

Aus dem Institut für Phytopathologie  
der Christian-Albrechts-Universität zu Kiel

**Functional characterization of *AtGLP5* and *AtSUC7*,  
and their role in plant defense and development  
trade-offs in *Arabidopsis thaliana***

Dissertation  
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**List of Abbreviations**

ABA	Abscisic acid
AGPPase	ADP-glucose pyrophosphatase/phosphodiesterase
ANOVA	Analysis of variance
<i>APR6</i>	<i>ACTIN-RELATED PROTEIN 6</i>
<i>BAK1</i>	<i>BRASSINOSTEROID INSENSITIVE 1-associated kinase 1</i>
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CaMV	Cauliflower mosaic virus
CDB	Czapek Dox Broth
cDNA	Complementary DNA
cfu	Colony-forming unit
CK	Cytokinin
CO <sub>2</sub>	Carbon dioxide
<i>COII</i>	<i>CORONATINE INSENSITIVE 1</i>
Col-0	Columbia-0 ( <i>Arabidopsis thaliana</i> ecotype)
Col-2	Columbia-2 ( <i>Arabidopsis thaliana</i> ecotype)
Ct	Threshold cycle
CTAB	Cetyltrimethylammonium bromide
CWDEs	Cell wall degrading enzymes
DAMPs	Damage-associated molecular patterns
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post-inoculation
e.g.	<i>Exempli gratia</i>
<i>EDS1</i>	<i>Enhanced disease susceptibility 1</i>
EDTA	Ethylenediaminetetraacetic acid
<i>EIN2</i>	<i>Ethylene-insensitive protein 2</i>
<i>ELF7</i>	<i>EARLY FLOWERING7</i>
et al.	<i>Et alii</i>
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
FAO	Food and Agriculture Organization of the United Nations
<i>FCA</i>	<i>FLOWERING CONTROL LOCUS A</i>
Fig.	Figure

<i>FLC</i>	<i>FLOWERING LOCUS C</i>
<i>FLD</i>	<i>FLOWERING LOCUS D</i>
flg22	Flagellin22
<i>FLK</i>	<i>FLOWERING LOCUS K-homology (KH)-domain</i>
<i>FLS2</i>	<i>Flagellin-sensitive 2</i>
<i>FPA</i>	<i>FLOWERING LOCUS PA</i>
<i>FRI</i>	<i>FRIGIDA</i>
<i>FRL1</i>	<i>FRIGIDA LIKE 1</i>
<i>FT</i>	<i>FLOWERING LOCUS T</i>
<i>FY</i>	<i>FLOWERING LOCUS Y</i>
GA	Gibberellic acid
GFP	Green fluorescent protein
GLP	Germin-like protein
<i>GST1</i>	<i>Glutathione S-transferase 1</i>
GUS	β-glucuronidase
GWAS	Genome-wide association
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
hpi	Hours post-inoculation
HR	Hypersensitive response
<i>HUA2</i>	<i>ENHANCER of AG-4 2</i>
i.e.	<i>Id est</i>
IAA	Indoleacetic acid
IBA	Indole-3-butyric acid
IRS	<u>I</u> ncreased <u>R</u> esistance to <u>S</u> . <i>sclerotiorum</i>
JA	Jasmonic acid
JA-Ile	Jasmonic acid-isoleucine
<i>JMJ27</i>	<i>JmjC DOMAIN-CONTAINING PROTEIN 27</i>
KO	Knockout
LB	Luria-Bertani medium
<i>LD</i>	<i>LUMINIDEPENDENS</i>
<i>LFY</i>	<i>LEAFY</i>
<i>LHP1</i>	<i>LIKE HETEROCHROMATIN PROTEIN 1</i>
<i>MADS</i>	<i>MCM1-agamous-ARG80-deficiens-SRF</i>
MAMPs	Microbial-associated molecular patterns
MAPK	Mitogen-activated protein kinases
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MFS	Major facilitator superfamily
miRNA	MicroRNA

List of Abbreviations

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MS medium	Murashige and Skoog medium
MST	Monosaccharide transporter-like
MYB	MYELOBLASTOSIS
<i>N. benthamiana</i>	<i>Nicotiana benthamiana</i>
NASC	Nottingham Arabidopsis Stock Centre
NCBI	National Center of Biotechnology Information
<i>NCED3</i>	<i>NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3</i>
NLRs	Nucleotide-binding and leucine-rich repeat receptors
<i>NPR1</i>	<i>NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1</i>
OA	Oxalic acid
OD <sub>600</sub>	Optical density at 600 nm wavelength
OE	Overexpression
OxO	Oxalate oxidase
<i>PAD4</i>	<i>Phytoalexin deficient 4</i>
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA	Potato dextrose agar medium
<i>PDF1.2</i>	<i>PLANT DEFENSIN 1.2</i>
<i>PDGLP1</i>	<i>Plasmodesmata-located germin-like protein 1</i>
<i>PEP</i>	<i>PEPPER</i>
<i>PIE1</i>	<i>PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1</i>
<i>PR</i> genes	Pathogenesis-related genes
PRRs	Pattern recognition receptors
<i>Pst</i> DC3000	<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000
PTI	PAMP-triggered immunity
qRT-PCR	Quantitative reverse transcription PCR
QTL	Quantitative trait locus
R genes	Resistance genes
RAM	Root apical meristem
<i>REF6</i>	<i>RELATIVE OF EARLY FLOWERING 6</i>
<i>RLKs</i>	<i>Receptor-like kinases</i>
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rounds per minute
RT	Room temperature
<i>S. sclerotiorum</i>	<i>Sclerotinia sclerotiorum</i>
SA	Salicylic acid
<i>SAG101</i>	<i>Senescence associated gene 101</i>

<i>SAG24</i>	<i>Senescence associated gene 24</i>
SAM	Shoot apical meristem
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
SE	Sieve element
<i>SOC1</i>	<i>SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1</i>
SOD	Superoxide oxidase
SSR	Sclerotinia stem rot
SUCs	Sucrose Carriers
<i>SUF4</i>	<i>SUPPRESSOR OF FRIGIDA 4</i>
SUTs	Sucrose Transporters
SWEETs	Sugar Will Eventually Be Exported Transporters
Tair	The Arabidopsis Information Resource
TAL	Transcription activator-like
T-DNA	Transfer DNA
TF	Transcription factor
TM	Transmembrane
TMV	Tobacco Mosaic virus
Tris/HCl	Tris(hydroxymethyl)aminomethane-hydrochloride
UV	Ultraviolet
<i>V. longisporum</i>	<i>Verticillium longisporum</i>
<i>VIN3</i>	<i>VERNALIZATION INSENSITIVE 3</i>
<i>VIP4</i>	<i>VERNALIZATION INDEPENDENCE 4</i>
<i>VSP2</i>	<i>VEGETATIVE STORAGE PROTEIN 2</i>
<i>WIN3</i>	<i>HOPW-1-INTERACTING 3</i>
Ws	Wassilewskija ( <i>Arabidopsis thaliana</i> ecotype)
WT	Wild-type

