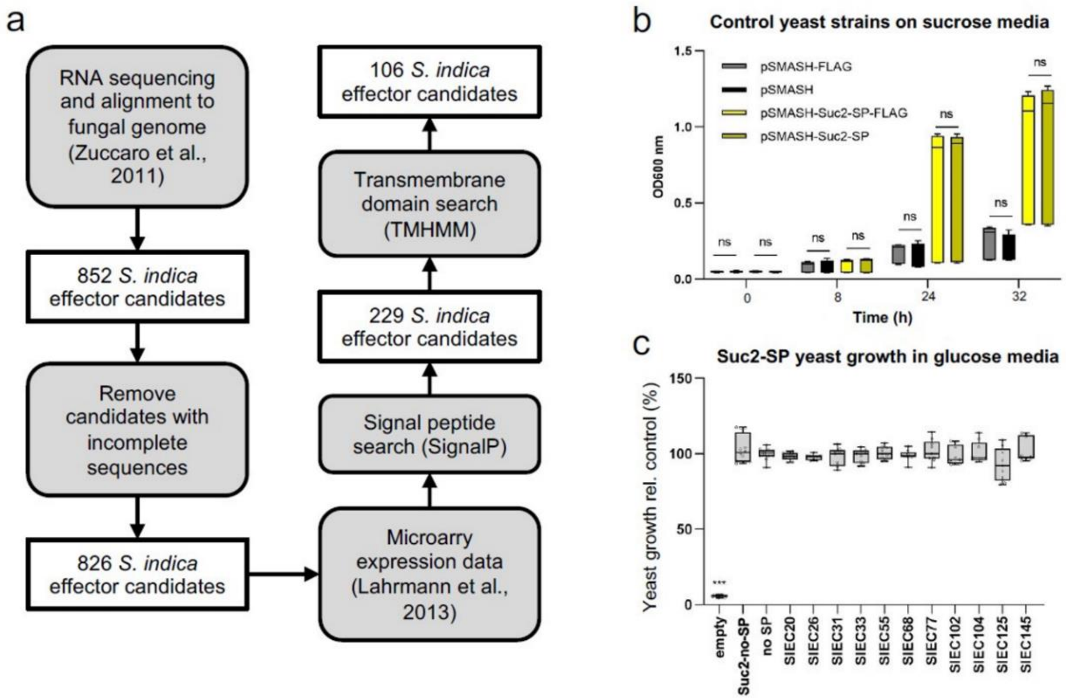
Supplementary Figures

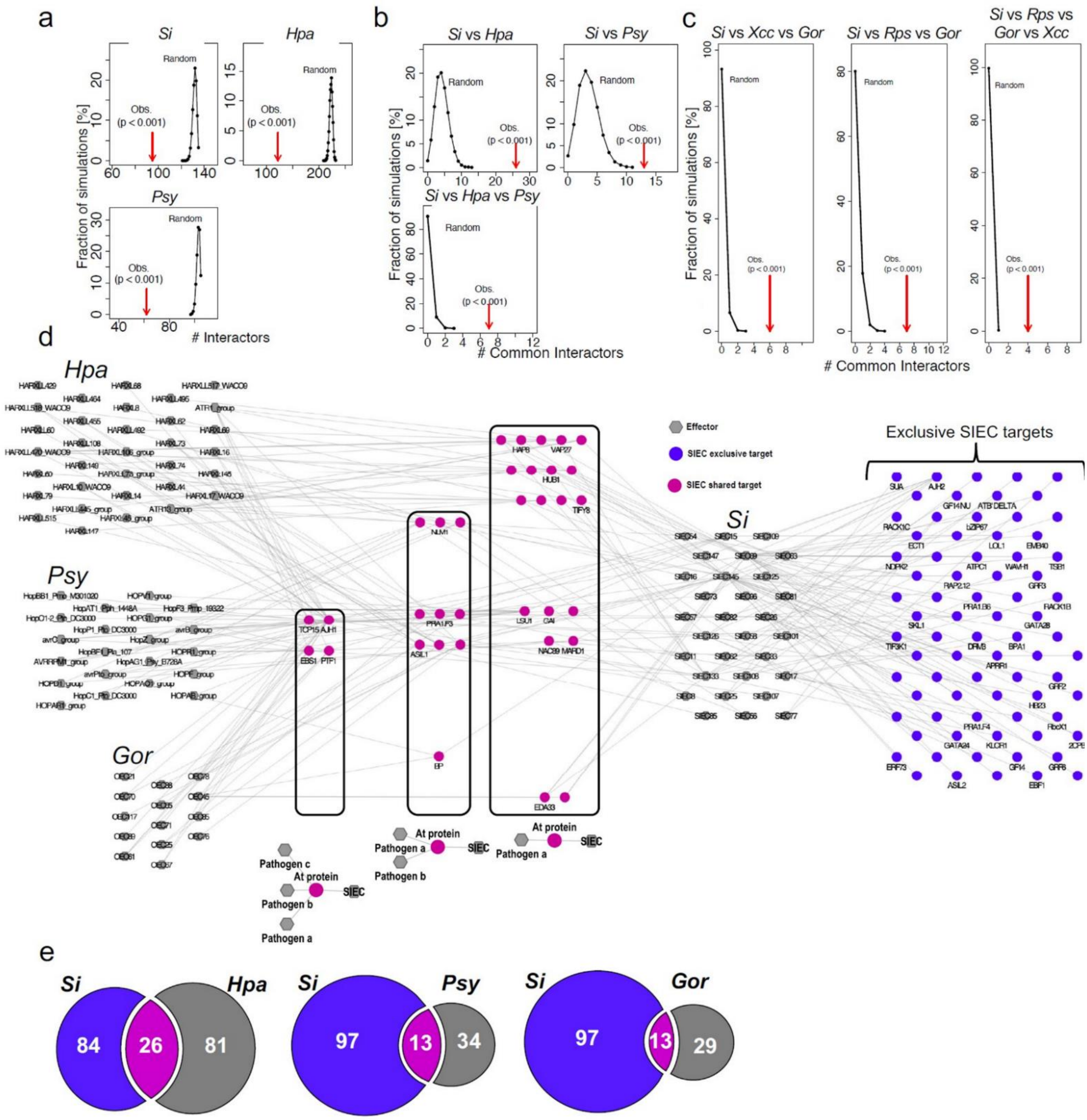


Supplementary Figure 1. In silico identification of SIECs and confirmation of effector secretion. Related to Figure 1.

(a) Bioinformatic pipeline to identify *Si* candidate effector proteins. RNA was harvested from *Si*-colonised Arabidopsis roots 3 and 10 days after inoculation.

(b) Confirmation of yeast signal sequence trap (YSST) specificity on sucrose media. Error bars represent min to max from n=3 biological replicates. Statistical difference was calculated by paired t-test.

(c) Growth of yeast cells after transformation with *pSMASH-SIECs* or control vectors confirms no effect of SIEC expression on yeast growth in non-selective glucose media. Error bars represent min to max of n=3 biological replicates. Statistical differences determined by two-tailed, unpaired t-test. (b-c) All box plots indicate minimum to maximum values, the 25th to 75th percentile with lines indicating the median of the data. *** indicates a p-value of < 0.001



Supplementary Figure 2. Comparative interactomics (8k_space). Related to Figure 2.

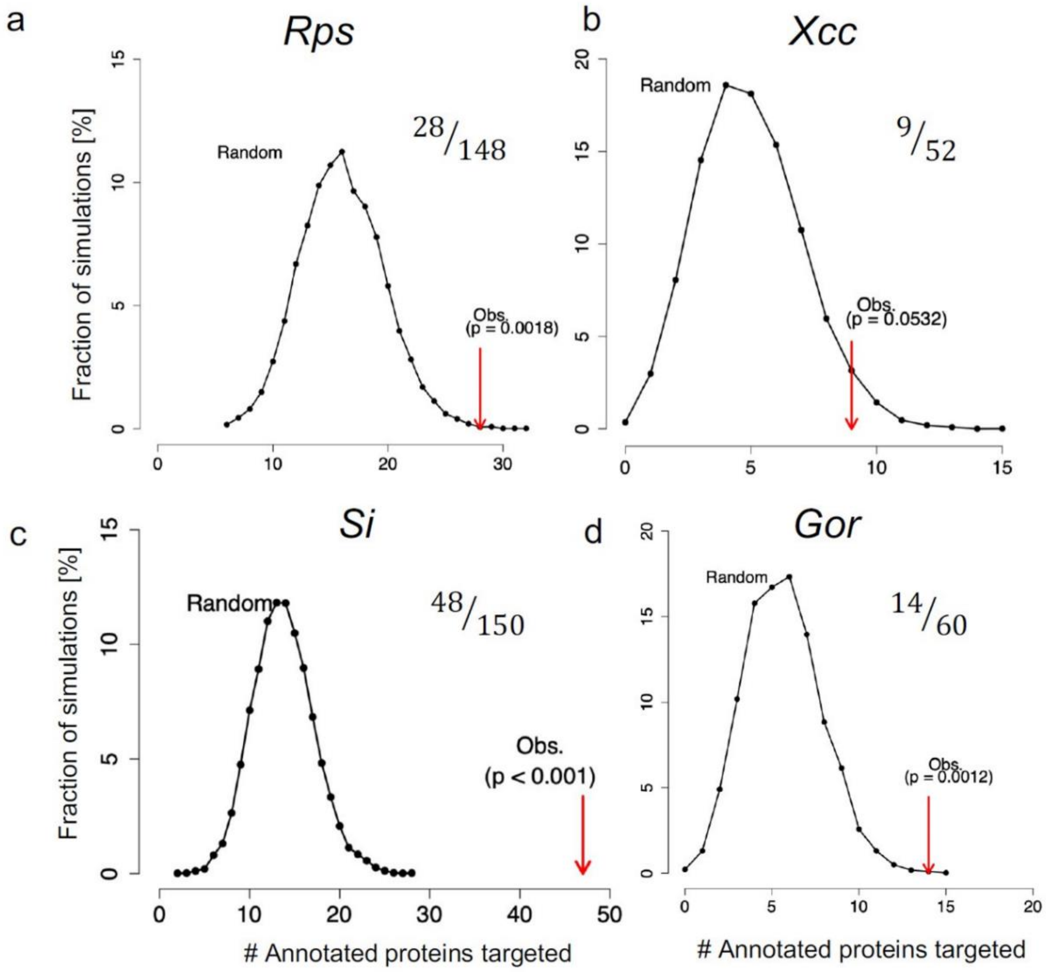
(a) Distribution of the number of simulated interactors of effectors from *Si*, *Hpa*, and *Psy* vs. the observed number.

(b) Distribution of the number of random simulated common interactors between *Si*, *Hpa* and *Psy* vs. the observed number of common interactors.

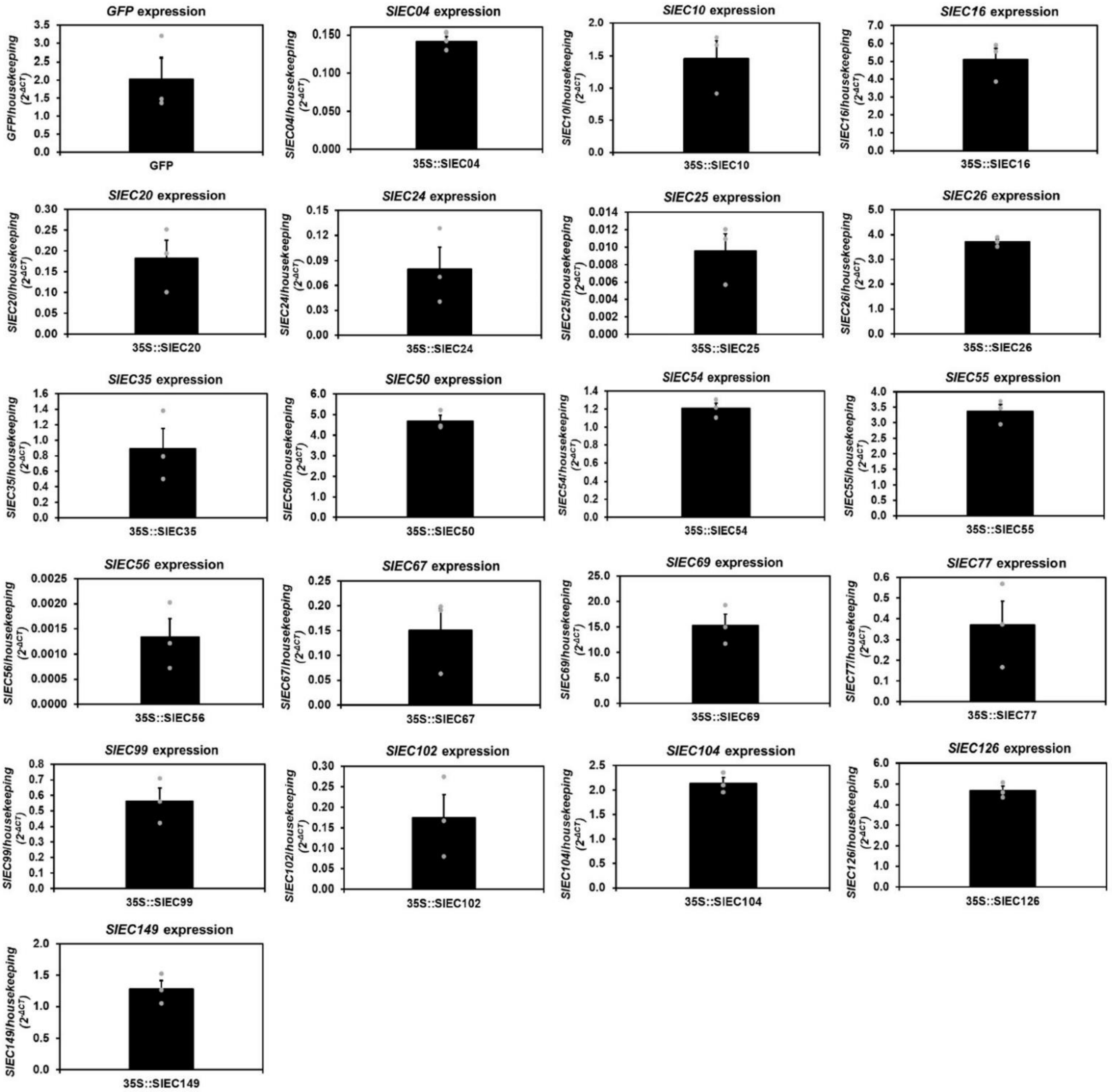
(c) Distribution of the number of random simulated common interactors between *Si*, Xcc, *Gor* and *Rps* in three and four-way convergence analysis.

(d) Classification of SIEC target proteins as either exclusive to *Si* (blue) or shared with at least 1 pathogen effector (magenta) from *Hpa*, *Psy* or *Gor*. Shared nodes are hierarchically displayed according to the number of microbes with interacting effectors.

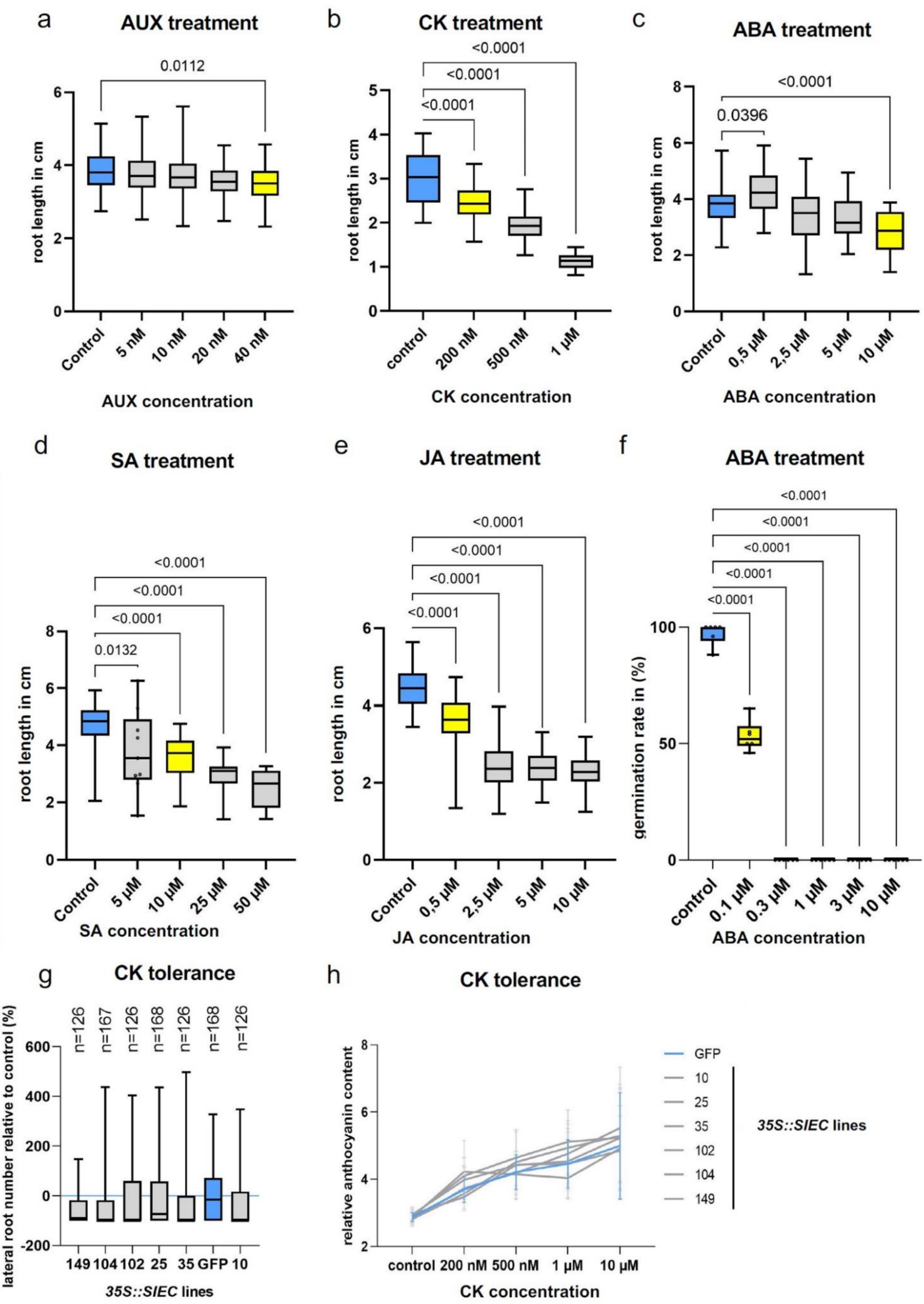
(e) Overlap between SIEC targets and pathogen effector targets from *Hpa*, *Psy* and Gor.