## Chapter 1

### General Introduction

Since the domestication of crops, occurred about 10,000 years ago, farmers have been confronted by a large number of pests and diseases, which caused starvation as well as social and economic upheavals (Bebber *et al.*, 2013). For example, the Irish potato famine (late-blight disease of potato) caused by *Phytophthora infestans* (oomycete) killed more than one million people, the Bengal famine (brown spot of rice) in India due to the fungus *Helminthosporium oryzae* had a death toll of between three and five million people (Bhattacharya, 2019). More recently, statistics from a global survey for assessment of crop losses for five major food crops, including rice, wheat, soybean, potato, and maize indicated that the estimated global crop yield losses caused by pathogens and pests range between 17% and 30% for all the five crops. That is 30% for rice, 21.5% for wheat, 21.4% for soybean, 17.2% for potato, and 22.5% for maize (Savary *et al.*, 2019). Although elevated CO<sub>2</sub> concentrations might increase the total aboveground biomass per plant of some crops like maize and wheat, most scientists believe that climate change is a significant threat to crop production (Kellner *et al.*, 2019; Prior *et al.*, 2005). The changing climate can influence the relationships among crops, pathogens, pests, and weeds (Grace *et al.*, 2019; Juroszek and Von Tiedemann, 2013; Ramesh *et al.*, 2017).

*Brassica napus* is an allopolyploid crop species (AACC) evolved by hybridization of two diploid species, *Brassica rapa* (A genome) and *Brassica oleracea* (C genome), about 6800 and 12,5000 years ago (An *et al.*, 2019). Rapeseed is a major vegetable oil (as edible oil or biodiesel) source, surpassing cottonseed in the early 2000s and ranking second in worldwide oilseed production behind soybeans (Carré and Pouzet, 2014). The global production of rapeseed oil reached nearly 68.2 million metric tons in 2019/2020, and the leading producing countries are Canada (27.9%), European Union (24.7%), China (19.2%), and India (11.3) (Mahsa Shahbandeh, 2020). Some commonly occurring rapeseed diseases are Phoma stem canker (blackleg) by *Leptosphaeria maculans* (Hwang *et al.*, 2016), stem rot by *Sclerotinia sclerotiorum* (Del Rio *et al.*, 2007), clubroot caused by *Plasmodiophora brassicae* (HWANG *et al.*, 2012), stem striping caused by *Verticillium longisporum* (Depotter *et al.*, 2016), Alternaria blight by *Alternaria* spp. (Meena *et al.*,

2016), and light leaf spot by *Pyrenopeziza brassicae* (Boys *et al.*, 2007).

## **1.1 Sclerotinia stem rot**

Sclerotinia stem rot caused by *S. sclerotiorum* is one of the most serious diseases of *B. napus*, causing yield losses from 10-20% and up to 80% in severely infected fields. The disease also affects oil quality by affecting the content of fatty acid (Qasim *et al.*, 2020).

### **1.1.1 Biology of *S. sclerotiorum***

*S. sclerotiorum* (Lib.) de Bary, the causative agent of Sclerotinia stem rot (SSR), is one of the most destructive fungal pathogens. It can colonize more than 400 plant species, including many economically important crops distributed in almost every continent of the world, particularly in cool and moist climatic regions (Boland and Hall, 1994). *S. sclerotiorum* was originally described as *Peziza sclerotiorum* in 1837 and finally accepted as *S. sclerotiorum* (Lib.) de Bary in 1981 according to the International Rules of Botanical Nomenclature (Bary, 1884; De Bary, 1887; Libert, 1837). The Sclerotinia genus also contains another two agriculturally important plant pathogen species, *S. minor* and *S. trifoliorum*. Geographical isolates of *S. sclerotiorum* from different areas and different plants have morphological variability and a high level of intraspecific phenotypic variability in mycelia growth rate and pigmentation (Kohn *et al.*, 1991).

The fungus *S. sclerotiorum* overwinters as a hard, black resting structure called sclerotia on infected tissues or in the soil, and as mycelium in dead or living plants (Sharma *et al.*, 2016). Sclerotia can stay viable in the soil for 3-8 years, possibly determined by the soil's biological component. In contrast, soil temperature, pH value, and moisture seem to have a minor effect on their survival (Adams and Ayers, 1979). This ability to survive under adverse conditions makes *S. sclerotiorum* one of the most successful plant pathogens. At certain times of the year, sclerotia can germinate carpogenically or myceliogenically, functioning either as soilborne or airborne pathogens determined by their intrinsic nature and the condition of the surrounding environment (Abawi and Grogan, 1979). Sclerotia that germinate carpogenically can form a small, disk- or cup-shaped apothecium. Subsequently, ascospores are released from the apothecia and can travel long distances by wind. They will germinate and initiate infection after landing on the aboveground senescent parts of susceptible hosts. Sclerotia that germinate myceliogenically can



directly infect plant tissues by producing hyphae (**Fig. 1**) (Agrios, 2005; Huang, 1985). Ascospores are accountable for almost all the infections caused by *S. sclerotiorum* (Bolton *et al.*, 2006).

The symptoms caused by *S. sclerotiorum* can be different due to the host species or the host parts that are infected and also affected by the environmental conditions. More than sixty names have been used to describe the plant diseases caused by *S. sclerotiorum* such as white mold of dry bean, stem rot of rapeseed, Sclerotinia blight of peanut (Porter *et al.*, 1997; Purdy, 1979; Tu, 1988; Williams and Stelfox, 1979). In sunflower, three diseases, including head rot, middle stalk rot, and Sclerotinia wilt, are caused by this pathogen (Nelson and Lamey, 2000).