Supplementary Figure 3. Representation of hormone annotated proteins in the 12k_space. Related to Figure 3. Observed (red arrow) vs simulated targeting of hormone annotated proteins for (a) *Rps*, (b) *Xcc*, (c) *Si*, and (d) *Gor.* Numbers (inset) represent the observed (top) # of annotated targets vs the total # of targets (bottom).



Supplementary Figure 4. Confirmation of SlEC expression in *35S::SlEC* lines. Related to Figure 4. qRT-PCR was performed on *35S::SlEC* lines to evaluate expression of respective candidate effectors. Expression of SlECs in *35S::SlEC* lines was calculated relative to housekeeping genes *UBQ10* and *ERF1α* (for primer sequences see Supplemental Table 7). Error bars represent the SEM from 3 technical replicates.



Supplementary Figure 5. Determination of hormone concentrations for hormone sensitivity assays.

Supplementary Figure 5. Determination of hormone concentrations for hormone sensitivity assays. Related to Figure 4.

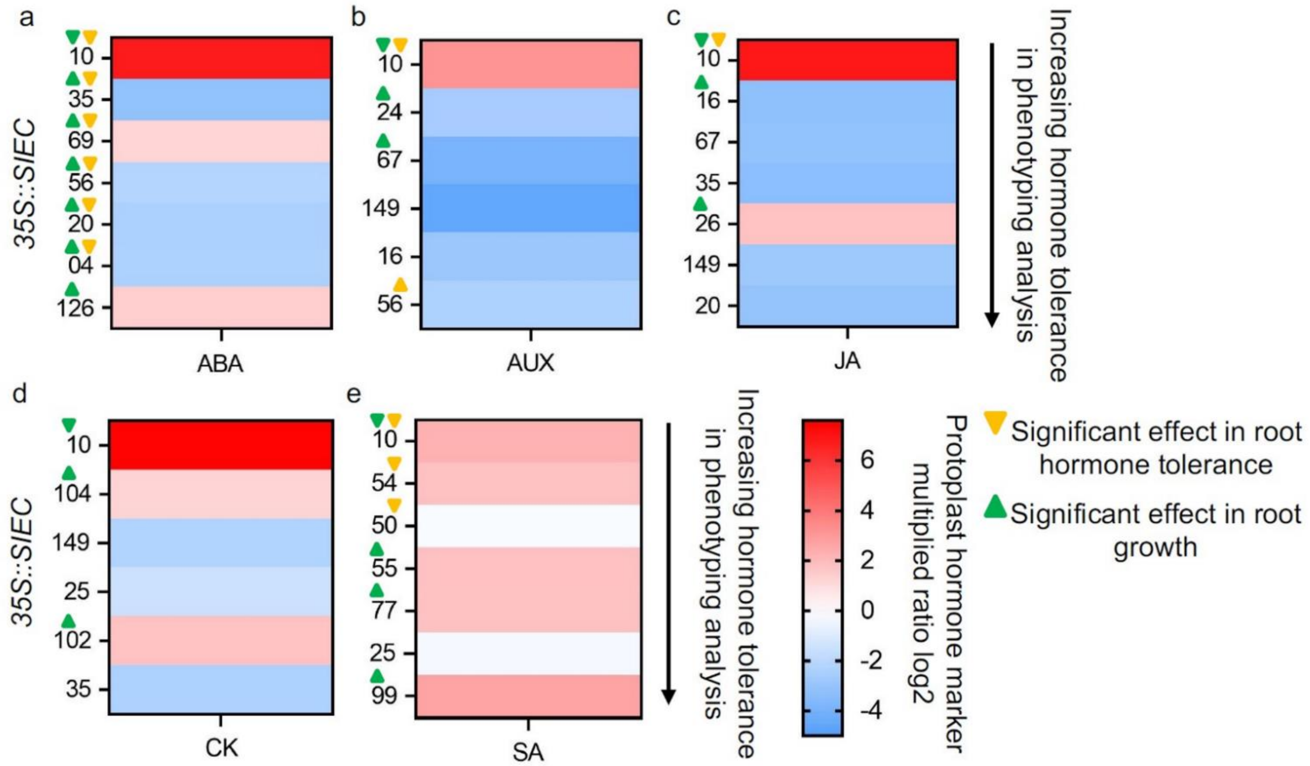
Primary root length of Col-0 seedlings after application of increasing concentrations of (a) AUX, (b) CK, (c) ABA, (d) SA and (e) JA. (f) Determination of seed germination rates (in % relative to untreated Col-0 seeds) after application of increasing concentrations of ABA. Yellow bars indicate the conditions used for plant hormone tolerance phenotyping.

(g) CK tolerance of *35S::SIEC* seedlings relative to *35S::GFP* control plants (blue) as determined by LRN after treatment with 200 nM CK.

(h) Determination of CK sensitivity of *35S::SIEC* seedlings as measured by anthocyanin content relative to fresh weight and chlorophyll content (blue) under continuous light and with increasing concentrations of CK across n=3 biological replicates.

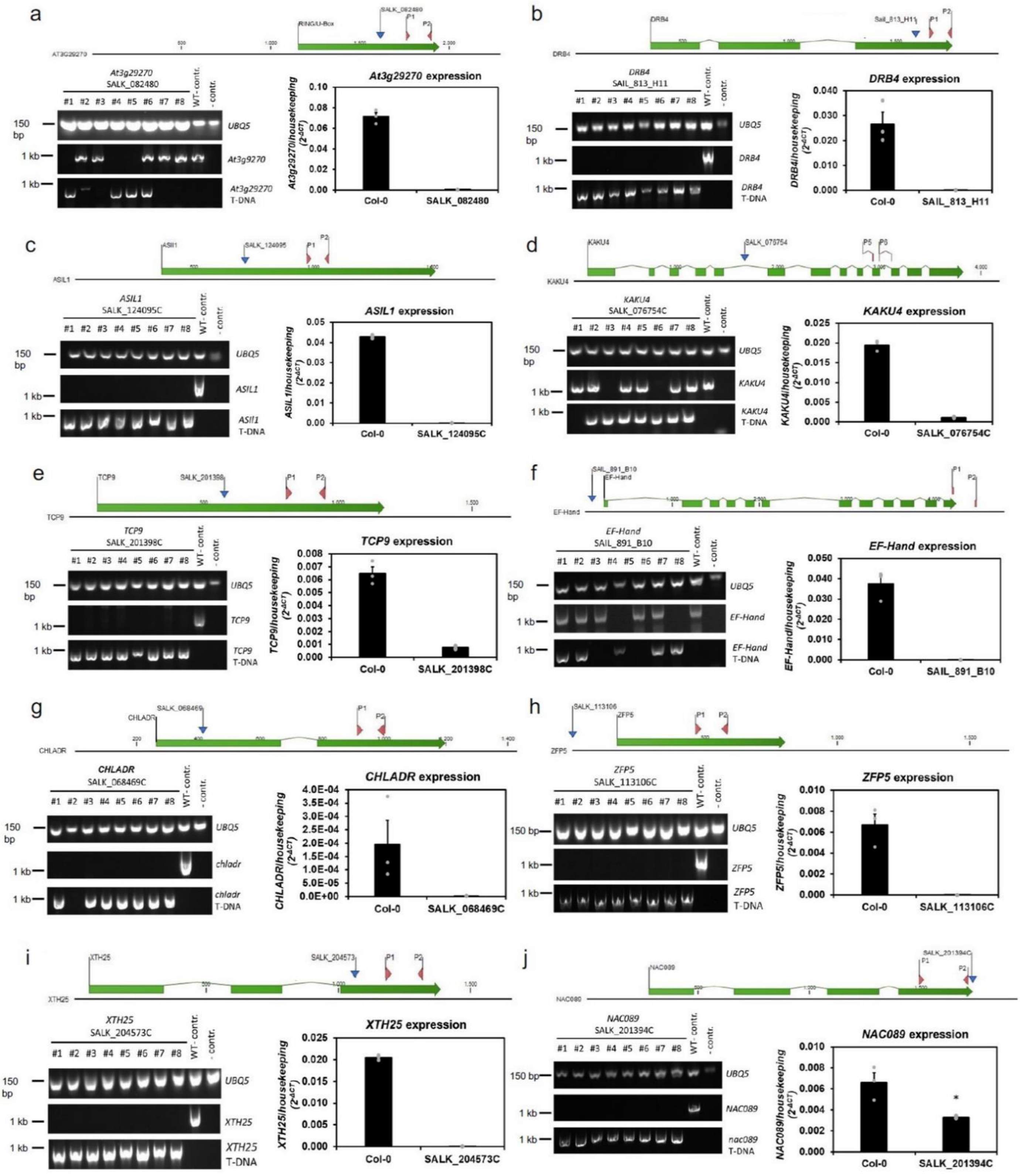
(a-g) All box plots indicate minimum to maximum values, the 25th to 75th percentile with lines indicating the median of the data.

(a-f) Error bars represent max to min values. Statistically significant differences were calculated by ANOVA: or two-tailed, unpaired t-test. Numbers above bars indicate p-values for respective comparisons (g, h) Error bars represent max to min from 3 biological replicates.



Supplementary Figure 6. Summary of multiplied $\log_2$ marker changes for lines which were tested for hormone tolerance phenotypes. Related to Figure 4.

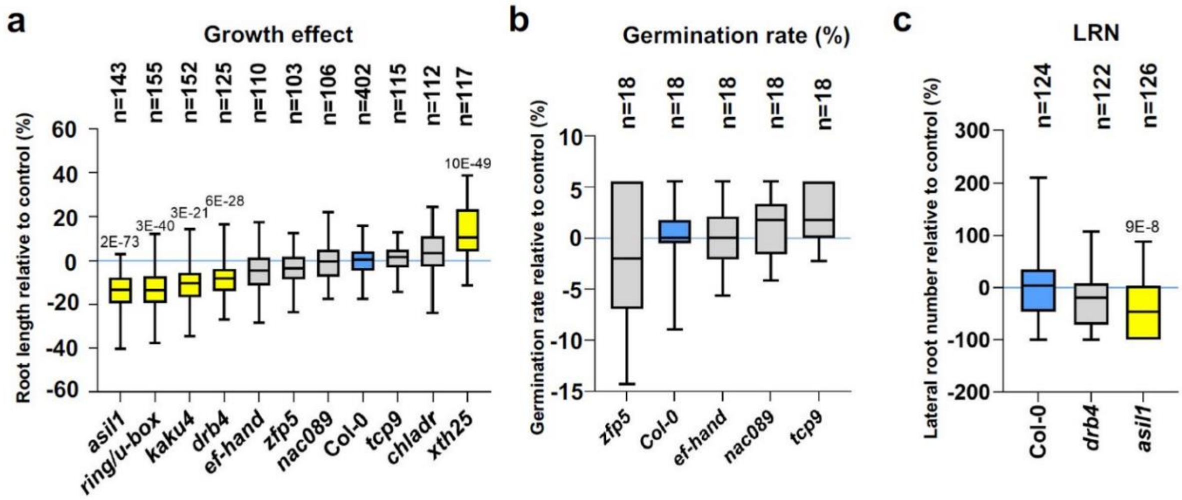
(a-e) Heatmaps showing $\log_2$ corrected multiplied ratios of hormone marker changes in mock and treated conditions, for lines which were tested for hormone tolerance in root growth assays for abscisic acid (a), auxin (b), jasmonic acid (c), cytokinin (d) and salicylic acid (e). Yellow triangles indicate the direction in which lines had statistically different responsiveness to respective hormones in root growth assays. Green triangles indicate the direction of statistically significant root length phenotypes.



Supplementary Figure 7. Characterisation of T-DNA insertion mutant lines of SIEC targets. Related to Figure 5.

Supplementary Figure 7. Characterisation of T-DNA insertion mutant lines of SIEC targets. Related to Figure 5.

(a-j) Individual T-DNA insertion mutants. Each panel shows on top: a schematic of the coding region of the target gene (green), with the position of the T-DNA insertion (blue triangle), and location of primers used in qRT-PCR (red). On the left hand side: the detection of a homozygous T-DNA insertion mutant plant by genotyping PCR, and on the right hand side: the measurement of target gene expression in homozygous T-DNA insertion mutant plants compared to Col-0 wild type as determined by qRT-PCR (for primer sequences see Supplemental Table 7). Data are presented as the mean +/- the SEM from 3 technical replicates.



Supplementary Figure 8. Growth, LRN and germination phenotypes of T-DNA insertion mutant lines of SIEC targets. Related to Figure 5.

(a) Primary root length of T-DNA insertion mutant plants of *Si* targets compared to Col-0 (blue). Yellow colour indicates significant growth differences compared to Col-0 plants.

(b) Rates of seed germination (in % relative to Col-0 plants) of T-DNA insertion mutant lines of *Si* targets.

(c) LRN of T-DNA insertion mutant lines of Si targets relative to Col-0.

(a-c) Yellow colour indicates significant differences compared to Col-0 according to two-tailed, unpaired t-test: numbers above plots indicate p-values for significantly different comparisons. Error bars represent max to min from at least n=3 biological replicates. All box plots indicate minimum to maximum values, the 25th to 75th percentile with lines indicating the median of the data.

Chapter 3 Discussion

This publication provides unique insight into host-signalling reprogramming by symbionts using effector proteins. Novel symbiont effector candidates were detected, and host protein interactions were investigated by yeast-two-hybrid analyses. Comparative interactomes between five different pathogens and the symbiont revealed plant protein targets specific for the *S. indica* symbiosis. The *S. indica* effector-hormone interactome further showed frequent hormone pathway targeting.

Functional effector analyses in protoplasts and in whole plants discovered remarkable data on their involvement in altering host hormone signalling as well as in conferring plant growth promotion. Moreover, effector-informed functional host protein analyses were able to assign previously unknown hormone signalling roles to effector targets. The paper appointed functions to symbiont effectors in increasing host fitness and demonstrated effector analyses as a useful tool for assigning plant protein functions and identifying connection points in complex plant signalling networks.

The publication provides extensive information on symbiont effectors, which on one hand suggests effectors as part of the molecular processes of symbiont conferred host benefits. On the other hand, it shows, that symbiont effector analyses might be used in the future to inform plant networks, as well as crop breeding for more stress resilient plants with improved yield.

CHAPTER 4 – Symbiont effectors modulate plant signalling and increase stress resilience

Chapter 4 introduction

We previously identified symbiont effector interactomes and their highly specific function in hormone signalling networks. Additionally, we revealed that hormone modulating effectors were able to increase plant root and hypocotyl growth. Therefore, previously unknown functions of *S. indica* effector proteins in regulating host benefits during plant-microbe interaction were uncovered.

This chapter aims to further investigate the role of symbiont effectors in increasing host fitness. Effector function in biotic and abiotic stress signalling in protoplasts and their interactome with stress-related plant proteins will be analysed. Whole plant assays of effector-expressing Arabidopsis plants were utilized to uncover effector effects on plant stress resilience. As a result, this chapter will provide first insights into putative molecular mechanisms, protein-protein interactions and signalling pathways contributing to increased host fitness activated by symbionts.

Symbiont effectors modulate plant signalling and increase stress resilience

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Abstract

Symbionts confer beneficial effects to their hosts, which often includes increased growth and biotic and abiotic stress resilience. How these benefits are activated on a molecular level is mostly unknown. We here present effector candidates of the fungal symbiont *Serendipita indica* (*Si*), which modulate plant stress signalling pathways. By mapping the *Si* effector interactome, we reveal frequent targeting of stress related host proteins, which are linked to the identified effector signalling functions. Functional and interaction data suggest molecular mechanisms mediating increased stress tolerance in the *Si*-Arabidopsis symbiosis. Moreover, *Si* effectors increase abiotic stress tolerance of Arabidopsis, as well as resistance to pathogen infection. Analysis of symbiont effectors might not only uncover signalling pathways that increase plant fitness but can identify genetic traits for crop improvement under changing climates.