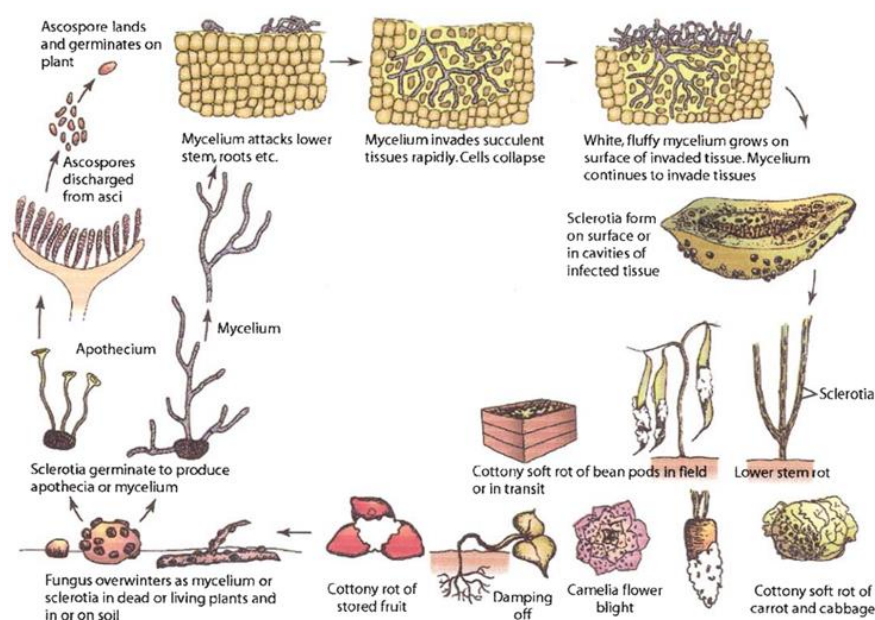


Fig. 1 Disease cycle and symptoms caused by *S. sclerotiorum*. (source: Agrios, 2005, p550)

### 1.1.2 Pathogenicity factors of *S. sclerotiorum*

As a necrotrophic pathogen, *S. sclerotiorum* has evolved competent strategies to penetrate and absorb nutrients from the dead tissues for growth and reproduction (van Kan, 2006). Mechanically, it can release phytotoxins such as oxalic acid (OA) and multiple cell wall degrading enzymes (CWDEs), including cellulases, xylanases, pectinases, aspartyl proteases, and acidic proteases (Huang *et al.*, 2008; Poussereau *et al.*, 2001; Yajima and Kav, 2006). These extracellular lytic enzymes can undermine the plant cell walls under acidic ambient pH conditions contributed by OA (Sharma *et al.*, 2016). Efficient pathogenesis by *S. sclerotiorum* requires OA since UV-irradiated *S. sclerotiorum* mutants deficient in OA production cannot produce sclerotia and

were nonpathogenic on dry beans (Godoy *et al.*, 1990). OA is the most ubiquitous low molecular weight organic acid produced by bacteria, fungi, plants, and animals with multiple functions (Palmieri *et al.*, 2019). It can also cause stomatal dysfunction in plant leaves via interfering with the ABA pathway (Guimaraes and Stotz, 2004). Moreover, it can inhibit host defenses by suppressing the plant oxidative burst during the initial infection stage and subsequently induce host programmed cell death (PCD) response for successful infection (Kim *et al.*, 2008; Williams *et al.*, 2011). Furthermore, OA also plays a vital role in balancing bulk calcium levels in plant tissues to guard the developing hyphae by chelating Ca<sup>2+</sup> ions from the degraded cell wall and forming calcium oxalate crystals (Heller and Witt-Geiges, 2013). Over the years, OA is regarded as the pathogenicity determinant and has received the most attention. In the last decade, however, multiple transcriptomes and bioinformatic analyses of *S. sclerotiorum* genome sequences at the molecular level have revealed many genes encoding small secretory proteins necessary for the complete life cycle of *S. sclerotiorum*. Some of them are functionally independent of OA, indicating a much more sophisticated infection process of *S. sclerotiorum* than previously thought. For example, disruption of Ss-Caf1 protein, which encodes a putative Ca<sup>2+</sup>-binding EF-hand motif, resulted in a defect of both plant pathogenicity and regular sclerotia establishment but a higher accumulated level of OA than the wild-type strain (Xiao *et al.*, 2014). The dysfunction of another Cerato-platanin protein SsCP1 in *S. sclerotiorum* notably reduced its virulence without influencing the production of OA (Yang *et al.*, 2018). Some of the other genes are involved in fungal morphology and development, and some can suppress host defense, acting as specific effectors (as summarized in **Table 1**) (Liu *et al.*, 2018a; Liu *et al.*, 2018b; Seifbarghi *et al.*, 2020; Wang *et al.*, 2016).

### **1.1.3 Disease management**

In the fields, plant diseases caused by *S. sclerotiorum* have historically been challenging to manage due to its broad range of host species and the long-term persistence of sclerotia. Currently, the principal application to control this pathogen is probably through integrated long-term management utilizing a combination of cultural practices, varietal resistance, synthetic chemicals, and biological control practices, targeting distinct aspects of the disease triangle, that is, the host, the pathogen, and the environment (Peltier *et al.*, 2012).

Table 1 List of some characterized pathogenicity factors of *Sclerotinia sclerotiorum*.