Introduction

As sessile organisms, plants cannot escape environmental fluctuations and need to adapt to stress events. Theses stresses can be abiotic, for example drought, cold, heat, high light, salts and other harmful soil components (Zhang et al. 2022), or biotic stresses caused by pathogens and pests, which often occur in combination in nature (Suzuki et al. 2014). Together with soil properties, these environmental conditions restrain the use of land for crop production and negatively affect crop productivity (Zhang et al. 2022). During stress conditions extensive hormone signalling is required for plant response, adaptation and defence (Verma et al. 2016; Hou and Tsuda 2022; Ku et al. 2018). Strongly interconnected hormone signalling pathways regulate all aspects of plant life under changing environments (Hou and Tsuda 2022; Emenecker and Strader 2020; Khan et al. 2020; Vega et al. 2019).

Increasing crop stress resistance is interconnected with essential plant processes and particularly to growth-related pathways. Therefore increased stress resistance often leads to growth reduction (Zhang et al. 2022; Zhang et al. 2020). Hormones like abscisic acid (ABA) and auxin which are known to be involved in stress response and development are important factors in this regulation. Auxin was frequently shown to act downstream of ABA to regulate growth processes (Emenecker and Strader 2020). Another process during stress response is reactive oxygen species (ROS) production including superoxide anions, H_2O_2, hydroxyl radicals and singlet oxygen, which is also associated with ABA signalling. However, under osmotic stress ROS accumulation can take place independent of ABA accumulation (Zhang et al. 2022). During abiotic stress plant response and development are controlled by extensive network crosstalk between ROS and hormone signalling pathways. These processes modulate transcriptomic, metabolic and proteomic networks to ensure plant survival (Devireddy et al. 2021). ROS production occurs in response to both biotic and abiotic stress and is sensed by neighbouring cells to activate defence pathways and fast spreading of the signalling in the whole plant. Rapid signal transduction through the whole plant for adaptation to stresses and defence involves Ca^{2+} and ROS signalling but also hydraulic and electric waves (Fichman and Mittler 2020). ROS and salicylic acid (SA) signalling regulate each other during biotic stress to fine tune effective pathogen arrest and immune responses (Lukan and Coll 2022). However, pathogens can use production of hormones, manipulation of hormone signalling pathways and other regulatory mechanisms to affect host responses. For instance, after recognition of the pathogen *Pseudomonas syringae*, SA- and ABA-mediated pathways trigger stomata closure to prevent entry of the bacteria. Virulent *Pseudomonas syringae* transfers the metabolic effector and phytotoxin coronatine (COR) into the host. COR mimics the active form of jasmonic acid (JA) to stimulate stomata re-opening and JA biosynthesis (Ku et al. 2018). *Hyaloperonospora arabidopsidis* uses its effector HaRxL44 to interact with the transcriptional Mediator subunit MED19a, which positively regulates immunity, to degrade the host protein in a proteasome-dependent manner. *P. sojae* represses ethylene (ET) biosynthesis with effector PsAvh238 (He et al. 2020). Yet, hormone modulation during host colonisation is not a unique trait of pathogenic interactions. Arbuscular mycorrhizal fungi (AMF), for example, reportedly alter JA-, SA- and ABA-signalling in hosts for colonisation and maintenance of mutualistic associations. Moreover, Nod factors of rhizobia, that suppress host immunity and induce nodule formation, modulate SA-, CK- and auxin-signalling (Nishad et al. 2020). However, while microbes modify various hormonal pathways for infection and colonisation, beneficial microbes are thought to additionally alter hormone signalling to confer host benefits.

Several beneficial microbes were shown to influence plant hormones for example to increase tolerance to cold or freezing stress (Acuña-Rodríguez et al. 2020). Various processes are manipulated by AMF to protect plants against abiotic stresses including improved phytohormone profile, photosynthesis rate, uptake and accumulation of mineral nutrients and osmoprotectants. Additionally, AMF can up-regulate antioxidant enzyme activity, which can be induced during different stresses (Begum et al. 2019). AMF and rhizobacteria mediated modification of root morphology, plasticity and increase in plant yield is connected to plant CK signalling (Cosme and Wurst 2013). Endophytic fungi increase plant salt stress resistance by inducing systemic resistance, levels of metabolites, antioxidants and modulation of phytohormones. They enhance transcription of the high Affinity Potassium Transporter 1 (HKT1) and the inward-rectifying K^+ channels KAT1 and KAT2, that regulate Na^+ and K^+ homeostasis (Gupta et al. 2021). Additionally, AMF improve plant growth and yield during these stress conditions. By hormone modulation, for instance ABA or stimulation of osmolytes, AMF alter water regulation in plants during drought. Other beneficial effects of AMF include increased resistance to extreme temperatures and toxic metals (Latef et al. 2016).

The symbiont *Serendipita indica* (*Si*) affects stress responses signalling pathways like Ca^{2+} and ROS production in hosts (Baltruschat et al. 2008; Vadassery and Oelmüller 2009). It increases tolerance to both biotic and abiotic stresses, while at the same time stimulating growth to reduce the tolerance-growth trade-off observed in plant stress response (Qiang et al. 2012). The symbiont influences plant ROS levels and modulates hormone pathways to affect both plant stress tolerance and development (Xu et al. 2018; Nath et al. 2016). The molecular nature of these processes is not entirely clear. Microbial effectors, especially *Si* effectors (SIECs) strongly affect hormone signalling by targeting hormone network members in a specific manner (Akum et al. 2015; Osborne et al. 2023; Shen et al. 2018). *Si* effector hormone data and phenotyping of *SIEC* expressing plants revealed beneficial growth effects of hormone modulating SIECs (Osborne et al. 2023), thus uncovering the function of effectors in increased plant growth. Since *Si* is known to improve diverse plant stress responses, the following analyses focus on biotic and abiotic stress response function of SIECs.

More specifically we aim to identify effector proteins of *Si*, which are involved in increased plant stress resilience. Protoplast analyses indicated molecular functions of SIECs in stress signalling pathways and the SIEC interactome revealed frequent targeting of stress regulating host proteins. Moreover, Arabidopsis plants expressing selected effectors had an improved abiotic stress tolerance. Pathogen assays showed increased resistance conferred by symbiont effectors. This study uncovered host proteins and signalling networks altered by SIECs to increased environmental stress resilience and fitness in plants.

Results

SIECs modulate stress and hormone signalling in protoplasts

SIEC functional analysis in protoplasts identified SIEC that affected the expression of four different stress markers for heat stress or immunity (Figure 1A, B, Supplementary Table 1). Two stress markers responsive to heat treatment *(pDREB2a::LUC* and *pHSP81.1::LUC*) and two PTI markers (*pFRK1::LUC* and *pPHI1::LUC*) were used to investigate SIEC function in biotic and abiotic stress responses. 82 out of 106 (77%) tested SIECs in all four pathways showed an effect in at least one. More SIECs induced expression of the heat signalling markers, while for the PTI markers suppressions were observed more often (Figure 1B). SIECs often had function on individual markers, with 69 of 82 (84%) SIECs altering only one or two markers (Figure 1C). This specificity can also be observed regarding the two different treatments. 38 SIECs exclusively modulated heat signalling and 16 affected PTI marker expression, indicating overall 66% (54 of 82 SIECs) were distinctive for either biotic or abiotic stress signalling pathway modulation (Figure 1D). Effector functions were validated and 40% and 60% of SIECs were confirmed to alter one heat and one PTI marker, respectively (Figure 1E, Supplementary Table 2). The results reveal a function of symbiont effector proteins in plant stress response and suggest a role in conferring improved biotic and abiotic stress resilience. However, SIECs affecting PTI might additionally have a role in immunity suppression for colonisation, as *Si* needs to overcome immune response in the course of host colonisation (Jacobs et al. 2011).

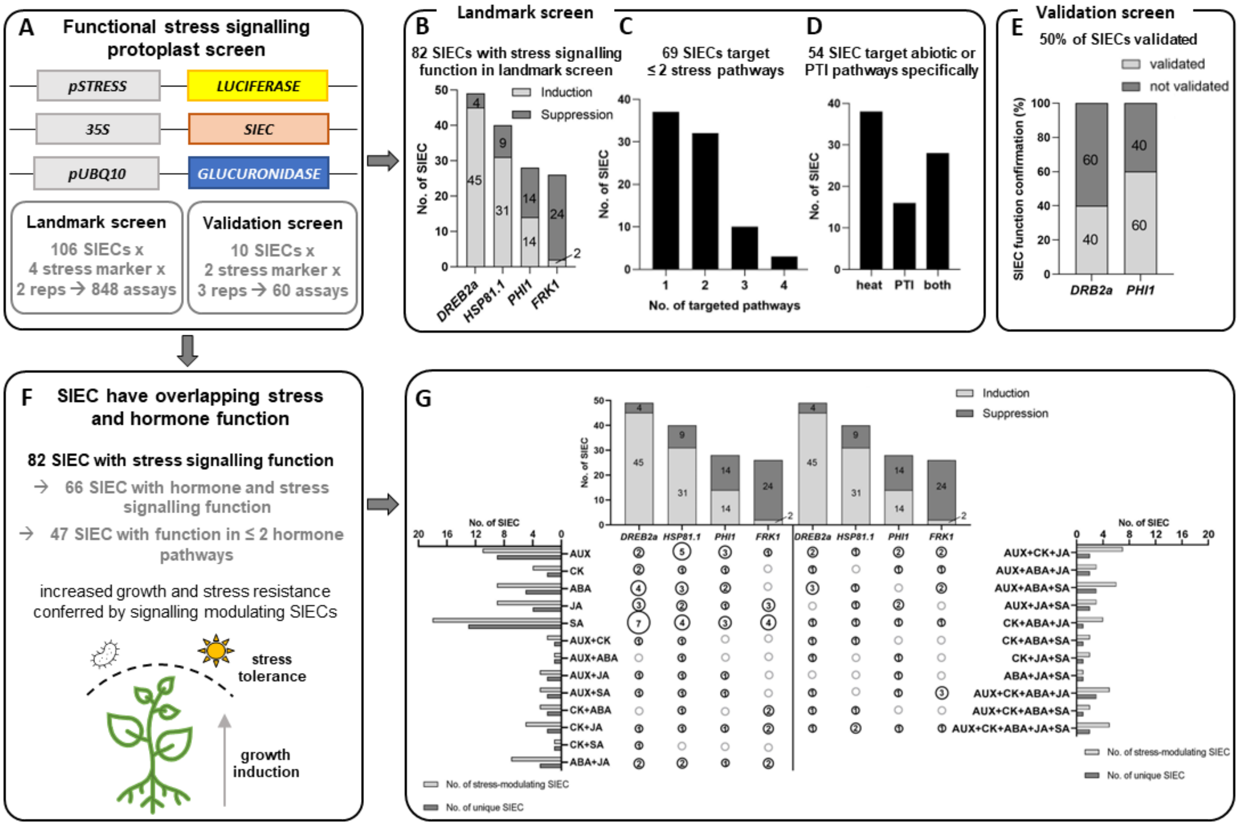


Figure 1. SIECs modulate stress and hormone signalling pathways.
SIEC function on abiotic stress and immunity marker expression in protoplasts. **A** Overview of initial stress marker screen in protoplasts. **B** Number of SIECs inducing or repressing heat stress (*DREB2a* and *HSP81.1*) or PTI marker (*PHI1* and *FRK1*) expression calculated from multiplied ratios of mock and treatment conditions. **C** Number of SIECs modulating one or multiple marker pathways indicating marker-specific SIEC functions. **D** Number of SIEC modulating a specific or both stress signalling pathways indicating pathway-specific SIEC functions. **E** Confirmation of SIEC function in modulating *pDREB2a::LUC* and *pPHI1::LUC* markers, respectively. **F** Summary of SIECs with overlapping stress and hormone signalling functions. **G** Number of SIECs with overlapping hormone and stress functions. Top bar chart shows number of SIECs modulating stress markers. Bar charts on the left and right indicate the number of SIEC additionally altering individual or multiple hormone markers. Number of SIECs affecting different combinations of hormone markers and the individual stress pathways is listed between the different bar charts. AUX – auxin, CK – cytokinin, ABA – abscisic acid, JA – jasmonic acid, SA – salicylic acid.

To elucidate molecular mechanisms, *Si* might use for conferring beneficial effects, SIECs were analysed for an overlapping function in hormone as well as stress signalling pathway modulation (Figure 1F, G). Of 82 SIECs with stress marker modulating function, 66 SIECs (80%) also had hormone signalling effects (Figure 1F, G). Most of them only affected one hormone pathway in addition to modulating stress signalling. This is not surprising, since SIEC functions in hormone signalling pathways were previously shown to be very specific (Osborne et al. 2023).

When analysing stress and hormone function of SIECs in depth, the affected signalling pathways appear interconnected. Interestingly, SIECs that affected PTI markers (*PHI1* and *FRK1*) mostly modulated JA signalling (Supplementary Figure 1A). However, this connection is not always specific.

Often multiple hormones are altered by a similar number of SIECs, for example by SIECs that affected expression of the *DREB2a* marker (Supplementary Figure 1A). SIECs specifically modulating immunity markers similarly affected all hormone markers (Supplementary Figure 1B). SIECs only altering heat response, also changed SA, JA and auxin and to lesser extent ABA and CK regulation (Supplementary Figure 1B).

The analyses did not show a clear correlation between specific hormone and stress signalling functions of SIECs, but rather suggest broader interdependencies with hormone signalling pathways. This might reflect the involvement of all hormones in very diverse aspects of plant stress response (Fahad et al. 2015; Khan et al. 2020; Ku et al. 2018). Therefore, it is also unclear how these symbiont effectors modulate signalling pathways and change phenotypic traits. Interactome analyses might uncover some of the processes SIECs affect in host plants.

Symbiont effectors interact with stress responsive plant proteins

We previously employed yeast-two hybrid analyses to identify SIEC targeted proteins in Arabidopsis and map the effector interactome. It revealed an over-representation of targeted plant proteins involved in hormone signalling (Osborne et al. 2023). Since hormones are known stress regulators, we mapped the GO terms for every SIEC target where 'stress' appeared as a character string and combined this with functional data identified in the *pSTRESS* marker screen (Figure 1 B). 20 of the 33 SIECs targeted stress response related proteins. 55 of the 156 (35%) effector targets were involved in plant stresses (Figure 2 A, B). These SIEC targets have GO-terms associated with biotic stress, mostly regulation of defence response or abiotic stress. For the abiotic stress targets salt stress response was found most frequently. 15 SIECs interacted with 16 host proteins that had functions in plant defence responses including GO-terms for regulation of defence response, defence response to other organism and innate immunity response. 8 SIECs interacted with 13 Arabidopsis proteins involved in abiotic stress response such as salt, heat and osmotic stress and response to water deprivation (Figure 2A, B). Additionally, the 25 targets of 12 SIECs had assigned GO-terms for other stresses (Figure 2A, B, C). All SIECs, which target these proteins (except for SIEC73) modulated stress (Figure 2A) or hormone (Figure 2B) markers in protoplasts. Therefore, SIEC activities might be mediated by their interactions with the identified host proteins.

Interestingly, we observed an overlap between SIEC stress response in protoplasts and assigned target GO-terms. For example, SIEC145 modulated the heat stress marker *pHSP81.1::LUC* (Supplementary Table 1) and interacted with multiple host proteins involved in heat and water deprivation responses. SIEC15 and SIEC17 interact with proteins connected to defence responses. SIEC17 showed immunity marker regulation (Supplementary Table 1), and both altered markers for the biotic defence related hormones SA and JA in protoplasts. Similar observations were made for other SIEC-target combinations (Figure 2A, B).