<b>Genes</b>	<b>Full name</b>	<b>Functions</b>	<b>References</b>
	oxalic acid (OA)	acidification, chelation, ROS, PCD	Cessna <i>et al.</i> , 2000; Guimaraes and Stotz, 2004
	Cellulases, xylanases, pectinases, aspartyl protease, acidic proteases	degrade the plant cell wall, facilitate pathogen invasion	Huang <i>et al.</i> , 2008, Poussereau <i>et al.</i> , 2001, Girard <i>et al.</i> , 2004
<i>Pka1</i>	Protein kinase A	sclerotial development	Jurick Ii <i>et al.</i> , 2004
<i>Sac1</i>	Adenylate cyclase	sclerotial development, infection cushion formation	Jurick Ii <i>et al.</i> , 2004
<i>Pph1</i>	Type 2A Ser/Thr phosphatase catalytic subunit PP2Ac	growth, sclerotial development	Erental <i>et al.</i> , 2007
<i>Rgb1</i>	Type 2A Ser/Thr phosphatase B subunit	sclerotial development, virulence, infection cushion formation	Erental <i>et al.</i> , 2007
<i>Smk1</i>	ERK-type MAP kinase	sclerotial development	Chen <i>et al.</i> , 2004
<i>Cna1</i>	Catalytic subunit calcineurin-encoding gene	sclerotial development, virulence	Harel <i>et al.</i> , 2006
<i>Pac1</i>	pH-Responsive transcription factor	sclerotial development, virulence	Rollins, 2003
<i>Cry1</i>	Cryptochrome family CRY-DASH ortholog	apothecial morphogenesis	Veluchamy and Rollins, 2008
<i>Axp</i>	Arabinofuranosidase/ $\beta$ -xylosidase	catalyze the hydrolysis of glycosidic bonds	Yajima <i>et al.</i> , 2009
<i>Pg1</i>	Polygalacturonase 1	virulence	Bashi <i>et al.</i> , 2012
<i>Nep1</i>	Necrosis and ethylene-inducing peptides	induce necrosis and cell death	Dallal Bashi <i>et al.</i> , 2010
<i>Nep2</i>	Necrosis and ethylene-inducing peptides	induce necrosis and cell death, cAMP signaling	Dallal Bashi <i>et al.</i> , 2010
<i>Ssp1</i>	Development-specific protein	sclerotial development	Li and Rollins, 2010
<i>Ssp2</i>	Development-specific protein	sclerotial development	Li and Rollins, 2010
<i>Nox1</i>	NADPH oxidase	sclerotial development, virulence, ROS regulation	Kim <i>et al.</i> , 2011
<i>Nox2</i>	NADPH oxidase	sclerotial development, ROS regulation	Kim <i>et al.</i> , 2011
<i>Iil</i>	Integrin-like protein	suppress host JA/ET signaling pathway	Zhu <i>et al.</i> , 2013
<i>Sl2</i>	Cell wall protein	sclerotial development, cellular integrity	Yu <i>et al.</i> , 2012
<i>Hex1</i>	Woronin body major protein	maintenance of cellular integrity	Yu <i>et al.</i> , 2012
<i>Gpd</i>	Glyceraldehyde-3-phosphate dehydrogenase	sclerotial formation	Yu <i>et al.</i> , 2012
<i>Ggt1</i>	$\gamma$ -Glutamyl transpeptidase	timing of sclerotial development	Li <i>et al.</i> , 2012
<i>V263</i>	Hypothetical secreted protein	virulence	Liang <i>et al.</i> , 2013
<i>Sod1</i>	Cu/Zn superoxide dismutase	sensitivity to ROS stress, sclerotia development	Xu and Chen, 2013
<i>Pth2</i>	Peroxisomal carnitine acetyltransferase	sclerotial, apothecial, and appressorial development, OA accumulation	Liberti <i>et al.</i> , 2013
<i>Shk1</i>	Histidine kinases	hyphal growth, sclerotial formation	Duan <i>et al.</i> , 2013
<i>Scfe1</i>	Sclerotinia culture filtrate elicitor 1	evoke MAMP-triggered immune responses	Zhang <i>et al.</i> , 2013
<i>Cut</i>	Cutinase	trigger defense responses	Zhang <i>et al.</i> , 2014

Table 1 continued

<b>Genes</b>	<b>Full name</b>	<b>Functions</b>	<b>References</b>
<i>Oah1</i>	Oxaloacetate acetylhydrolase	required for OA accumulation, virulence	Liang <i>et al.</i> , 2015a
<i>Scat1</i>	Type A catalase	hyper branching of hyphae, growth rate, sclerotia size	Yarden <i>et al.</i> , 2014
<i>Naca</i>	Nascent polypeptide-associated complex $\alpha$	sclerotial maturation	Li <i>et al.</i> , 2015
<i>Mads</i>	MADS-box proteins	growth rate, virulence	Qu <i>et al.</i> , 2014
<i>Caf1</i>	Secreted protein	appressorium formation	Xiao <i>et al.</i> , 2014
<i>Odc2</i>	Oxalate decarboxylase	appressorium development, OA accumulation	Liang <i>et al.</i> , 2015b
<i>Cvnh</i>	Cysteine-rich protein	virulence, sclerotial development	Lyu <i>et al.</i> , 2015
<i>Pemg1</i>	Elicitor-homologous protein	virulence, cushion number, CWDEs activities	Pan <i>et al.</i> , 2015
<i>Bi1</i>	Putative BAX inhibitor-1	sensitivity to heat and ER stress, virulence	Yu <i>et al.</i> , 2015
<i>Mat</i>	Mating-type gene	carpogenic germination, ascospore production	Doughan and Rollins, 2016
<i>Ssvp1</i>	Cysteine-rich protein	virulence, plant energy metabolism	Lyu <i>et al.</i> , 2016b
<i>Frk1</i>	Atypical forkhead (FKH)-box-containing protein	hyphal growth, virulence, sclerotial formation	Fan <i>et al.</i> , 2017
<i>Xyl1</i>	Endo- $\beta$ -1, 4-xylanase	sclerotia production, apothecium germination, virulence	Yu <i>et al.</i> , 2016
<i>Rhs1</i>	Rearrangement hot spot repeat-containing protein	morphology, virulence, appressoria formation	Yu <i>et al.</i> , 2017
<i>Foxe2</i>	Forkhead-box transcription factor	apothecia formation	Wang <i>et al.</i> , 2016
<i>Sop1</i>	Microbial opsin homolog gene	sclerotial development, virulence, stress, fungicide resistance	Lyu <i>et al.</i> , 2016a
<i>Smk3</i>	lt2 mitogen-activated protein kinase ortholog	sclerotia production, hyphae formation, growth rate	Bashi <i>et al.</i> , 2016
<i>Cp1</i>	erato-platanin protein	interact with PR1, virulence	Yang <i>et al.</i> , 2018
<i>Nsd1</i>	ATA-type IVb zinc-finger transcription factor	sexual and asexual development, appressoria formation	Li <i>et al.</i> , 2018a
<i>Ams2</i>	ATA transcription factor	appressoria formation, virulence, chromosome segregation	Liu <i>et al.</i> , 2018b
<i>Sfh1</i>	ATA-box, SNF5 transcription factor	growth, ROS accumulation, virulence	Liu <i>et al.</i> , 2018a
<i>Ste12</i>	ownstream transcription factor of MAPK pathway	mycelial growth, appressoria formation, pathogenicity	Xu <i>et al.</i> , 2018
<i>Scd1</i>	Scytalone dehydratase	hyphal branching, sclerotial formation, resistance to irradiation	Liang <i>et al.</i> , 2018
<i>Thr1</i>	Trihydroxynaphthalene reductase	hyphal branching, sclerotial formation, resistance to irradiation	Liang <i>et al.</i> , 2018
<i>Sm1</i>	Cerato-platanin protein	hyphae development, infection cushion formation	Pan <i>et al.</i> , 2018
<i>Pks13</i>	Polyketide synthase	appressorium pigmentation	Li <i>et al.</i> , 2018b
<i>Svf1</i>	Survival factor 1 homolog	oxidative stress sensitivity, appressorium formation	Yu <i>et al.</i> , 2019
<i>Trr1</i>	Thioredoxin reductase	oxidative stress tolerance, virulence, sclerotial development	Zhang <i>et al.</i> , 2019a
<i>Fdh1</i>	Formaldehyde dehydrogenase	sclerotial development, interact with SsNsd1	Zhu <i>et al.</i> , 2019
<i>Qdo</i>	Quercetin dioxygenase gene	flavonol degradation	Chen <i>et al.</i> , 2019a
<i>Cad</i>	Cinnamyl alcohol dehydrogenase	linked to the NADPH oxidase pathways	Zhou <i>et al.</i> , 2020
<i>Os4</i>	Putative MAPK kinase kinase	virulence, fungicide sensitivity, phosphorylation of SsHog1	Li <i>et al.</i> , 2020a
<i>Ppt1</i>	Phosphopantetheinyl transferase	hyphal growth, sclerotial development, virulence	Li <i>et al.</i> , 2020b