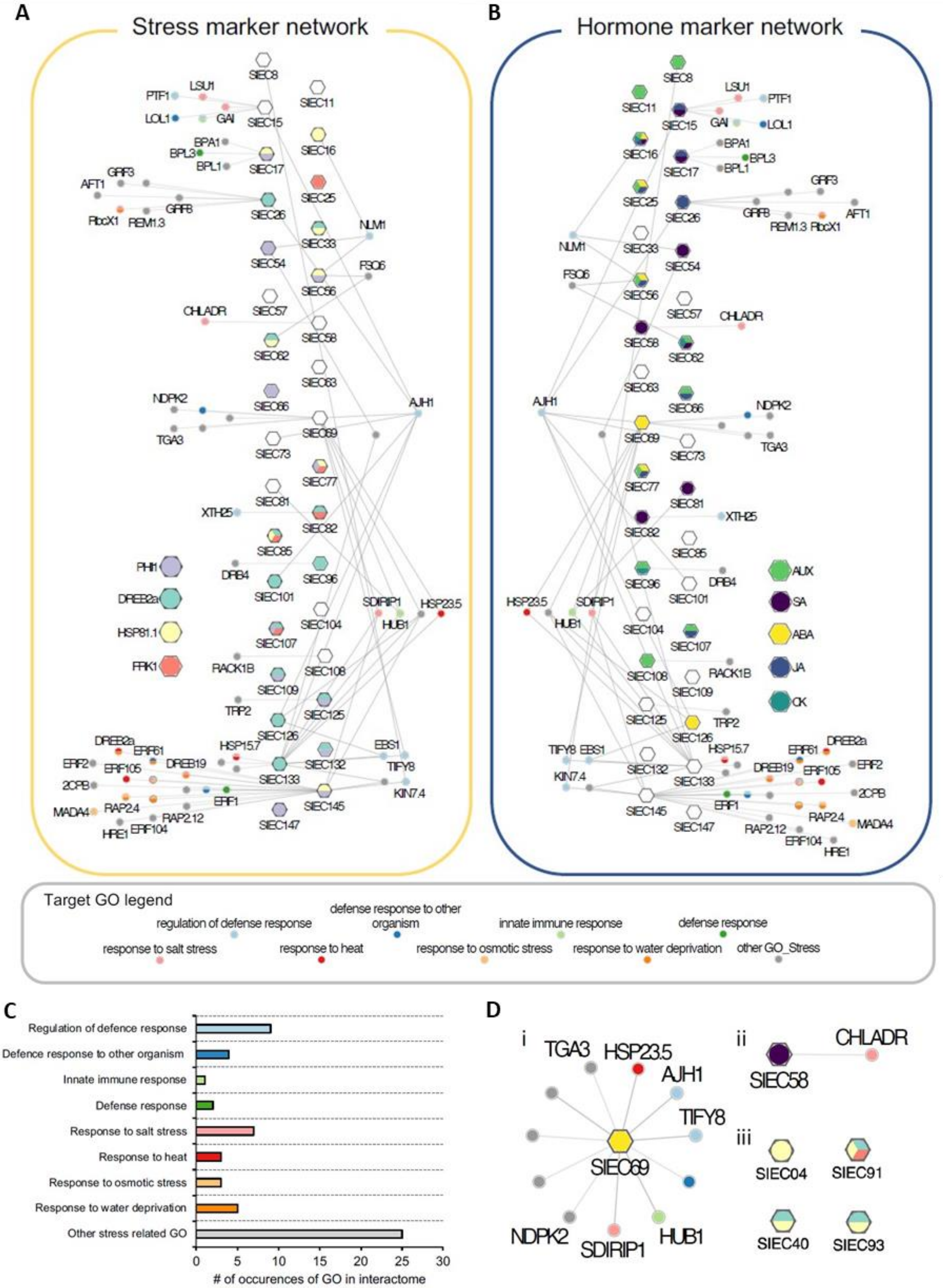


Figure 2. Interactome of SIECs and targeted Arabidopsis proteins with stress response functions.

Figure 2. Interactome of SIECs and targeted Arabidopsis proteins with stress response functions. **A** Interactome between stress marker modulating SIECs and stress responsive host proteins. **B** Interactome showing hormone marker function of SIECs targeting stress response host proteins. GO terms of targeted host protein are indicated below the networks. **C** Stress-related GO-terms occurring in the SIEC interactome. **D** Subnetwork of SIECs used in abiotic stress phenotyping; (i) SIEC with multiple stress targets, (ii) SIEC with one stress related target, (iii) SIEC without known stress related targets

Taken together, the network analysis revealed symbiont effectors interacting with stress related host proteins. The connections between SIEC functions identified in protoplasts (Figure 1B) (Osborne et al. 2023) and known target functions provide first insights in molecular mechanisms of symbiont-conferred stress resilience. To further analyse SIEC functions in plant stress tolerance, 6 SIECs with diverse stress and hormone-modulating functions in the protoplast assays and representing 3 interaction categories were chosen for abiotic stress phenotyping assays (Figure 2D).

SIECs increase Arabidopsis abiotic stress tolerance

To analyse abiotic stresses effects in seedlings, mannitol and NaCl concentrations, at which root trait changes in response to the stress application could be observed, was determined. NaCl in plant growth media induces mild salt stress response in Arabidopsis seedling causing reduction of root growth by restraining metabolic processes (Hongqiao et al. 2021). Mannitol stimulates osmotic stress similar to drought in plants by decreasing the osmotic potential of the media therefore decreasing plant growth (Lipavsk and Vreugdenhil 1996). The two treatments were used to analyse whether *SIEC* expression influenced root responses and could increase plant tolerance to abiotic stresses.

Sensitivity assays using a range of mannitol and NaCl concentration revealed 50 mM mannitol and 100 mM NaCl to reduce root length by 50% (Supplementary Figure 2A, B, Supplementary Table 3). However, 50 mM of NaCl were sufficient to significantly reduce root growth, while lower amounts of NaCl increased root growth (Supplementary Figure 2B), which is consistent with previous reports (Hongqiao et al. 2021). Expression of *SIECs* in respective plants was verified by qRT-PCR using SIEC-specific primers. All *35S::SIEC* seedlings used in the abiotic stress phenotyping assays expressed the expected *SIEC*. The GFP control used in each qRT-PCR displayed no unspecific amplification (Supplementary Figure 3, Supplementary Table 4) confirming primer specificity to identify *SIEC* expression.

Plants with confirmed *SIEC* expression were used to analyse root length under salt stress and mannitol treatment. The decrease in primary root length between mock and stress treatment was calculated for each line and compared relative to the root reduction of *GFP* expressing seedlings. Additionally, lateral root density was employed as an indicator for plant drought stress tolerance (Zhan et al. 2015).

During mannitol stress plants increased lateral root development, therefore the lateral root density increased while their distance decreases. Hence, lateral root number and distance was assessed with and without treatment. All abiotic stress effects are shown in percent relative to the response of the control plants, where positive values point to an increase and negative values a decrease of the analysed root trait.

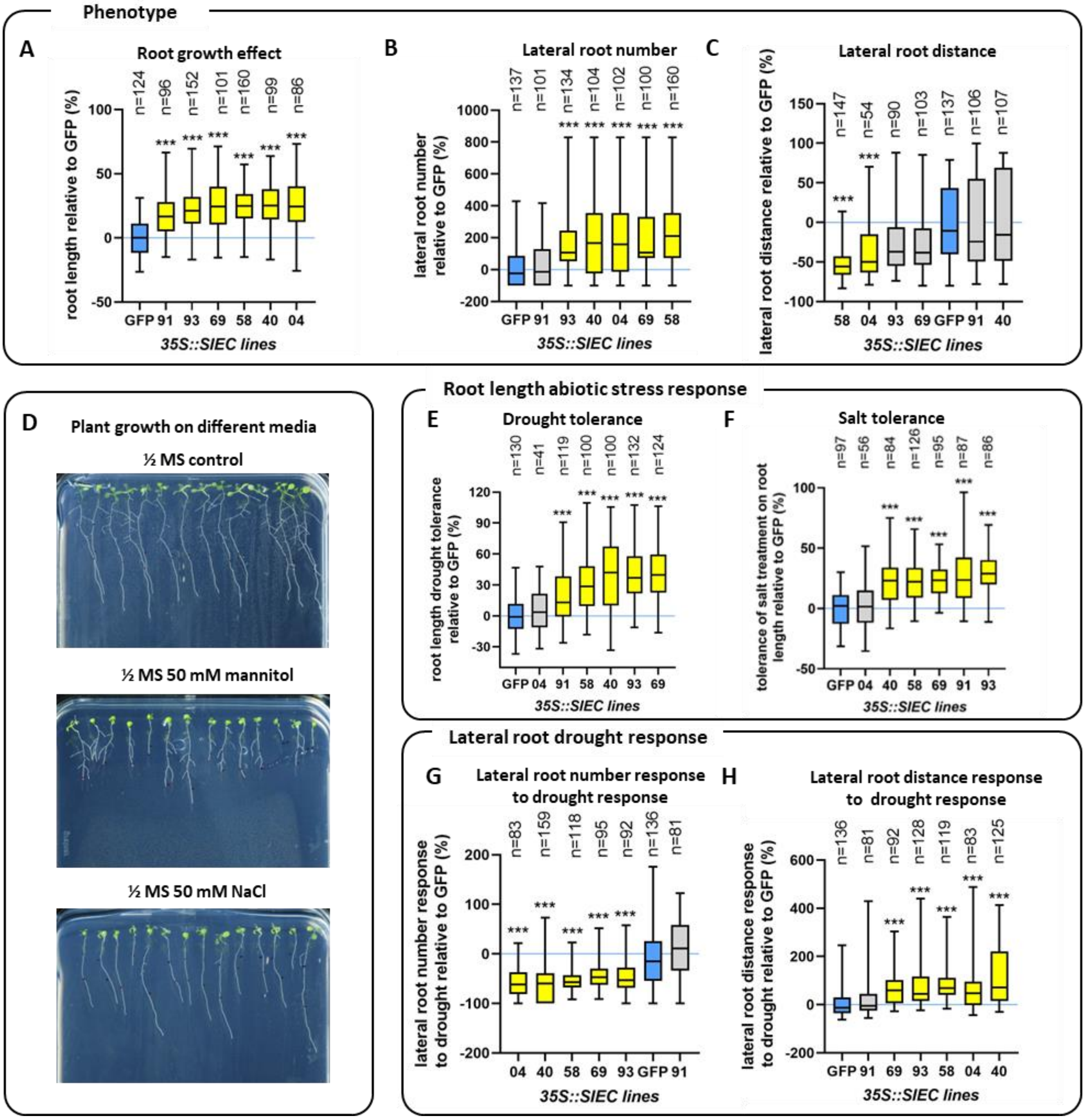


Figure 3. Drought and salt stress phenotyping of *SIEC* expressing seedlings.

Figure 3. Drought and salt stress phenotyping of *SIEC* expressing seedlings.
Primary root length was measured in 14-day old seedlings treated with mannitol, NaCl or mock. Root responses are shown relative to *GFP* expressing plants (%). **A** Effect of *SIEC* expression on root length. **B** Effect of *SIEC* expression on lateral root number. **C** Effect of *SIEC* expression on lateral root distance. **D** Representative pictures of plant growth on different stress treatment media. **E** Effect of mannitol and **F** NaCl on root length of *SIEC* overexpressing seedlings relative to the GFP control plants (%). **G** The number of lateral roots was counted in mannitol and mock-treated plants. **H** Lateral root distances were calculated by dividing lateral root numbers by root length. Both graphs show relative root trait changes in response to mannitol treatment compared to GFP control plants (%). Positive values indicate increases of root traits as compared to GFP plants. GFP expressing controls are shown in blue; significant effects are indicated in yellow. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; statistical significance was calculated using a two-tailed, unpaired t-test of at least three replicates. Sample size (n) is indicated above each box.

All *SIEC* expressing plants developed longer roots than the *GFP* expressing control (Figure 3A). All (except *SIEC91*-expressing lines) had more lateral roots than GFP control plants as well (Figure 3B), but the distance between lateral roots was only reduced in *SIEC04* and *SIEC58*-expressing seedlings (Figure 3C, Supplementary Table 5).

Remarkably, an abundance of SIECs improving root stress responses were identified. Six SIECs were analysed for stress modulation. All significantly altered root trait response to abiotic stresses compared to the control, therefore the SIECs modulated plant phenotypic stress reaction. These findings were consistent with their activities found in protoplast assays (Supplementary Table 1) and in line with the reported stress response function of their interacting targets in Arabidopsis (Figure 2D). For both mannitol and salt treatment all (but SIEC04) SIECs increased the tolerance of primary root growth to the stresses as compared to GFP control plants (Figure 3E, F). When examining lateral roots all, except for SIEC91, showed a reduced number and increased distance between lateral roots after mannitol treatment (Figure 3G, H, Supplementary Table 6). Longer roots and reduced lateral root formation of *SIEC* expressing plants in response to abiotic stress, implies that these seedlings were less affected by the treatments.

Taken together, the analyses revealed a growth promoting effect of SIECs (Figure 3A) that displayed stress response functions in protoplasts or interaction with stress related host proteins (Figure 2D). Effector expression might accelerate root development, as indicated by enhanced lateral root development in 14-day old seedlings (Figure 3B). This could not be observed for SIEC91, which seems to affect root length but not lateral root development (Figure 3A, B). Because of this possible developmental promotion by SIECs, lateral root distance was used to identify specific effects on lateral roots. Only *SIEC58*- and *SIEC48*-expressing plants had a reduced distance/increased density of lateral roots (Figure 3C), uncovering additionally amplified lateral root development independent of the advanced root growth.

Analyses of SIEC signalling function in protoplasts and network analysis of interacting host proteins pointed to effector candidates that increased plant stress resilience. The results partly revealed the molecular nature of increased stress resistance during host-microbe interaction by symbiont effector signalling and host protein modulation. Following these abiotic stress response assays, another phenotyping experiment was conducted to reveal SIEC function in plant immunity and pathogen defence.

Symbiont effectors modulate plant pathogen resistance

Protoplast assays identified SIECs modulating PTI markers (Figure 1B, Supplementary Table 1). In addition, SIECs altered hormone signalling pathways involved in biotic stress response for example SA and JA signalling (Figure 1G) (Osborne et al. 2023). 16 SIECs exclusively modulated flg22 responsive markers, while 28 were involved in both immunity and heat stress signalling (Figure 1D). Furthermore, SIECs interacted with host proteins with known functions in immunity and plant defence response (Figure 2A, B). To explore SIEC functions in plant pathogen defence all available *SIEC* expressing Arabidopsis plants were used. Pathogen assays involved three pathogens, *Phytophthora parasitica*, *Botrytis cinerea* and *Rhizoctonia solani* to investigate pathogen root and leaf colonisation effects in *SIEC* expressing plants.

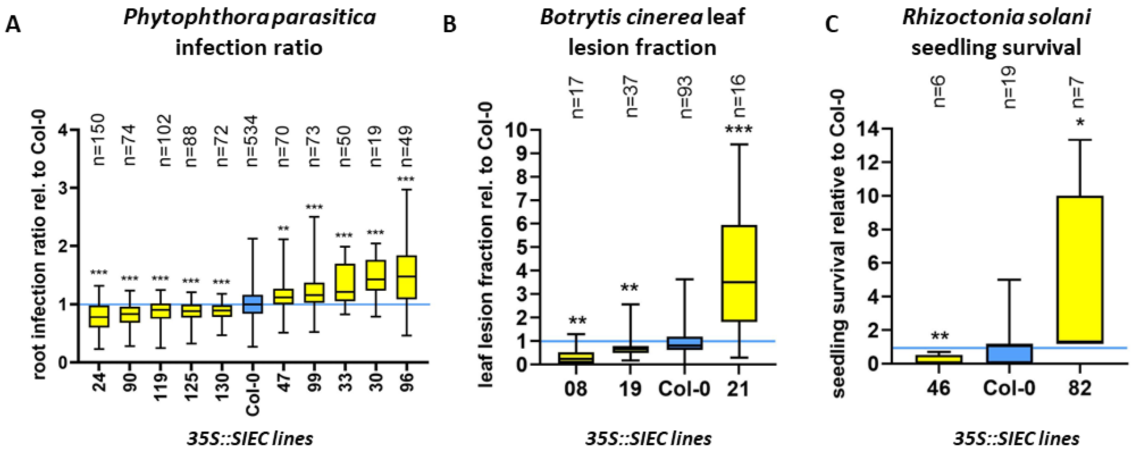


Figure 4. Pathogen infection response of *SIEC* expressing plants.
SIEC expressing plants were analysed after infection with pathogens. Only significant results are shown in this figure relative to Col-0 infection for **A** *Phytophthora parasitica* root infection ratios, **B** *Botrytis cinerea* leaf lesion fraction, and **C** *Rhizoctonia solani* seedling survival after root infection. The Col-0 control is indicated in blue; significant effects are indicated in yellow. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; statistical significance was evaluated by two-tailed, unpaired t-test of al least two biological replicates. Sample size (n) is indicated above each box.

Phenotyping revealed SIEC effects, which both increased and reduced infection after pathogen colonisation of different *SIEC*-expressing plants. Most SIECs (10) affected *Phytophthora parasitica* infection, of which five were reducing root infection and five caused an increase in pathogen colonisation of roots (Figure 4A, Supplementary Table 7). Three and two SIECs were affecting *Botrytis cinerea* and *Rhizoctonia solani* infection respectively. In both pathogen assays one SIEC stimulated stronger disease symptoms, while *B. cinerea* leaf lesions were reduced by SIEC08 and SIEC19 and SIEC82 increased seedling survival following *R. solani* infection (Figure 4B, C, Supplementary Table 8, 9).

Most SIECs affected root colonisation by *P. parasitica*, which might reflect an overlap in infection strategies and that both microbes target root tissue (Jacobs et al. 2011; Meng et al. 2014). Effects on root colonisation were only analysed for this pathogen. SIEC function in plant immunity against leaf colonisation by *Botrytis* could hint to a function of SIECs in induced systemic resistance (ISR), also known to be activated by *Si* (Stein et al. 2008). During the ISR process signalling for increased defence response and resistance is initiated from the root to other plant organelles (Choudhary et al. 2007). Significant effects for *R. solani* infection were probably less often observed, because the sample size was the smallest. Interestingly, SIECs had very specific functions. Each identified SIEC with significant influence on pathogen infection only affected one pathogen, independent of its effect on the infection outcome. The experiment identified 8 SIECs, which increased pathogen tolerance, and 7 that increased pathogen infection (Figure 4A-C). Hence, SIECs might be used by the symbiont to suppress immunity and colonise hosts as proposed before (Akum et al. 2015; Nostadt et al. 2020) as well as to induce resistance against pathogens.