Some cultural ways consist of the selection of field, crop rotation, zero tillage (Garza *et al.*, 2002; Mueller *et al.*, 2002), using cover crops (Koike *et al.*, 1996), early planting date (Sulc and Rhodes, 1997), row spacing (Huang and Hoes, 1980), and a balanced fertilizer application (Hu *et al.*, 1999). Fungicides have shown to be more successful in controlling SSR (Sumida *et al.*, 2015). Several kinds of fungicides have proved to be efficacious for the management of *S. sclerotiorum*. They include benzimidazoles, quinone-oxidoreductase inhibitors, succinate dehydrogenase inhibitors, anilinopyrimidines, dicarboxamides, and demethylation inhibitors (Derbyshire and Denton-Giles, 2016). Biological control is another promising disease management strategy. Mycoparasite capable of colonizing the hyphae and sclerotia of *S. sclerotiorum*, such as *Coniothyrium minitans*, is a commercial biocontrol product called Contans (Hedke *et al.*, 2001). However, due to the limited efficiency, adverse impact on our environment, and the emergence of fungicide resistance, these methods are far from satisfactory. So far, higher levels of *S. sclerotiorum* resistance have lacked in the main crop sources. However, the inheritance of resistance against *S. sclerotiorum* is quantitative, and several partially resistant traits have been identified (Denton-Giles *et al.*, 2018; Zhao and Meng, 2003). Through multiple crosses of (Zhongyou 821 × 84004) × Zhongshuang No.4, Wang *et al.* were able to select a distinguished semi-winter *B. napus* variety Zhongshuang No. 9 with relatively higher resistance to *S. sclerotiorum* (Han-zhong *et al.*, 2004). While traditional breeding is time-consuming and has its inferiorities regarding alleles with added impacts, the identification and cloning of peculiar genes through genetic engineering will be a practical tool in selecting the specific traits desired and generate plants with superior SSR resistance (Ulukan, 2009).

## **1.2 Plant defense response**

Due to their sessile nature, plants cannot escape from adverse environmental conditions and are frequently confronted with both abiotic (e.g., heat, cold, flood, drought, and nutrition) and biotic (e.g., herbivores, nematodes, pathogens, and parasitic plants) stress factors (Rejeb *et al.*, 2014). To adapt to the environment and protect themselves against these potential phytopathogenic pathogens, plants have developed effective and multilayered self-defense strategies and sophisticated signaling pathways that allow the signal perception, transduction, and further triggering downstream defense responses (Conrath *et al.*, 2015).

### 1.2.1 Plant innate immunity

Preformed structural features like cuticle, trichomes, stomata, and the cell wall can act as the outer passive defensive barriers (Luo *et al.*, 2010; Melotto *et al.*, 2008; Stenglein *et al.*, 2005; Underwood, 2012). Actively, plasma membrane-localized pattern recognition receptors (PRRs) can sense microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) such as flagellin, bacterial elongation factor-Tu (EF-Tu), fungal chitin, and host-derived damage-associated molecular patterns (DAMPs) such as Plant Elicitor Peptide 1 (Pep1) and oligogalacturonides (OGs) to stimulate defense reactions, named PAMP-triggered immunity (Dressano *et al.*, 2020; Howlader *et al.*, 2020), to impede further colonization (Matzinger, 2007; Nürnberger and Kemmerling, 2006). According to the zigzag model of plant defense, some successful pathogens can overcome PTI by emitting special effector proteins, resulting in effector-triggered susceptibility (ETS). However, some plants can directly or indirectly recognize these specific effectors and propel a more powerful response designated as effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI is triggered when the cytosolic resistance proteins indirectly perceive these effectors. These recognition complexes activate downstream signals that initiate plant defense and usually involve a hypersensitive cell death response (HR) surrounding the infection sites to limit the invasion of the pathogen (Thordal-Christensen, 2020). The first cloned *R* gene, *Hm1* from maize, encodes an NADPH-dependent *Helminthosporium carbonum* (HC) toxin reductase that can detoxify HC toxin secreted by *Cochliobolus carbonum* (Johal and Briggs, 1992). Since then, various *R* genes in plants have been characterized. Through a comprehensive review, Kourelis *et al.* concluded that most *R* genes encode surface receptors, such as receptor-like kinases (RLKs) or intracellular receptors such as nucleotide-binding leucine-rich repeat receptors (NLRs). Most of them function either through the perception of PAMPs or loss of susceptibility (Kourelis and Van Der Hoorn, 2018). The PTI and ETI pathways also involve numerous defense-signaling genes like mitogen-activated protein kinases (MAPK), as well as transcription factors (TFs) and signaling molecules like phytoalexins (Ali *et al.*, 2018). A MAPK cascade is minimally composed of a MAPK (or MPK), a MAPKK (MAPK kinase), and a MAPKKK (MAPK kinase kinase) to transduce defense signals from the immune receptors to the downstream proteins. The MAPK module MEKK1-MKK4/MKK5-MPK3/MPK6 was responsible

for the positive regulation of plant defense (Asai *et al.*, 2002), whereas the MEKK1-MKK1/2-MPK4 cascade shows negative regulation of defense (Takagi *et al.*, 2019). WRKY transcription factors are critical components of plant immune signaling, acting as repressors and activators in many plant processes (Birkenbihl *et al.*, 2017; Chen *et al.*, 2019b; Rushton *et al.*, 2010). WRKY protein function via interactions with a diverse array of proteins such as MAPK cascades and other WRKY transcription factors or even themselves (Han *et al.*, 2019).

### **1.2.2 Plant defense against necrotrophic fungal pathogens**

Plant pathogens are divided into three types according to the nature of their trophic interaction with the host plants. Necrotrophic pathogens obtain nourishment from destroyed cells, while biotrophic pathogens require living host tissue to survive. Hemi-biotrophic pathogens behave initially as biotrophs (usually during the somatic growth stage) and then as necrotrophs in a later stage (usually at the spore production stage) (De Silva *et al.*, 2016). A study about the interaction between the latent period (i.e., the period between infection and the onset of sporulation), trophic type, and host resistance suggested that the duration of this period of these three trophic types are significantly different. The necrotrophs displayed the shortest latent periods to gain access to all the necrotic tissue resources and complete their infection cycle rapidly (Précigout *et al.*, 2020). Necrotrophic pathogens, including fungal, bacterial, or oomycete species, can suppress host defense and facilitate colonization (Laluk and Mengiste, 2010). Some of the economically important fungal necrotrophs are *Botrytis cinerea*, *S. sclerotiorum*, *Alternaria* spp., *Pectobacterium carotovorum* (formerly *Erwinia carotovora*), and *Fusarium graminearum*.

The development and tolerance to different stress elements of plants are modulated by diverse cellular signaling molecules such as plant hormones (Munné-Bosch and Müller, 2013). Among them, SA, JA, and ET play critical roles in coordinating conversation in the course of the recognition of pathogen attacks between different cells. Although other plant hormones such as IAA, CK, GA, ABA, and BR are also involved in regulating plant defense, their role in plant-microbe interaction is less well studied (Bari and Jones, 2009). Each of these phytohormones can activate specific downstream target genes leading to diverse signaling events. In general, SA induces the thioredoxin-catalyzed monomerization of NPR1 and its subsequent

relocation into the nucleus. Nuclear NPR1 can then bind to different WRKY transcription factors and activate expression of *PR* genes like *PR1*, *PR2*, *PR5* and trigger systemic acquired resistance (SAR) to biotrophic microbes (Kovacs *et al.*, 2015). By contrast, JA/ET-dependent defenses activate plant defensins like *PDF1.2* and *VSP2* and trigger Induced Systemic Resistance (Misra *et al.*, 2019), and are more effective against pathogens with necrotrophic lifestyles (van Loon *et al.*, 2006). A considerable work using molecular, biochemical, and genomic tools has demonstrated antagonistic crosstalk between SA and JA-/ET signaling pathways (Li *et al.*, 2019a). For example, *WRKY70* acts as a positive regulator of SA-responsive genes and a negative regulator of JA-associated genes, incorporating signals from these reciprocally antagonistic ways (Li *et al.*, 2004). So far, the metabolism and signaling transduction of SA, JA, and ET have been intensively studied. However, in nature, the interplay between these molecules is highly complicated, and the mechanisms underlying the crosstalk still require further investigation (Li *et al.*, 2019a).

### **1.3 Germin and germin-like proteins (GLPs)**

Germin proteins were originally recognized in wheat embryos as a protein marker of wheat development with different isoforms (Dunwell *et al.*, 2008; Lane *et al.*, 1993). They are evolutionary conserved universal glycoproteins. Based on the conservation of nine amino acid residues, they are grouped into the cupin superfamily. This “germin box” would form a core  $\beta$ -barrel shape (Barman and Banerjee, 2015; Dunwell *et al.*, 2004).

#### **1.3.1 Classification and structure of GLPs**

Phylogenetic analyses of some GLPs from various crop species have divided these GLPs into ten different subgroups, suggesting a complex evolutionary significance within a species (**Fig. 2**). AtGLP5, together with HvGER6 from barley and other eight members, belongs to the GER6 subfamily (Barman and Banerjee, 2015). Woo *et al.* showed that the barley germin protein contains six  $\beta$ -jellyroll monomers locked into a homohexamer, refined at a resolution of 1.6 Å (**Fig. 3**) (Woo *et al.*, 2000). This tightly arranged structure enables germin proteins to be resistant to various denaturing agents such as high temperature, SDS, and extreme pH conditions (Carter and Thornburg, 1999; Patnaik and Khurana, 2001).



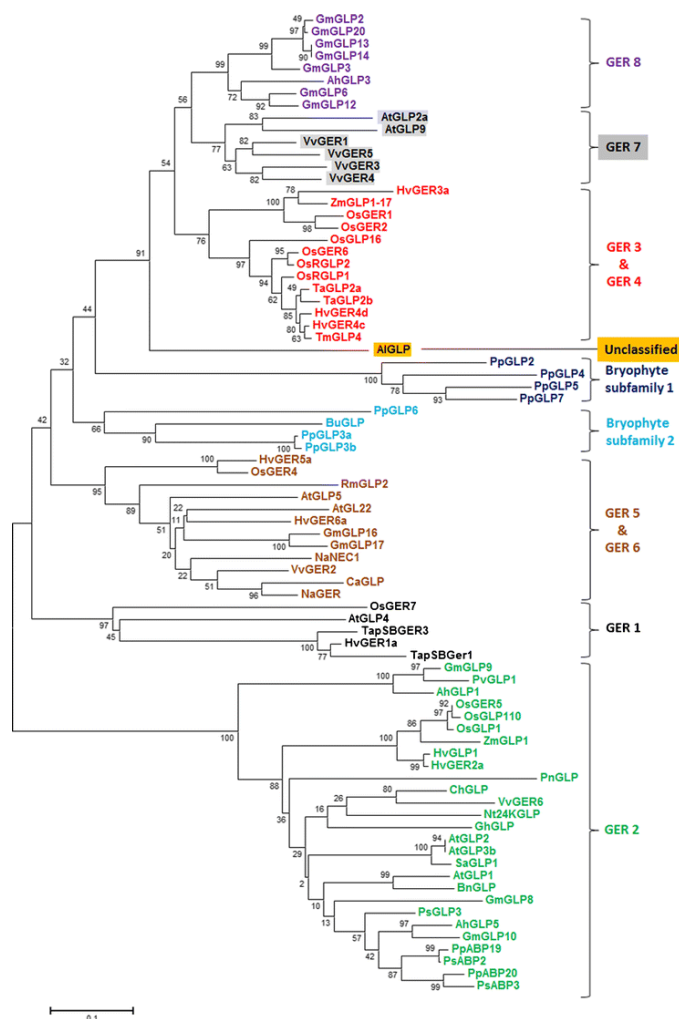


Fig. 2 Phylogenetic tree of 80 GLPs from a total of 26 plant species, including *Hordeum vulgare*, *A. thaliana*, *Oryza sativa*, *Glycine max*, etc. (source: Barman and Banerjee, 2015)