Discussion

Serendipita indica effectors increase plant abiotic stress tolerance to drought and salt stress

Climate change increases abiotic stresses like drought and threatens crop production and the availability of food (Abobatta 2020). During drought various plant processes are affected including hormone signalling, photosynthesis, gene transcription and protein synthesis. Under drought conditions plants need to balance water content and photosynthesis. Therefore, photosynthesis together with gas exchange is decreased. As a result, metabolic processes are reduced which limits crop growth and productivity (Abobatta 2020). Salinity is often observed in arable areas due to improper management of irrigation and drainage. In costal regions rain, cyclones and wind can add NaCl, which often leads to soil sodicity and alkalinity as major limiting factors in agriculture (Chinnusamy et al. 2006). Salinity induces ion toxicity, osmotic stress, nutrient deficiency and oxidative stress while also causing unfavourable conditions for beneficial microbes. Hight salt contents in soil inflict osmotic stress and limit water uptake (Chinnusamy et al. 2006). During salinity, similarly to drought, various plant processes are affected that decrease crop production. Mechanisms during salt stress affect ion homeostasis, antioxidant regulation, and polyamine production (Abobatta 2020).

Under stress conditions plants can adapt their morphology to ensure survival. During drought the root architecture is altered for example by increased growth to absorb water available in deeper soil. Plants can adjust their osmotic potential by accumulation of organic and inorganic substances such as sugars, amino acids and ions and activate antioxidant systems since oxidative stress is common during drought (Fang and Xiong 2015). Stress response regulating hormones include ABA, auxin, brassinosteroids, CKs, ET, gibberellins, JA, SA, and strigolactones. For effective response to stresses extensive crosstalk between hormone and ROS signalling are necessary (Devireddy et al. 2021). *Si* massively modulates hormone signalling of its hosts (Xu et al. 2018), at the same time, *Si* is known to increase host stress resistance by diverse mechanisms (Abdelaziz et al. 2017; Baltruschat et al. 2008). *Si* induces freezing-stress related genes for proteins involved in lipid and ion transport, metabolism and hormone signalling (Jiang et al. 2021). Similarly, *Si* upregulates expression of drought related genes to moderate stress effects like reduced photosynthetic efficiency and degradation of chlorophyll (Sun et al. 2010). These beneficial stress effects of *Si* that increase host abiotic stress tolerance are frequently connected to modulations of hormone signalling pathways (Xu et al. 2018). However, it was previously not known whether symbiont effector proteins are involved in these processes.

The hormone and stress marker protoplast analyses (Figure 1) suggest that *Si* effectors recruit hormone signalling to modulate plant stress response. In addition, SIECs might modulate multiple signalling pathways or act at contact points of multiple networks. Therefore, analysing SIECs could reveal previously unknown crosstalk points between stress and hormone signalling networks. Phenotyping revealed SIECs with functions in abiotic stress response (Figure 3E-H) and in root morphology and development of plants (Figure 3A-C).

In the abiotic stress phenotyping assays SIECs were chosen based protoplast and interaction data. All SIECs in the abiotic phenotyping assays had hormone and/or stress function in protoplast assays, often in one or both heat stress markers. All tested *35S::SIEC* plants expressed *SIECs* (except for SIEC91) had ABA function in protoplasts (Figure 2D). SIECs chosen based on their target interaction were SIEC69 and SIEC58. SIEC69 interacted with multiple stress related proteins (Figure 2D, i) and modulated ABA, auxin and JA signalling markers in protoplasts (Osborne et al. 2023). SIEC58 had ABA and SA function (Osborne et al. 2023) and interacted with CHLADR (AT1G54870) (Figure 2D, ii), which is involved in detoxification and stress response (Sunkar and Zhu 2004). Both SIECs, that were chosen because of their targets, did not have stress marker function in protoplasts. The other four tested SIECs altered heat stress response in protoplasts for at least one (SIEC04) or both heat stress markers (SIEC40, SIEC91, SIEC93) (Figure 2D, iii, Supplementary Table 1). The data suggests a possible specificity of SIECs to different phenotypical and morphological responses of the root to stress. SIEC04, for instance, only affected lateral root response to mannitol, while no primary root growth effect was observed under any of the applied stresses (Figure 3E-H). Consequently, symbiont effectors might modulate host plasticity to distinct stresses or only affect specific response of plant tissues or roots zones. These exclusive functions could depend on SIEC interaction partners and where in the stress signalling pathways the effectors regulate plant responses.

The two heat stress responsive markers in protoplast assays were based on *DRBE2a* and *HSP81.1*. While it is responsive to heat treatment, the transcription factor DREB2a interacts with elements involved in cold and drought stress-responsive gene expression. Overexpression of *DREB2a* induced drought stress tolerance in Arabidopsis. It regulates expression of water and cold stress-responsive genes (Sakuma et al. 2006). SIECs regulating this heat responsive signalling pathway in protoplasts might therefore additionally be involved in cold stress responses. *HSP81.1* is heat inducible but also responsive to auxin treatment (Yabe et al. 1994). However since heat and drought or salt stress are often occurring together in nature, there might be connected signalling pathways or response mechanisms (Pandey et al. 2017). As a result, SIECs modulating HSP81.1 signalling in protoplasts were also revealed to affect other abiotic stress tolerances of Arabidopsis (Figure 3E-H). Another important signalling factor is ABA. This hormone is a major regulatory component in abiotic stress responses by integrating extrinsic signals and redirecting plant development to cope and survive environmental stresses. ABA signalling is involved in response to drought, low or high temperature and salinity (Hong et al. 2013). For this function it crosstalks with other phytohormones. JA and ABA, for example, are mediating plant salt response (Kang et al. 2005), while root growth is regulated together with ET (Luo et al. 2014) and plant development depends on ABA-auxin interactions (Emenecker and Strader 2020). Especially under osmotic stresses like drought or high salinity conditions ABA is an important factor for adaptation (Yoshida et al. 2014). Interestingly, most of the SIECs that increased abiotic stress tolerance (Figure 3E-H) modulated ABA signalling (Osborne et al. 2023). Thus, protoplast assays were efficient in indicating SIEC roles in plant hormone and stress signalling. Moreover, the interactome analyses provided information on effector candidates and their targets in abiotic stress tolerance. Consequently, the analyses might reveal targets for breeding stress tolerant crops in the future.

Symbiont effectors increase host pathogen resistance

It is known that symbionts, like pathogens, need to suppress immunity to successfully colonise host plants (Jacobs et al. 2011), for which they rely on effector proteins (Lo Presti et al. 2015). Few symbiont effector proteins are comprehensively analysed, and these studies often focus on plant immunity and colonisation. The AMF *Rhizophagus irregularis*, for example, modulates ET signalling, utilizing the effector-like protein SP7 to interact with the host protein ERF19. Since ET signalling is involved in defence, this interaction might suppress defence for colonisation (Plett and Martin 2015). *Si* effector candidates were discovered to modulate host immunity or are potentially involved in colonisation strategies. PIIN_29 is an effector-like small-secreted protein with sequence homology to E3 ligases that is induced during host interactions. It was suggested that this protein increases host colonisation by suppressing plant defence and ROS generation (Plett and Martin 2015; Zhang 2014). PIIN_08944 interfered with SA immunity pathways and suppressed PTI marker, SA-related defence gene expression and ROS burst in hosts (Akum et al. 2015). The *Si* effector Dld1 potentially enhances micronutrient accessibility for the microbe and interferes with ROS homeostasis to aid colonisation (Nostadt et al. 2020). These studies clearly revealed immunosuppressive functions of symbiont effector proteins. Consistent with this, we confirmed possible defence suppressing activities of some effector candidates, as they enhanced susceptibility to pathogens. However, phenotyping of *SIEC* expressing plants additionally revealed SIECs that increased pathogen resistance. SIECs could reduce root and leaf infection of Arabidopsis by *Phytophthora parasitica* and *Botrytis cinerea* respectively and one SIEC increased seedling survival after *Rhizoctonia solani* infection (Figure 4).

Phytophthora spp. are oomycetes that can cause great damages in agriculture and natural ecosystems. *Phytophthora parasitica* is a soilborne pathogen with a broad host range. It serves as a model organism of oomycete pathogens and is used to investigate pathology and disease-control strategies (Meng et al. 2014). Gray mold diseases in more than 200 host plants are caused by *Botrytis cinerea*. It is a necrotrophic ascomycete that kills host cells with toxins and ROS to subsequently feed on plant tissues. While *B. cinerea* shares virulence factors with other phytopathogens, it also displays strain-specific virulence factors and possible host adaptation (Choquer et al. 2007). Another soil borne pathogen with a wide host range is *Rhizoctonia solani*. The microbe is a necrotrophic fungus causing seedling blight, root rot and hypocotyl rots (Ajayi-Oyetunde and Bradley 2018).

When infecting *SIEC* expressing plants with these pathogens, significant differences in severity of disease outcomes could be observed (Figure 4A-C). All SIECs that affected pathogen infection had a function in hormone and/or stress signalling in protoplasts or interact with stress related Arabidopsis proteins (Table 1) (Osborne et al. 2023).

Table 1 SIEC affecting pathogen infection of plants, their function in protoplast and target function. Information about SIEC function in hormone signalling pathways and SIEC targets was obtained from Osborne et al. 2023. GO biological process information of targets can be found at The Arabidopsis Information Resource (TAIR) https://www.arabidopsis.org

SIEC	Hormone function in protoplasts	Stress function in protoplasts	SIEC-target with GO-term function
08	auxin/JA/SA		KIN7.4 (AT4G39050) defence response
19	JA	Heat/Immunity	
21	SA	Heat	
24	auxin/JA/CK	Heat/Immunity	
30		Immunity	
33		Heat	
46	auxin	Heat	
47	auxin/JA	Heat	
82	SA	Heat/Immunity	XTH25 (AT5G57550) defence response to fungi and bacteria
90	JA		
96	SA/CK	Heat	DRB4 (AT3G62800) dsRNA binding in viral silencing, defence response to virus
99	auxin/SA	Heat/Immunity	
119	SA	Immunity	
125	SA	Heat/Immunity	TRP2 (AT4G27070) oxidative stress, auxin and tryptophan biosynthesis
130	ABA/auxin/SA	Heat/Immunity	

Interestingly, identified SIECs often modulated hormone signalling in protoplasts. Respective hormones are involved in pathogen defence and plant immunity, like auxin, SA and JA (Hou and Tsuda 2022; Pieterse et al. 2012). While hormones like auxin are not typically linked to plant pathogen defence, vast crosstalk between hormone pathways connects auxin and cytokinin signalling to immune response by balancing growth and defence response (Naseem et al. 2015). Both SA and JA signalling pathways are known as major regulators of plant defence (Hou and Tsuda 2022), but all phytohormones are interconnected in complex networks to adapt plant processes in response to microbe interactions. Additionally, hormone crosstalk ensures optimal use of resources for growth and survival in an efficient manner (Pieterse et al. 2012). Therefore, some SIECs might modulate hormone signalling during pathogen infection but not directly affect severity of infection. Instead, they could improve other host traits like growth and development, which was not analysed in these assays.

For all three pathogen infection assays, SIECs were identified that did not benefit the plant but increased infections when expressed in Arabidopsis (Figure 4A-C). During identification of symbiont effector candidates different *Si* host colonisation stages were considered and it is possible, that some of the identified effectors are involved in immunity suppression to establish *Si* symbioses (Osborne et al. 2023). Interestingly, no SIEC was identified with effects on multiple pathogens (Figure 4A-C). Since the three analysed pathogens have distinct colonisation strategies (Meng et al. 2014; Choquer et al. 2007; Ajayi-Oyetunde and Bradley 2018), it is possible that SIECs alter specific immunity and defence pathways of plants. This corresponds with observed specificity of SIEC function in hormone (Osborne et al. 2023) and stress signalling pathways in protoplasts (Figure 1C, D). SIECs with a role in pathogen infection specifically interacted with proteins involved in distinct defence pathways. For example, SIEC96 interacted with DRB4 which is involved in viral silencing (Jakubiec et al. 2012).

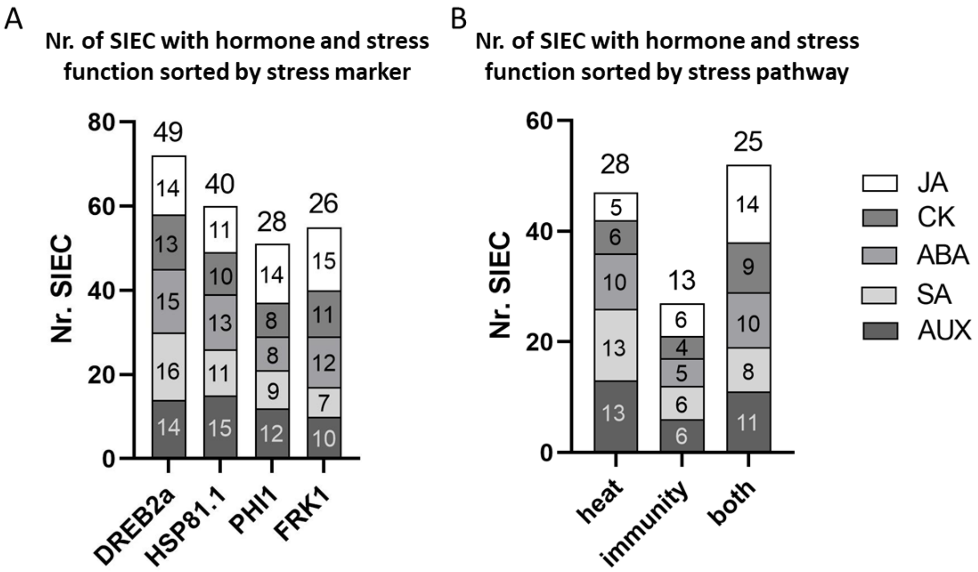
Symbiont effector proteins are known for their function in hormone modulation, a function that was shown to be necessary for successful colonisation of plants (Akum et al. 2015; Miwa and Okazaki 2017). Therefore, they were proposed to have similar function in colonisation and suppression of host immunity as pathogen effectors (Miwa and Okazaki 2017). Here it was proven, that this is not always the case. Even if some SIECs might be relevant in the colonisation processes, a large number of symbiont effectors are involved in signalling pathways that increase biotic and abiotic stress resilience as well as promoting growth of Arabidopsis.

Conclusion and outlook

While immunity regulation was a known function of SIEC proteins, their function in abiotic stress signalling was formerly not recognised. Here we not only revealed SIECs with immune suppressing functions but also those, which increase pathogen resistances in plants. *SIEC* expressing plants additionally displayed increased tolerance to abiotic stresses. While the tested symbiont effectors frequently increased resistance to both salt and drought, SIECs identified for modulating plant pathogen response were very specific. The results confirmed that symbiont effectors have additional functions compared to pathogen effectors. They demonstrate a previously unknown role of symbiont effectors in conferring beneficial effects independent of increased nutrient availability or transfer by the fungus. These assays indicated that hormone modulation by SIECs could be a mechanism to improve host stress resistance. Growth promoting effects could be shown for some SIECs. Therefore, this study revealed the manifold functions of symbionts effectors in plants and how they are used as a mechanistic tool by microbes to improve host traits and fitness. Moreover, SIEC analyses uncovered new contact points of host-symbiont interactions and hormone-stress signalling network connections within plants. These results might be used to further analyse signalling networks participating in the beneficial outcome of *Si* symbioses.

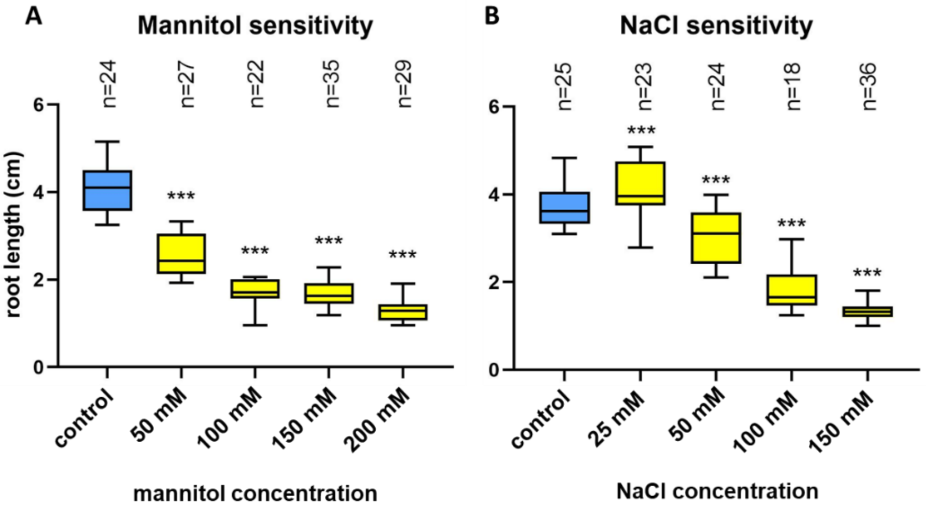
Investigating the mode of action of SIECs can assist in the identification of plant stress resistance mechanisms. Underlying genetic traits could be breeding targets and tools for investigating signalling pathways and network connection points participating in stress adaptive hormone signalling and plant stress responses. Increasing crop stress resistance is an important aim of agriculture by crop breeding, use of fertilizers, and pathogen protection reagents. Due to the climate change, extreme weather conditions like drought, heat and flooding as well as the emergence and spread of pathogens are increasing. It demands for crops with an improved adaptation to such conditions while generating enough yield to ensure overall food security.

Supplementary Figures



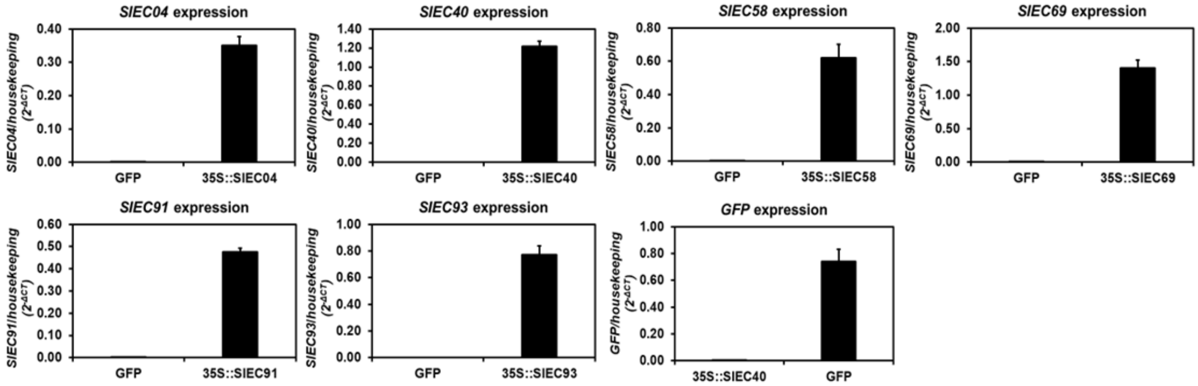
Supplementary Figure 1. Number of SIECs with stress and hormone function.

A Number of SIECs that alter stress marker expression and have additional hormone functions. **B** Number of SIEC that specifically modulate stress signalling and, in addition, hormone pathways. The number above each bar indicates the total number of SIECs which is lower than the sum as individual SIECs can affect multiple hormone and stress pathways. AUX – auxin, CK – cytokinin, ABA – abscisic acid, JA – jasmonic acid, SA – salicylic acid



Supplementary Figure 2. Mannitol and NaCl sensitivity assays.

A Effect of ascending concentrations of mannitol and **B** NaCl on primary root length of 14-day old seedlings. Root length of control plants are indicated in blue. (***) $p < 0.001$; statistical significance was evaluated by ANOVA. Sample size (n) is indicated above each box.



Supplementary Figure 3. qRT-PCR-based quantification of *SlEC* expression in *SlEC*-expressing Arabidopsis seedlings.

RNA of roots of 14-day old seedlings was used to analyse *SlEC* expression by qRT-PCR. *SlEC*-specific primers were used to confirm expression in *p35S::SlEC* lines relative to housekeeping genes *UBQ5* and *EF1α*. GFP-expressing plants served as negative control. Data shown is based on three technical replicates of RNA extracted from a pool of *p35S::SlEC* seedlings.

Supplementary Tables

Supplementary Table 1 SIEC multiplied ratios in protoplast screens for stress signalling markers. Yellow indicates marker inductions by the SIEC, blue indicates marker suppressions. pEG201 ev is the empty vector control used for comparison and analysing function of the effectors in the four signalling pathways. DREB2a: *pDREB2a::LUC* heat stress marker, HSP81.1: *pHSP81.1::LUC* heat stress marker. PHI1: *pPHI1::LUC* immune response marker, FRK1: *pFRK1::LUC* immune response marker.

SIEC	DREB2a/heat	HSP81.1 / heat	PHI1 / flg22	FRK1 / flg22
pEG201 ev	1	1	1	1
SIEC3	8.4823093	8.3236185	0.574777	0.108135
SIEC4	0.9716698	0.3820871	0.872131	0.608424
SIEC6	0.9596513	0.6423038	1.249409	0.882421
SIEC7	2.2797881	1.9227938	0.784142	0.784945
SIEC8	1.5010093	1.2793738	0.772517	0.71956
SIEC10	231.33268	5.7957262	106.4925	10.41782
SIEC11	0.7617246	0.6383113	0.919764	1.083513
SIEC12				
SIEC14	1.5144329	0.8019849	0.7009	0.782828
SIEC15	1.1557755	0.8969423	1.040455	0.506628
SIEC16	0.819008	0.3220452	1.617248	0.559542
SIEC17	0.854676	0.4046479	0.401398	0.523796
SIEC19	2.4456606	4.0392722	0.575085	0.250442
SIEC20	1.6290773	0.8571871	0.452567	0.304633
SIEC21	2.2043029	1.1629333	0.734259	0.950984
SIEC24	4.089641	1.7932427	0.478048	0.468553
SIEC25	0.7628547	0.5339775	0.683672	0.225746
SIEC26	2.7581058	0.5288665	1.327122	1.01908
SIEC27	1.7951411	1.440367	0.926979	0.914957
SIEC30			0.464821	1.07619
SIEC31	2.7448795	6.2230755	0.937198	1.583037
SIEC33	15.207284	8.7560265	1.342142	1.762936
SIEC35	10.9202	1.0799484	0.654962	0.154518
SIEC36	2.2246036	2.2102294	0.529952	0.445954
SIEC37	1.1135409	1.8097529	1.130548	1.186153
SIEC39	2.0926063	0.6389881	1.494608	1.066379
SIEC40	3.0473507	2.1468908	0.829845	1.138553
SIEC45	5.8743973	3.0951099	0.525193	0.544164
SIEC46	0.8450396	6.2083535	0.965137	
SIEC47	0.6107929	0.3280383	0.616714	0.818165
SIEC48	0.4161609	0.5430589	0.425644	1.031132
SIEC49	1.0809514	4.6645281	1.79824	1.449219
SIEC50	0.7031975	0.3398083	0.470892	0.786717
SIEC52	0.4584718	0.5654065	0.699613	0.857562
SIEC54	0.6143679	0.8028078	0.365384	0.722001

SIEC55	1.039721	1.8529739	2.997149	1.09581
SIEC56	0.5247151			1.123857
SIEC57	1.0565506	0.6111408	0.787559	1.121068
SIEC58	0.7595667	0.9741645	0.808202	0.961049
SIEC59	1.0721355	1.1964935	0.66468	0.564434
SIEC62	2.681417		1.445148	1.085205
SIEC63	1.0038982	1.1448382	1.665215	1.499369
SIEC64	0.9677017	2.9202922	1.615553	1.137531
SIEC65		0.4431657	0.09265	0.266045
SIEC66	1.2149104	1.0996211	0.407649	0.68947
SIEC67	1.7406561	0.5667042	0.134934	0.453137
SIEC68	1.5609471	1.2094109	1.368478	0.816638
SIEC69	1.725405	1.4430728	1.32982	0.990309
SIEC73	1.7537815	1.5147179	1.190892	0.746679
SIEC74	1.3643868	1.1366099	0.982877	0.59132
SIEC77	1.1076538			0.431386
SIEC80	1.3756743	0.9356451	0.609263	0.287859
SIEC81	1.2411587	0.7577009	0.669467	0.6744
SIEC82	0.4597968	0.7109088	1.231238	0.435194
SIEC84	4.674641	10.264696	1.406662	0.135046
SIEC85	4.8942408	9.7236427	1.169844	0.356874
SIEC88	2.0474921	1.947779	0.508614	0.355682
SIEC89	38.820435	2.2045005	53.46884	4.313931
SIEC90	1.1467887	1.0042034	1.097209	0.867914
SIEC91	10.958931	16.858393	0.714334	0.29085
SIEC92	1.8744673	0.9627922	0.273905	0.377558
SIEC93	3.9889912	6.5316081	0.984956	0.521519
SIEC95	4.8170741	4.3187081	1.203293	0.525633
SIEC96	2.0847569	0.5135325	0.807565	1.2689
SIEC98	0.7775555	1.5450717	0.757418	0.909333
SIEC99	1.6578832	2.9014083	6.747929	0.864201
SIEC100	1.5396963	2.7998076	1.907786	0.736661
SIEC101	3.6419682	1.6800428	1.976108	0.869211
SIEC102	3.4321437	3.4297569	1.904324	0.675512
SIEC103	1.7146712	0.1903728		
SIEC104	1.6792352	1.3810434	0.911251	0.745101
SIEC105	0.5841751	1.9714943	1.676163	1.255187
SIEC107	3.1284937	1.0236826	2.060759	0.331372
SIEC108	1.4790853	0.6176722	1.597508	0.60531
SIEC109	2.9802015	0.994233	2.216686	0.897991
SIEC111	0.5936687	1.6592589	2.026546	1.696965
SIEC112	0.9438287		1.714335	0.578989
SIEC113	14.130859	1.9797277	1.630774	0.684441
SIEC114	4.0159039	2.4174491	1.223273	0.8554
SIEC116	1.2347306	1.1217516	0.729441	0.177229

SIEC117	1.7187187	8.5494618	0.896358	0.755297
SIEC119	1.6733493	0.906509	0.391392	0.147201
SIEC120	2.3460172	1.2452482	0.794019	0.595754
SIEC121	2.054108	1.8120041	1.821065	0.485841
SIEC122	5.9147406	8.796469	1.820659	0.652034
SIEC123	0.7953929	3.4331243	2.230488	1.48255
SIEC124	4.2015497	13.973189	0.910838	0.700233
SIEC125	2.1538657	0.9698723	0.281767	1.105459
SIEC126	2.5421146	1.5658323	0.922368	1.04081
SIEC128	2.3402182	1.6247355	2.092791	1.173167
SIEC130	0.4587677	0.7267112	0.941643	0.147003
SIEC131	2.5400828	0.5089168	1.181772	1.155128
SIEC132	2.2515209	0.9101482	0.383489	0.871404
SIEC133	2.2492657	0.6111259	1.682036	1.071337
SIEC134	3.1905263	3.5511667	4.10423	1.211763
SIEC135	0.6688719	2.2186837	1.76912	1.252505
SIEC136	6.5173778	19.194963	1.305119	1.006671
SIEC137	1.0974019	2.0706469	0.5876	0.28965
SIEC138	2.0178448	0.8157482	0.842681	0.805234
SIEC141	1.4792172	0.4152825	0.577694	0.676828
SIEC142	4.4659996	4.6965752	0.686671	0.636278
SIEC144	0.8169758	1.1199224	3.945889	1.863969
SIEC145	0.6675186	0.3554298	3.498773	1.182615
SIEC147	1.534985	0.8000455	4.59633	1.798275
SIEC149	15.858193	3.0825053	2.058881	0.246034
SIEC150	2.4149891	1.4090999	1.707467	0.6898

Supplementary Table 2 Confirmation protoplast screen.

			ORIGINAL			REP1			REP2			REP3	
	effector	*SIE (treated) / ev (treated)*	*SIE (mock) / ev (mock)*	Original (ratio multiplied)	*SIE (treated) / ev (treated)*	*SIE (mock) / ev (mock)*	*ratios multiplied**	*SIE (treated) / ev (treated)*	*SIE (mock) / ev (mock)*	*ratios multiplied**	*SIE (treated) / ev (treated)*	*SIE (mock) / ev (mock)*	*ratios multiplied**
DREB2a	SIEC122	1.8177088	3.2539539	5.9147406	0.9325136	1.5014519	1.4001244	0.8827654	1.5982453	1.4108756	1.2101004	1.9369397	2.3438915
	SIEC136	1.8007888	3.6191795	6.5173778	1.1299243	1.6015989	1.8096856	1.3679802	2.0609054	2.8192778	1.0136017	1.4137274	1.4329564
	SIEC3	2.5204761	3.3653599	8.4823093	1.1242588	1.6753824	1.8835635	1.0646301	0.9206974	0.9802021	1.4012859	1.920173	2.6907114
	SIEC35	2.9831168	3.6606679	10.9202	0.4999354	0.7461677	0.3730357	0.305928	0.5278818	0.1614938	0.2578688	0.4918091	0.1268222
	SIEC91	2.6885021	4.076222	10.958931	1.2398319	1.6431942	2.0372846	1.6673623	3.0063379	5.0126544	1.4312203	2.5832555	3.6972077
	SIEC113	2.7529536	5.132981	14.130859	1.0814985	1.7069097	1.8460203	1.0535841	1.6747658	1.7645066	0.8377103	1.1894001	0.9963727
	SIEC33	3.6888905	4.1224547	15.207284	1.0404499	1.6248652	1.6905908	1.1154129	1.9267544	2.1491267	1.2878951	1.967114	2.5334365
	SIEC149	3.704807	4.2804371	15.858193	0.534197	0.9138312	0.4881659	0.3292594	0.6949009	0.2288026	0.280322	0.6690694	0.1875549
	SIEC89	7.1631357	5.419475	38.820435	10.982205	18.835447	206.85473	2.6551856	5.4280159	14.41239	3.3621533	7.9949558	26.880267
	SIEC10	15.184899	15.234391	231.33268	10.79264	28.763749	310.43677	2.0499011	6.108744	12.522321	2.5640602	6.7845437	17.395979
PHI1	SIEC65	0.2471462	0.3748808	0.0926503	0.2073148	0.1448816	0.0300361				0.0945387	0.1440459	0.0136179
	SIEC67	0.3581027	0.3768026	0.134934	0.4228072	0.3876403	0.1638971	0.2130942	0.2464919	0.052526	0.2465873	0.2815959	0.069438
	SIEC92	0.5415702	0.5057607	0.2739049	0.4947397	0.6665467	0.3297671	0.4801973	0.2940039	0.1411799	0.273523	0.2212193	0.0605086
	SIEC125	0.3582921	0.7864157	0.2817666	0.5382392	0.4270606	0.2298607	0.5552009	0.6550532	0.3636862	0.3624572	0.4034592	0.1462367
	SIEC144	2.2478798	1.7553824	3.9458887	1.2600202	1.3501494	1.7012155	0.6884289	0.5943655	0.4091784	0.5129121	0.6303197	0.3232986
	SIEC134	1.6346275	2.5108048	4.1042304	0.7019369	0.8329018	0.5846445	0.6753084	0.5655732	0.3819364	0.7981589	0.9105679	0.7267778
	SIEC147	2.5417279	1.8083486	4.5963301	0.4641423	0.5610483	0.2604063	0.7807023	0.7370745	0.5754357	0.8089029	0.7902646	0.6392473
	SIEC99	2.3436787	2.8792041	6.7479295	1.0541148	0.8670667	0.9139878	0.4776739	0.9330123	0.4456756	0.4457891	0.8413762	0.3750764
	SIEC89	5.3289914	10.033575	53.468835	1.6878282	1.6662299	2.8123097	0.7184678	2.7850404	2.0009617	0.522666	1.1085534	0.5794031
	SIEC10	6.9519369	15.318399	106.49254	3.2635795	3.4722642	11.33201	0.7313936	4.1253868	3.0172814	1.1866473	4.5844208	5.4400906

Supplementary Table 3 Data of root abiotic stress sensitivity assays.

mannitol root sensitivity				
mannitol concentration	average root length (cm)	SE	n	p-value
control	4.08	0.12	24	
50 mM	2.55	0.09	27	2.0173E-14
100 mM	1.73	0.06	22	1.94731E-21
150 mM	1.67	0.05	35	1.6763E-28
200 mM	1.28	0.04	29	1.41806E-29

NaCl root sensitivity				
NaCl concentration	average root length (cm)	SE	n	p-value
control	3.75	0.10	25	
25 mM	4.14	0.13	23	0.019343513
50 mM	3.04	0.13	24	4.74352E-05
100 mM	1.84	0.12	18	2.39124E-15
150 mM	1.33	0.03	36	2.46081E-34

Supplementary Table 4 qRT-PCR data of *SIEC* expressing Arabidopsis seedling pools.