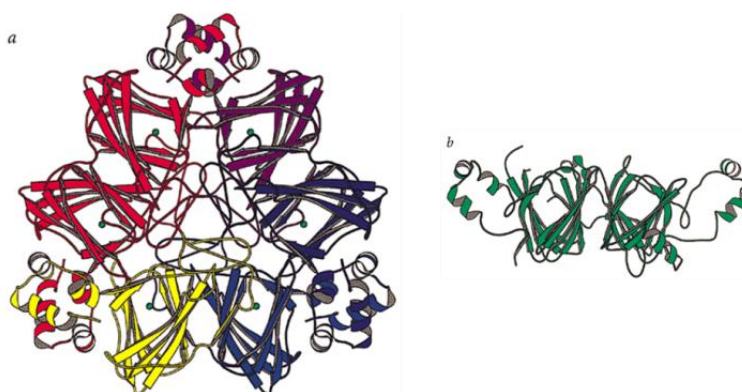


Fig. 3 The structure of the germin homohexamer (a) and the phaseolin monomer shows the close structural similarity with the germin dimer (b). (source: Woo et al., 2000)

### 1.3.2 Functions of GLPs in plants

In 1993, wheat germin was identified to possess strong sequence similarity with barley oxalate oxidase and further proved to be an oxalate oxidase (OxO), which can convert oxalates into

hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>) (Lane *et al.*, 1993). Because of this activity, germin proteins might be beneficial in plant defense by clearing oxalate, a known pathogenicity factor of oxalate producing pathogens like *S. sclerotiorum* (Magro *et al.*, 1984). Meanwhile, it can produce H<sub>2</sub>O<sub>2</sub>, a critical messenger in signal transduction during biotic and abiotic stresses (Stone and Yang, 2006). In recent years, many studies into germin proteins have been carried out in plants due to their great potential in plant disease-resistance breeding. For example, transgenic soybean and sunflower overexpressing wheat *gf-2.8* gene successfully enhance plant disease resistance against *S. sclerotiorum* (Donaldson *et al.*, 2001; Hu *et al.*, 2003).

Despite the high sequence similarity with germin proteins, germin-like proteins, however, lack this oxidase activity. Instead, some of them exhibit a superoxide oxidase (SOD) activity that can convert superoxide anions into H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>. In 1999, Yamahara *et al.* first reported the isolation of an extracellular Mn-SOD protein from the moss, which turns out to be a germin-like protein (Yamahara *et al.*, 1999). At the same time, another group determined a tobacco nectar protein Nectarin I as a germin-like protein, but could not observe oxidase activity or any other enzymatic function (Carter *et al.*, 1999). Based on Yamahara's discovery, they then demonstrated that Nectarin I also possesses SOD activity (Carter and Thornburg, 2000). Until now, there are about 20 germin-like proteins confirmed with SOD activity. Apart from SOD activity, another germin-like protein HvGLP1 is an ADP-glucose pyrophosphatase/phosphodiesterase (AGPPase), which catalyzes the lysis of several NDP-monosaccharides (Rodríguez-López *et al.*, 2001). Peruvianin-I, sharing a high identity with GLPs, represented the only germin-like protein with proteolytic activity, suggesting that it is a cysteine peptidase (Cruz *et al.*, 2020; da Cruz *et al.*, 2019).

Apart from biochemical properties, Germin-like proteins also participate in plant growth and resistance against pathogens. For example, the rice *germin-like protein 2-1* (*OsGLP2-1*) regulated seed development through ABA and GA signaling pathways (Wang *et al.*, 2020b). Overexpression of *OsGLP2-1* results in a higher level of H<sub>2</sub>O<sub>2</sub> both before and after pathogen infection and quantitatively enhances plant resistance to rice blast and bacterial blight associated with the JA signaling pathway (Liu *et al.*, 2016). Three new members of *AhGLPs* in peanut are induced under several conditions, including different hormonal, biotic, and abiotic stresses (Wang *et al.*, 2013).

The expression of a sugar beet germin-like protein BvGLP1 in *A. thaliana* also increases H<sub>2</sub>O<sub>2</sub> level in the plant and improves plant resistance to both *Rhizoctonia solani* and *V. longisporum* (Knecht et al., 2010). More recently, GhABP10, a germin-like protein from *Gossypium hirsutum*, participates in plant resistance against verticillium and fusarium wilt disease (Pei et al., 2019). In addition to conferring anti-biotic and abiotic stresses, expression of the *Capsicum chinense* *CchGLP* gene in transgenic tobacco showed higher tolerance to heavy metals such as Cadmium and Aluminium (Cedillo-Jimenez et al., 2020).

The *A. thaliana* genome encodes at least 12 germin-like proteins (Carter et al., 1998). They are expressed in different tissues at different developmental stages (Membré et al., 2000). *AtGLP4* is highly transcribed in various tissues such as seedlings, leaves, stems, floral organs, and it can be stimulated by auxin treatment and can bind auxin in vivo. However, the affinity is very low (Yin et al., 2009). Analysis of the *AtGLP13* promoter showed that *AtGLP13* was uniquely expressed in vascular bundles, principally in phloem tissues (Yang et al., 2013). *A. thaliana* plasmodesmata-located germin-like protein 1 (PDGLP1) was primarily expressed in the root, and overexpression of *AtPDGLP1* disrupted root development probably by regulating the phloem-mediated distribution of supplies between the primary and lateral root meristems (Ham et al., 2012).

#### **1.4 Plant sucrose transporters**

In plants, sugars play important roles as nutrients and signal molecules and regulate various biological procedures in plant growth and development, such as germination, seedling development, floral induction, and the onset of senescence (Yoon et al., 2020). Sucrose, which is the main carbohydrate for carbon partitioning, is synthesized in the mesophyll and exported from photosynthetic source leaves to sink organs such as roots, young leaves, reproductive tissues, and seeds via the phloem. The long-distance transport of sugars comprises different crucial steps depending on membrane sugar transporters (**Fig. 4**) (Durand et al., 2018; Geiger, 2020).

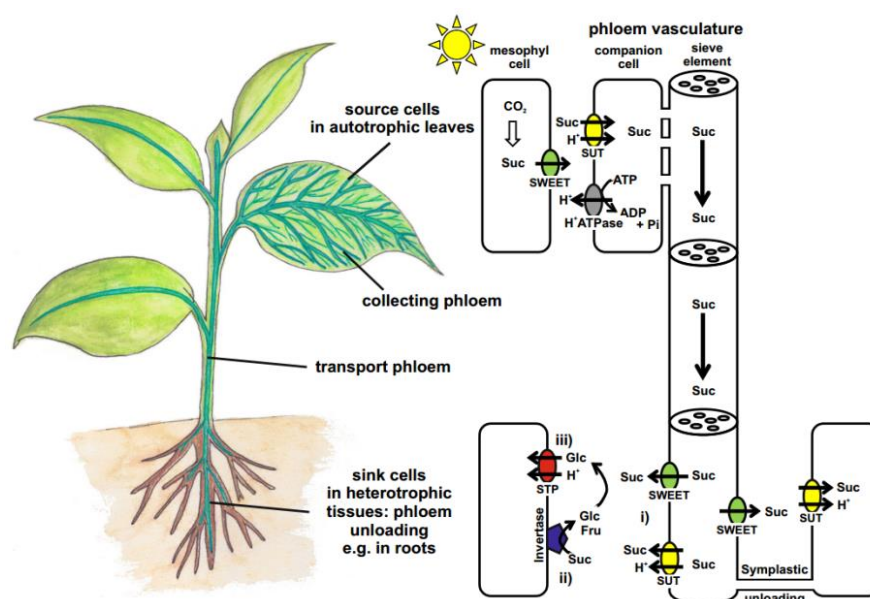


Fig. 4 Long-distance transport of sucrose from source to sink tissues. (source: Geiger, 2020)