							Exp. Rel. to Housekeeping				Fold change				
Sample	UBQ5 C_T	EF1a C_T	Average Geomean	Gene of interest C_T	ΔCT	Average	2^-ΔCT	Average	SD	SE	ΔΔCT	2^-ΔΔCT	AVERAGE	SD	SE
GFP	17.80	18.24	18.04	35.58	17.54	17.84	0.0000053	0.00	0.00	0.00	-0.31	1.24	1.01	0.21	0.12
GFP	17.87	18.52		36.15	18.11		0.0000035				0.27	0.83			
GFP		17.98		35.92	17.88		0.0000041				0.04	0.98			
35S::SIEC04	17.72	16.92	17.44	18.83	1.39	1.52	0.3819361	0.35	0.05	0.03	-16.46	89862.72	82535.10	10945.27	6319.25
35S::SIEC04	17.87	17.47		19.19	1.75		0.2973173				-16.09	69953.42			
35S::SIEC04		16.89		18.86	1.42		0.3731230				-16.42	87789.15			
GFP	17.80	18.24	18.04	40.00	21.96	21.96	0.0000002	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00
GFP	17.87	18.52		40.00	21.96		0.0000002				0.00	1.00			
GFP		17.98		40.00	21.96		0.0000002				0.00	1.00			
35S::SIEC40	18.22	17.61	17.99	17.67	-0.32	-0.28	1.2518099	1.22	0.10	0.06	-22.28	5109980.45	4964549.53	400412.52	231178.28
35S::SIEC40	18.26	18.07		17.85	-0.14		1.1052581				-22.11	4511745.17			
35S::SIEC40		17.55		17.62	-0.37		1.2914816				-22.33	5271922.97			
GFP	17.80	18.24	18.04	40.00	21.96	21.96	0.0000002	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00
GFP	17.87	18.52		40.00	21.96		0.0000002				0.00	1.00			
GFP		17.98		40.00	21.96		0.0000002				0.00	1.00			
35S::SIEC58	17.85	17.21	17.62	18.44	0.82	0.71	0.5646302	0.62	0.14	0.08	-21.14	2304862.18	2534353.06	573043.07	330846.57
35S::SIEC58	18.04	17.81		18.57	0.95		0.5172920				-21.01	2111624.01			
35S::SIEC58		16.86		17.97	0.36		0.7806260				-21.60	3186572.97			
GFP	17.80	18.24	18.04	34.38	16.34	16.34	0.0000120	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00
GFP	17.87	18.52		34.38	16.34		0.0000120				0.00	1.00			
GFP		17.98													
35S::SIEC69	17.70	17.54	17.66	17.31	-0.35	-0.48	1.2748616	1.40	0.21	0.12	-16.69	106048.97	116770.91	17623.93	10175.18
35S::SIEC69	17.86	17.64		17.30	-0.37		1.2881273				-16.71	107152.47			
35S::SIEC69		17.46		16.94	-0.72		1.6482756				-17.06	137111.30			
GFP	17.80	18.24	18.04	37.03	18.99	20.70	0.0000019	0.00	0.00	0.00	-1.71	3.28	1.48	1.57	0.91
GFP	17.87	18.52		39.20	21.16		0.0000004				0.45	0.73			
GFP		17.98		40.00	21.96		0.0000002				1.26	0.42			
35S::SIEC91	17.66	17.53	17.63	18.70	1.06	1.07	0.4781804	0.48	0.03	0.02	-19.64	816043.83	813310.29	47692.00	27534.99
35S::SIEC91	17.73	18.02		18.79	1.16		0.4478659				-19.54	764310.31			
35S::SIEC91		17.16		18.62	0.99		0.5036895				-19.71	859576.73			
GFP	17.80	18.24	18.04	40.00	21.96	21.96	0.0000002	0.00	0.00	0.00	0.00	1.00	1.00	0.00	0.00
GFP	17.87	18.52		40.00	21.96		0.0000002				0.00	1.00			
GFP		17.98		40.00	21.96		0.0000002				0.00	1.00			
35S::SIEC93	18.20	20.54	19.40	19.55	0.15	0.39	0.8992521	0.77	0.12	0.07	-21.81	3670813.21	3149548.06	477991.62	275968.59
35S::SIEC93	18.49	21.02		19.82	0.42		0.7461990				-21.54	3046039.53			
35S::SIEC93		19.99		19.98	0.58		0.6692166				-21.38	2731791.43			
35S::SIEC40	17.96	18.11	18.09	27.60	9.51	9.68	0.0013716	0.00	0.00	0.00	-0.17	1.13	1.03	0.27	0.16
35S::SIEC40	18.44	18.94		28.24	10.16		0.0008757				0.47	0.72			
35S::SIEC40	17.44	17.62		27.47	9.38		0.0014998				-0.30	1.23			
GFP	17.78	18.22	18.03	18.25	0.22	0.46	0.8568109	0.74	0.16	0.09	-9.46	704.19	607.71	133.04	76.81
GFP	18.31	18.86		18.88	0.85		0.5547584				-8.83	455.94			
GFP	17.08	17.93		18.34	0.31		0.8067064				-9.37	663.01			

Supplementary Table 5 Data of plant phenotyping on control media.
Yellow colours indicate significant increase, while blue indicates significant decrease of the measured trait.

	primary root growth effect			
	mean (%)	SE (%)	n	mean p-value t.test of at least 3 replicates
GFP	1.86517E-14	1.26	124	
SIEC4	25.61	2.15	86	2.825758E-22
SIEC40	25.89	1.94	99	1.324787E-24
SIEC58	24.72	1.20	160	2.116418E-34
SIEC69	24.69	1.90	101	2.812361E-23
SIEC91	17.00	1.67	96	1.081308E-14
SIEC93	21.74	1.32	152	5.233778E-26
	lateral root number control effect			
	mean (%)	SE (%)	n	mean p-value t.test of at least 3 replicates
GFP	0	10.51	137	
SIEC4	201.8899157	24.4106	102	0.003282424
SIEC40	191.0343966	24.6691	104	0.001563259
SIEC58	237.752149	16.1727	160	0.000441104
SIEC69	206.7881773	23.9566	100	0.00081206
SIEC91	24.43634607	12.0884	101	0.512646414
SIEC93	146.3914059	15.9092	134	0.000914933
	lateral root distance control effect			
	mean (%)	SE (%)	n	mean p-value t.test of at least 3 replicates
GFP	-2.17831E-15	4.02285	137	
SIEC4	-33.92176347	5.68794	54	0.018734149
SIEC40	5.048910198	5.86318	107	0.412074469
SIEC58	-53.42271681	1.55651	147	7.63059E-06
SIEC69	-15.22907497	5.20166	103	0.208860729
SIEC91	2.160108701	5.7377	106	0.446140352
SIEC93	-17.64883006	5.13813	90	0.182541977

Supplementary Table 6 Data of plant response to abiotic stress treatment.
Yellow colours indicate significant increase, while blue indicates significant decrease of the measured trait.

	primary root growth tolerance mannitol			p-value t.test				
	mean (%)	SE (%)	n	repeat 1	repeat 2	repeat 3	repeat 4	
GFP	2.40E-15	1.52	130					
SIEC4	4.636	3.39	41	0.4924	0.9083	0.02906		
SIEC40	38.614	3.31	100	3.33E-09	6.2E-07	1.2E-05		
SIEC58	32.022	2.90	100	4.31E-05	2E-05	4.7E-12		
SIEC69	40.219	2.41	124	1.22E-11	1.5E-05	2.4E-14	1.3E-08	
SIEC91	19.829	2.24	119	2.67E-07	0.01429	0.00771	0.0028	
SIEC93	39.801	2.34	132	6.49E-12	3.4E-06	3.5E-07	7E-14	
	primary root growth tolerance NaCl			p-value t.test				
	mean (%)	SE (%)	n	repeat 1	repeat 2	repeat 3	repeat 4	
GFP	-5.42064E-15	1.61	97					
SIEC4	2.50	2.72	56	0.007	0.03346	0.10565		
SIEC40	20.59	2.07	84	7.08E-10	1.8E-05	0.03364		
SIEC58	22.30	1.40	126	1.69E-06	6.2E-09	0.01357	6.1E-11	
SIEC69	22.63	1.35	95	4.24E-07	2.6E-06	9.5E-11		
SIEC91	28.63	2.77	87	1.56E-10	0.00024	4.6E-05		
SIEC93	30.62	1.82	86	4.81E-09	1.2E-06	3.1E-10		
	lateral root number drought effect			p-value t.test				
	mean (%)	SE (%)	n	repeat 1	repeat 2	repeat 3	repeat 4	repeat 5
GFP	1.08E-14	4.79	136					
SIEC4	-58.520	3.09	83	0.0002	0.0019	0.00069	0.0026	2.6E-06
SIEC40	-56.933	3.12	159	6.74E-06	0.00077	0.00145	3.1E-05	7.4E-09
SIEC58	-53.957	1.92	118	0.0001	1.2E-08	8.3E-06	9.9E-05	
SIEC69	-46.226	2.88	95	0.036	1.3E-05	1E-05		
SIEC91	7.803	6.19	81	0.267	0.06746	0.49328		
SIEC93	-45.3828	3.31	92	0.046	0.00105	8.9E-06		
	lateral root distance drought effect			p-value t.test				
	mean (%)	SE (%)	n	repeat 1	repeat 2	repeat 3	repeat 4	repeat 5
GFP	-5.01E-15	4.52	136					
SIEC4	84.759	13.18	83	0.005	5E-05	0.0026	0.04382	0.00071
SIEC40	137.139	12.96	125	0.008	7.3E-06	6E-08	3.4E-05	
SIEC58	80.931	6.04	119	0.005	8.7E-06	6.6E-10	1.4E-08	
SIEC69	77.739	9.02	92	0.0001	0.01125	7.6E-08		
SIEC91	15.357	7.51	81	0.118	0.65637	0.77924		
SIEC93	80.844	9.81	129	2.11E-07	0.03748	0.00404	0.00052	

Supplementary Table 7 Data of *Phytophthora parasitica* root infection ratio relative to Col-0. Yellow colours indicate significant increase, while blue indicates significant decrease of infection.

				p-value t.test		
	mean	SE	n	repeat 1	repeat 2	repeat 3
Col-0	1	0.012	534			
SIEC24	0.783	0.020	150	0.005525847	0.000146	3.89805E-05
SIEC30	1.463	0.081	19	0.010538698	0.0066101	
SIEC33	1.347	0.050	50	1.20168E-05	0.0041146	
SIEC47	1.144	0.036	70	0.017149977	0.045329	
SIEC90	0.815	0.023	74	0.00079625	7.474E-05	
SIEC96	1.485	0.069	49	1.23004E-06	0.0017658	
SIEC99	1.225	0.041	73	0.007891628	0.0081914	
SIEC119	0.864	0.021	102	0.009730085	0.0061554	
SIEC125	0.872	0.018	88	0.004276309	0.0098652	
SIEC130	0.883	0.017	72	0.033456479	0.0041928	

Supplementary Table 8 Data of *Botrytis cinerea* leaf infection ratio relative to Col-0. Yellow colours indicate significant increase, while blue indicates significant decrease of infection.

				p-value t.test	
	mean	SE	n	repeat 1	repeat 2
Col-0	1	0.1095566	93		
SIEC08	0.3569993	0.0935652	17	0.012319243	0.0035436
SIEC19	0.7024866	0.069239	37	0.020957972	0.0110765
SIEC21	3.9358267	0.6256503	16	5.71454E-05	0.0189199

Supplementary Table 9 Data of *Rhizoctonia solani* seedling survival relative to Col-0. Yellow colours indicate significant increase, while blue indicates significant decrease of survival.

				p-value t.test	
	mean	SE	n	repeat 1	repeat 2
Col-0	1	0.2523198	19		
SIEC46	0.214364	0.0765155	6	0.030848795	0.0277072
SIEC82	4.2200328	1.3788517	7	0.032626878	0.0024321

Supplementary Methods

Plant treatment concentrations

Treatment concentrations were chosen based on at the smallest concentration with a significant effect on plant primary root length. Stronger treatment could impact phenotypes very intensely and conceal effects of *SIEC* expression.

Supplementary Table 1. Final stress treatment concentrations used in plant root phenotyping.

Treatment	**Concentration**
Mannitol	50 µM
NaCl	50 µM

Pathogens infection assays of *35S::SIEC* plants

Pathogen infection assays were performed by Yingqi Zhan and Prof. Dr. Weixing Shan at the College of Agronomy, Northwest A&F University Yangling, China.

For *Phytophthora parasitica* infection assays Arabidopsis *35S::SIEC* expressing plants were grown for 10 days vertically on sterile ½ MS, sucrose plates in 13 h light at 23 °C. For infection with *P. parasitica* transformant Pp1121 which expresses *GFP*, seedlings were then placed on media without sucrose (Wang et al. 2011). 5 mm diameter fresh *P. parasitica* mycelial plugs were cut from a culture grown on carrot agar at 23 °C for 3-5 days. The plugs were transferred onto the middle of the root for every seedling. 48 hours after inoculation *P. parasitica* hyphae growth was observed using a fluorescence microscope. Infected root and total root length was measured using ImageJ. Ratio of infection was calculated dividing infected length by total root length.

B. cinerea (strain Bc001) was isolated from tomato grown for 15 days at 16 °C on potato dextrose agar. For spore harvesting and removal of hyphae, mycelia of 3 ml potato dextrose broth were filtered through four layers of gauze. Spore solution was set to 5 X 105 spores/ml in potato dextrose broth. Leaves of 4-week-old Arabidopsis were detached and 5 µl spore solution dropped on the leaf for infection. Lesion diameter was analysed 48 h after infection. Lesion fraction size was calculated by dividing lesion diameter by leaf area.

For fungal infection, *R. solani* (strain HBZJ-5X) was grown for 7 days at 23 °C on potato dextrose agar. Arabidopsis seedlings were grown for 10 days vertically on ½ MS media with sucrose and then transferred in ½ MS media without sucrose. 5 mm mycelium plugs from *R. solani* grown on potato dextrose agar media were put on each seedlings middle of the root. Seedlings were kept at 23 °C and 11 h light for 12 days and seedling survival monitored at 3, 6, 9 and 12 days after inoculation. Rate of seedling survival was calculated by dividing the number of surviving seedlings by the absolute number of inoculated plants. For all pathogen infection assay calculations of *35S::SIEC* plants were set relative to the infection or survival rates of wildtype Col-0 Arabidopsis.

Protoplast *pSTRESS* marker assays

Protoplast screening was performed as described in (Lehmann et al. 2020; Osborne et al. 2023). To analyse SIEC function in signalling pathways stress-pathway markers were used: *pSTRESS::LUC* (heat marker: *pDREB2a::LUC* and *pHSP81.1::LUC*, immunity marker: *pPHI1::LUC* and *pFRK1::LUC*). Transformed cells were treated with mock (water), 0.1 mM flg22 (PTI signalling) or 1 h 37 °C (heat signalling).

Additional primers used in this project

All primers used in this project can be found online in supplementary information of the publication Osborne et al. 2023, see Data availability. Additionally used primers are listed in the following table: Supplementary Table 2.

Supplementary Table 2. Additional primers used in this project.

Name	Sequence
qRT-PCR SIEC expression	
SIE40fwd	ACCACTTTCATTTGCGACC
SIE40rev	AGTAAGACCGTCAGAAGCC
SIE91fwd	CATTTGTATCTTCTCCTTCGCC
SIE91rev	AAGCATCCTATCGCCACC
SIE93fwd	GGAACGGATGTGACGCT
SIE93rev	TCTCAAGTAAGACGCCTGTG
SIE58fwd	CTCCTACTCTTGATACGCCAG
SIE58rev	AGCATCCTTCTCCTCAATAGTG

Chapter 4 Discussion

This chapter presents further insight into the role of symbiont effectors in the confer of host benefits. The results revealed their function in plant stress signalling possibly by interacting with stress response regulating host proteins. Moreover, SIEC protoplast and interactome assays uncovered extensive interconnection of hormone and stress signalling networks. Functional analysis in whole plants showed symbiont effectors, which increased growth, biotic as well as abiotic stress resilience of plants. Therefore, symbiont effectors were confirmed as part of the molecular mechanisms to improve plant fitness by symbionts.

CHAPTER 5 – General discussion

5.1 The identified symbiont effector candidates are secreted proteins

During plant-microbe interactions, pathogens as well as symbionts secrete and transfer effectors to modulate host signalling and to facilitate colonisation. These effector can be small proteins (Hogenhout et al. 2009; Miwa and Okazaki 2017; He et al. 2020) and other molecules like small RNAs (Zanini et al. 2019). Effectors of the beneficial, endophytic fungi *Serendipita indica* (*Si*) were identified by RNA sequencing of colonised roots at different interaction stages. 106 candidates fulfilled defined effector criteria and were considered *Si* effector candidates (SIECs) (Osborne et al. 2023). Since mRNA was isolated at different *Si* colonisation stages this approach probably identified a large variety of effector proteins with diverse functionalities (Osborne et al. 2023).

Yeast-signal-sequence-trap confirmed secretion of a randomly selected set of identified SIECs in this project (Chapter 3, Figure 1a) and validated the functionality of the effector detection methods. However, secretion and translocation of effectors by filamentous microbes is not clearly understood. Oomycete effectors have shared amino terminal signatures like RXLR and LXLFLAK. These motifs were shown to mediate effector import by host cells, most likely via endocytosis. This uptake could be microbe independent and rather unspecific, which requires neither a microbe-derived translocation machinery nor a signal to trigger uptake by the host plant (Lo Presti and Kahmann 2017). In plant-fungus interactions, haustoria are often formed as fungal structures within plant cells that are surrounded by the plant plasma membrane. Exosomes and vesicles were detected between the haustorial cell wall and the plant plasma membrane and might be used by fungi to unconventionally secrete effectors (Lo Presti and Kahmann 2017).

Identification of fungal effectors is challenging *in silico*, because of their large variety. They can, for example, exceed a size of 300 amino acids (Rafiqi et al. 2013). However, bioinformatic protocols have been developed to predict protein effectors in fungal genomes (Sperschneider et al. 2018). Current studies suggest that fungal effectors have similarities in protein structure rather than in amino acid sequences. These structural traits might be essential for effector secretion and transfer (Lo Presti and Kahmann 2017). Recently, sequence-based bioinformatics was used to try and identify new fungal effector proteins. However, fungal effectors show high sequence polymorphisms which challenges prediction methods. Still, the structures of some effector proteins are available and were previously used to identify other candidates based on search for similar structural properties (Rozano et al. 2023). Thus, the identification of 106 new effector candidates (Osborne et al. 2023) and their subsequent structural analysis could guide future template-based bioinformatics for effector identification. A genomic study of *Si* revealed about 10% of the genome encode putative small secreted proteins including members of a specific gene family with DELD motifs at the C-terminus (Zuccaro et al. 2011). Members of this family were also identified and analysed during this project (Osborne et al. 2023; Roberts 2022). SIEC145, for instance, was confirmed as secreted protein (Chapter 3, Figure 1a) and is a member of the DELD motif protein family in *Si* (Roberts 2022; Osborne et al. 2023). SIEC145 contains a conserved domain of two helical, C-terminal structures predicted by AlphaFold (Roberts 2022; Varadi et al. 2022). Conserved structures like this might be part of mechanisms of effector transfer from *Si* to plant or could indicate specific effector functions. To relate protein structures to effector identities and functions, SIEC functions in plant signalling were investigated utilizing protoplast and whole plant assays.

5.2 SIECs alter hormone signalling in plants and promote growth

Hormones control every aspect of plant life from development (Brumos et al. 2018) to responses to the environment (Ku et al. 2018). Auxin and cytokinin (CK) are important developmental hormones (Ferreira and Kieber 2005; Brumos et al. 2018). Abscisic acid (ABA) is known for its prominent function in abiotic stresses (Hong et al. 2013), while salicylic acid (SA) and jasmonic acid (JA) are involved in biotic stress response (Hou and Tsuda 2022). Although hormones were formerly divided into developmental and stress functions, we now know, that these hormone signalling pathways are tightly connected and often act together in response to environmental conditions (Altmann et al. 2020; Ku et al. 2018).

Si colonisation modulates hormones signalling of hosts. While, crosstalk between SA and JA is exploited by pathogens to colonise hosts (Hou and Tsuda 2022), *Si* similarly utilizes JA signalling components to suppress immunity during successful colonisation (Jacobs et al. 2011). The *Si* effector PIIN_08944 was identified to reduce expression of PTI and SA-defence pathway genes to facilitate *Si* symbiosis by affecting SA-mediated immune response (Akum et al. 2015). However, hormone signalling was also connected to beneficial effects conferred by *Si*. *Si*-induced growth promotion was shown to require trans-zeatin biosynthesis and to depend on CK receptors CRE1/AHK2 in Arabidopsis (Vadassery et al. 2008). In addition, *Si* interferes with auxin synthesis and signalling in plants which contributes to increased root growth effects (Xu et al. 2018). ABA concentrations are altered in Arabidopsis roots during *Si* colonisation. Especially under stress conditions ABA signalling may work as a sensor in favour of beneficial symbiont interactions. Even though, *Si* is alters host hormone signalling to mediate beneficial effects (Xu et al. 2018), the molecular mechanisms of these processes are mostly unknown.

Our interactome studies revealed a pronounced targeting of the hormone network by *Si* (Chapter 3, Figure 3a). Symbiont effector interactions with hormone signalling proteins were more frequently detected than for pathogen effectors (Chapter 3, Figure 3d), indicating their importance in conferring host benefits.

In protoplast assays we identified abundant and highly specific symbiont effector functions in hormone signalling pathways (Chapter 3, Figure 4b, c). SIECs with hormone modulating functions in protoplasts were further expressed in whole plants. Plant traits were significantly affected by SIECs in response to hormone applications (Chapter 3, Figure 4f-k). This provided evidence that SIECs alter hormone signalling involved in root growth and development. Interestingly, SIEC functions in whole plants were confirmed with especially high confirmation rates for ABA response (Chapter 3, Figure 4f). The functional analysis and interactome of *Si* effectors, clearly reveals their involvement in the modulation of hormone signalling. Therefore, the results suggest effectors as part of the molecular mechanism that mediate increased host fitness.

In addition to their function in hormone signalling, various *SIECs* promoted plant growth when expressed in Arabidopsis. *SIEC* expression resulted in faster root and hypocotyl development compared to control plants (Chapter 3, Figure 4l,m; Chapter 4, Figure 3A) and no increase in nutrient acquisition was necessary. Moreover, some SIECs increased the number of lateral roots, while SIEC04 and SIEC58 enhanced lateral root density (Chapter 4, Figure 3B, C). Interestingly, some SIECs improved root growth while others increased hypocotyl length when over expressed in plants (Chapter 3, Figure 4l, m). Only five SIECs promoted growth in both plant tissues indicating that SIECs effects might be specific to different plant organs and tissues. However, not all tested SIECs promoted plant growth. SIEC10 and SIEC20 reduced seedling development (Chapter 3, Figure 4l, m). Although all analysed SIECs modulated hormone signalling in protoplasts, there was no obvious connection between a specific hormone function and observed growth effects (Osborne et al. 2023). During *Si* host colonisation a multitude of effector proteins is likely present at specific times of the interaction. Some of these effectors may regulate or interact with each other as reported for some small secreted fungal pathogen proteins (Ludwig et al. 2021). Thus, the effects observed for single effectors are in some cases probably different from their function in presence of the large *Si* effector repertoire (Zuccaro et al. 2011).

The results of the functional together with interactome analysis suggest that SIECs might mediate beneficial effects by their interaction with specific host proteins. For about half of the SIECs analysed in whole plants, host targets were identified in yeast-two-hybrid assays. These host targets are involved in hormone signalling, developmental and stress processes (Chapter 3, Figure 1b) (Osborne et al. 2023). SIEC126, for example, targets proteins with a role in plant development (Osborne et al. 2023) such as TCP9 (AT2G45680) (Nicolas and Cubas 2016). TCP9 also interacted with SIEC104. Therefore, the impact of the two effectors on growth promotion could be connected to the target function in different tissue. SIEC126, SIEC26 and SIEC69 interact with CSN5a (AT1G22920) and CSN5b

(AT1G71230), proteins involved in the regulation of plant development and growth as part of the COP9 (Constitutive photomorphogenesis 9) signalosome (CSN) (Gusmaroli et al. 2004). Interestingly, SIEC26 interacts with almost all members of the 14-3-3 family (General regulatory factor (GRF)-family) (Osborne et al. 2023). GRF proteins in Arabidopsis are scaffolding proteins, which are highly important for diverse regulatory and developmental processes also involving hormones like ABA, auxin and CK (Jaspert et al. 2011). Supported by these results, effectors seem to promote plant growth by interacting with their targets and especially by regulating hormone signalling pathways.

5.3 Symbiont effector proteins improve plant stress resilience

The molecular mechanisms behind symbiont-induced stress resilience are mostly unknown. The protoplast assays revealed *Si* effector function in PTI and heat stress responsive signalling pathways (Chapter 4, Figure 1B). Stress modulating SIECs had additional functions in hormone signalling (Chapter 4, Figure 1F, G, Supplementary Figure 1) and interacted with host proteins involved in biotic and abiotic stress responses (Chapter 4, Figure 2A, B).

SIECs tested for function in abiotic stress responses were chosen based on their hormone (especially ABA) and heat stress signalling effects in protoplasts. Additionally, SIEC58 and SIEC69 were found to interact with proteins involved in salt, heat or other abiotic stress responses in the yeast-two-hybrid assays (Chapter 4, Figure 2A, B, G) (Osborne et al. 2023). All selected SIECs increased abiotic stress tolerance of Arabidopsis seedlings (Chapter 4, Figure 3E-H). SIECs that improved plant resilience to several stresses and influenced multiple traits possibly affect fundamental stress pathways that are responsive to different environmental stimuli. Salt and drought stress can, for example, trigger similar response pathways, as both stresses cause osmotic stress and impair plant water uptake (Chinnusamy et al. 2006). Plant responses to both stresses includes, activation of anti-oxidant systems (Abobatta 2020). *Si* effector Deld1 was shown to affect oxidative stress and reactive oxygen species (ROS) homeostasis (Nostadt et al. 2020). The study linked this function to colonisation strategies, but this effector activity might play a role in improving host stress resistance. Furthermore, drought and high salt stress leads to ABA accumulation in roots, which regulates downstream gene transcription (Hong et al. 2013). Therefore, some SIECs might mediate ABA signalling or other mechanisms like antioxidant production at a level that can increases resistance to multiple stresses.

Various root-associated mutualists enhance plant pathogen resistance by activating induced systemic resistance (ISR) (Pieterse et al. 2014). ISR is a defence mechanism based on priming which results in a pronounced immune response upon subsequent pathogen attack. In this way beneficial microbes are able to stimulate an enhanced defensive capacity of hosts (Choudhary et al. 2007). In addition to ISR, plant pathogens trigger systemic acquired resistance (SAR). SAR priming seems to require enhanced

SA production while ISR was shown to involve JA and ET signalling (Choudhary et al. 2007). *Si*-mediated ISR enhances powdery mildew resistance in Arabidopsis and depends on NPR1 and JA signalling regulation. *Si* apparently primes JA-regulated defence genes independent of SA (Stein et al. 2008). Several SIECs affected JA signalling in protoplasts (Chapter 3, Figure 4b), suggesting possible function in ISR.

In collaboration with Jingqi Zhang and Prof. Weixing Shan at the College of Agronomy, Northwest A&F University Yangling, China, pathogen infection assays with *SIEC* expressing plants revealed SIEC functions in improving resistance to different pathogens (Chapter 4, Figure 4A-C). However, some SIECs were found to enhance susceptibility as they might have a function in overcoming host immunity to colonise host plants (Jacobs et al. 2011). It implies that symbionts like pathogens have evolved effectors to interfere with plant defence (Akum et al. 2015), which is also supported by overlapping host protein targets of pathogen and *Si* effectors (Chapter 3, Figure 2e). The analyses revealed, that SIECs can modulate specific host signalling pathways, which leads to increased plant stress resilience by symbionts.

5.4 SIECs informed uncovering of highly complex plant signalling networks

Microbial effector targeting of host proteins and host modulation involves complex processes. Some effectors from different pathogens can target the same host proteins, which can be viewed as “hubs” and are necessary targets for successful host infection. Redundancy of effectors might contribute to robustness of infection and pathogens possibly use this strategy to counteract host immunity (Białas et al. 2018). *Si* modulates hormone signalling and utilize effector proteins during colonisation (Nostadt et al. 2020; Akum et al. 2015; Schäfer et al. 2009a, 2009b). Our interactome analysis revealed Arabidopsis proteins targeted by SIECs (Chapter 3, Figure 1b). Protoplast assays were able to assign hormone modulating activity to these effectors (Chapter 3, Figure 4b). A subnetwork analysis combined interaction and functional data and revealed the targets of SIECs with hormone function. (Chapter 3, Figure 5a). While some SIEC targets share effector hormone function (Chapter 3, Figure 5b, Type I) or have a known role in a different hormone pathway (Chapter 3, Figure 5b, Type III), most target proteins are without known hormone function (Chapter 3, Figure 5b, Type II). Therefore, effectors might be a tool for the identification of individual plant protein function in complex signalling networks.

For this reason, I selected 10 effector targets to determine if target and SIEC functions coincided in hormone signalling. Using knockout mutants 7 of the 10 targets not only revealed previously unknown roles in hormone signalling (Chapter 3, Figure 5d-m) the analyses identified new contact points between hormone pathways in the highly complex hormone network. As a control the Type I interaction between SIEC107 and Arabidopsis 6b-interacting protein-like1 (ASIL1), which share auxin function, was used. ASIL1 is a plant-specific trihelix transcription factor especially involved in gene expression in seeds and was shown to represses expression of auxin signalling regulating genes (Gao et al. 2009). In phenotyping and protoplast assays *asil1* revealed auxin effects (Chapter 3 Figure 5f, k). Therefore, the positive control was successful and indicates that SIEC107 might exerts its function by ASIL1 interaction.

A Type III interaction was, for example, identified between SIEC69 and ZFP5. SIEC69 and its other targets had ABA function (Chapter 3, Figure 5c, iii), while the C2H2 zinc finger protein ZFP5 was previously only connected to CK (An et al. 2012) and gibberellin acid signalling (Zhou et al. 2013; Zhou et al. 2011). ZFP5 is involved in root hair formation by promoting expression of the transcription factor *CAPRICE* (*CPC*) (An et al. 2012). ABA was previously shown to be regulated by other ZFP family proteins to control seed germination and plant development (Joseph et al. 2014). Interestingly, seed germination assays in response to ABA revealed previously unknown ZFP5 functions corresponding to the interacting effector SIEC69 (Chapter 3, Figure 5i). Therefore, the results reveal an SIEC-informed connection between ABA, CK and gibberellin acid signalling by ZFP5.

To five proteins without known hormone function effector-informed assays assigned a role in hormone signalling. SIEC96 only interacted with DRB4 in yeast-two-hybrid assays and had auxin signalling function in protoplasts (Chapter 3, Figure 5c, vi). The dsRNA-binding protein 4 (DRB4) interacts with Dicer-like ribonuclease (DCL4) in antiviral response and was found to associate with viral RNA possibly to repress viral RNA translation and facilitate efficient antiviral plant response (Jakubiec et al. 2012). The analysis revealed DRB4 function in auxin signalling equivalent to the interacting SIEC96 (Chapter 3, Figure 5e,f k). Moreover, it uncovered a previously unknown relation between viral RNA silencing mechanisms and auxin signalling.

The stress related protein NAC089 is targeted by SIEC56 and SIEC62. While SIEC56 had JA function, SIEC62 had SA function in protoplasts (Chapter 3, Figure 5c, ii). Membrane-associated transcriptions factors (MTFs) like NAC089 were identified as key regulators of cell survival in ER-stress response (Yang et al. 2014). Pathogens can induce ER stress and it was shown that NAC089 is involved in *Plantago asiatica* mosaic virus infection (Gayral et al. 2020). The results of this project revealed NAC089 as member and connection point of the in SA and JA signalling network, which was informed by both interacting SIECs (Chapter 3, Figure 5d, g, m).

SIEC62 with SA signalling modulating function in protoplasts additionally targets a RING/U-BOX plant protein (Chapter 3, Figure 5c, ii). In Arabidopsis RING-finger domains bind metal-ligands and are one of the most frequently detected protein domains (Kosarev et al. 2002). The identified SIEC62 interacting protein belongs to the large RING-type ubiquitin ligase family (Stone et al. 2005). The *ring/u-box* analysis was able to confirm a function in SA signalling for another SIEC62 target (Chapter 3, Figure 5j), assigning a new member to the SA network.

An EF-hand protein was identified as target of SIEC25 which had ABA, auxin and JA modulating function in protoplasts (Chapter 3, Figure 5c, viii). Many environmental stimuli are first recognised by Ca^{2+} sensors which transfer the signalling and subsequent responses trough hormone pathways (Ranty et al. 2016). Several classes of Ca^{2+} binding proteins contain Ca^{2+}-binding EF-hand motif(s), which have diverse function in transcription, translation, protein- and nucleic-acid-binding (Day et al. 2002). Here, the previously unknown JA signalling function of an EF-hand protein was informed by SIEC25 (Chapter 3, Figure 5m).

The Teosinte branched1/Cincinnata/proliferating cell factor 9 (TCP9) was the target of five SIECs: SIEC16, SIE26 and SIEC66, which are JA modulating and SIEC69 and SIEC126 altering ABA signalling in protoplasts (Chapter 3, Figure 5c, i). TCP transcription factors are important regulators in plant development. However, it was also revealed that they are involved in hormone synthesis, transport and signal transduction (Nicolas and Cubas 2016). TCP9 could be an important interactor during microbe colonisation because it was a highly conserved target of symbiont as well as pathogenic effectors in the comparative interactome analysis (Chapter 3, Figure 2e). Here, TCP9 was revealed to be involved in JA signalling matching to three of the interacting SIECs and assigning a novel member to the hormone network (Chapter 3, Figure 5m).

Overall, combining SIEC interactome and functional analyses not only uncovered plant protein functions, but also contact points between highly complex and interconnected plant protein networks (Altmann et al. 2020). This project revealed SIEC-informed protein analysis as a highly successful tool for the annotation of protein networks. Based on the newly identified beneficial functions of SIECs, the results provide novel insight in hormone as well as stress response networks.

5.5 Conclusion and outlook

This project revealed 106 *Si* effectors, their interactome in Arabidopsis and function in hormone and stress signalling. It was uncovered, that symbiont effectors participate in molecular processes regulating host benefits. Based on the objectives of this study, the following conclusions can be made:

Objective I: Protoplasts and whole plant assays revealed symbiont effectors which function in the modulation of hormone as well as biotic and abiotic stress responsive plant pathways.

Objective II: Effector interactome and functional target analyses allowed to uncover novel protein functions in hormone pathways and indicate effectors as a tool for the investigation of highly complex signalling networks.

This project and future analyses of symbiont effectors will broaden our understanding of plant signalling pathways and how beneficial microbes alter them. This requires further functional and interaction analyses of symbiont effectors. Examining effectors of other beneficial microbes or *Si* effector functions in different host plants could reveal conserved molecular traits that activate beneficial host effects and will be an exciting research focus in future studies. These results will be highly important for crop breeding and potentially for new crop engineering technologies that aim at improving the adaptation of crops to changing environments and at increasing food security.

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