#### 1.4.1 Classification of sugar transporters in plants

With the completion of the *A. thaliana* genome, it was found that sugar transporters belong to large multigenic families comprising monosaccharide and disaccharide transporters (Doidy *et al.*, 2012). The monosaccharide transporter-like (Yadeta *et al.*, 2011) superfamily contains 53 members of the major facilitator superfamily (MFS) class of transporters, which can be grouped into seven distinct clades (Büttner, 2007). To date, the STP (Sugar Transporter Proteins)-subfamily represents the best-characterized family of hexose transporters contributing to the uptake of sugars into sink cells (Geiger, 2020). RNA-Seq analysis in cabbage (*Brassica oleracea*) indicated that a large number of *BoSTPs* exhibited organ-specific expression, and two (*BoSTP4b* and *BoSTP12*) of the 22 *BoSTP* genes were up-regulated in the clubroot-susceptible cabbage at 28 days after *Plasmodiophora brassicae* inoculation, indicating that they might participate in monosaccharide unloading and carbon partitioning during pathogen colonization (Zhang *et al.*, 2019b). The release of sucrose into the apoplast is promoted by the SWEETs transporters. Sucrose is then accumulated in the companion cell (CC) by the AtSUC2 H<sup>+</sup>/sucrose symporter and eventually moves to the sieve element (SE) through plasmodesmata (Durand *et al.*, 2018). The Sugar Will Eventually Be Exported Transporters (SWEETs) have critical roles in distinct procedures such as apoplastic phloem loading, seed filling, secretion of nectar, pollen nutrition, and susceptibility to pathogens (Anjali *et al.*, 2020; Chen *et al.*, 2012). They are often hijacked by

pathogens for sugar supply as a nutrition source, resulting in successful colonization (Gupta, 2020). Several studies reported that various transcription activator-like (TAL) effectors from *Xanthomonas oryzae* pv. *oryzae* such as Tal5 as well as a series of artificial TAL effectors can bind to promoters of rice SWEET genes (i.e., *OsSWEET11*, *OsSWEET12*, *OsSWEET13*, *OsSWEET14*, *OsSWEET15*) and upregulate these SWEET genes, leading to the promoted release of sucrose of host cells to support bacterial virulence (Streubel *et al.*, 2013; Zhou *et al.*, 2015). A similar study in cotton (*Gossypium* spp.) suggested that *GhSWEET10* was activated by the transcription activator-like (TAL) effector Avr6 from *Xanthomonas citri* subsp. *malvacearum* (*Xcm*) and conferred susceptibility to bacterial blight of cotton (Cox *et al.*, 2017). Sucrose transporters (SUTs), also called SUCs (Sucrose Carriers), belong to the MFS superfamily (Niño-González *et al.*, 2019). All described SUTs have a similar structure, with 12 predicted transmembrane (TM) domains and are postulated to form a large central cytoplasmic pore for sucrose, with N- and C-termini and 5 even-numbered loops located in the cytosol (Sauer, 2007).

#### 1.4.2 Plant sucrose transporters (SUTs)

SUTs have been classified based on sequence homology into three distinct clades (**Fig. 5**) (Lalonde *et al.*, 2004). Clade I consists of SUT1/SUC2 transporters, which are generally high affinity, low capacity sucrose transporters. To date, all transporters in Clade I are from dicots. Clade II contains what is often referred to as SUT4 transporters. They have a low affinity and high capacity for sucrose transporters. The remaining clade, clade III, contains SUT2/SUC3 proteins presented in both monocots and dicots. SUT members can contribute to several processes, including phloem loading and unloading, plant growth, circadian rhythms, responses to abiotic stress, and interactions with pathogens (Misra *et al.*, 2019). For instance, in the *Zea mays-Ustilago maydis* pathosystem, sucrose transporters from both organisms, ZmSUT1 and UmSrt1, compete for the apoplasmic sugar at the plant-fungus interface (Wittek *et al.*, 2017). Eighteen deduced SUT genes were identified in cotton (*Gossypium hirsutum*), expression analysis of these genes by qRT-PCR indicated that SUT genes may play essential roles in cotton fiber elongation. Some are involved in distinct regulatory networks to respond differently to abiotic stresses (such as heat, cold, drought, and salinity) and phytohormones (such as IAA, GA, and SA) (Li *et al.*, 2018c). The yield of sweet potato is tightly associated with the transport and allocation

of sugars from photosynthesis. *A.thaliana* transgenic plants overexpressing a novel sweet potato *IbSUT4* gene exhibited higher accumulation of sucrose in the roots than the leaves. They also flowered earlier than wild-type plants accumulating a higher level of the *FT* gene and *AtABI3*, an ABA response factor, suggesting that *IbSUT4* is involved in the abiotic stress response through the ABA signaling pathway (Wang *et al.*, 2020a).

*A. thaliana* encodes nine sucrose transporters, AtSUC1-9, and all of them are localized to the plasma membrane except AtSUC4, which is targeted to the tonoplast (Schneider *et al.*, 2012). AtSUC1-5 show low affinity, whereas, AtSUC8 and AtSUC9 show higher affinity to sucrose and maltose. Previously, *AtSUC6* and *AtSUC7* have been designated as pseudogenes without transporter activity (Sauer *et al.*, 2004). But later, AtSUC6 was demonstrated to exhibit transporter activity at extremely low pH values. Until now, AtSUC7 does not show any transporter activity (Rottmann *et al.*, 2018). The expression of *AtSUCs* can be observed in several organs and regulated by phytohormones, abiotic stress, photoperiod, and temperature. For example, *AtSUC1* is highly expressed in roots, pollen, and trichomes and is vital for regular male gametophyte function (Sivitz *et al.*, 2008). Moreover, the sucrose uptake activity of AtSUC1 is required for its induction in roots by exogenously applied sucrose (Lasin *et al.*, 2020). *AtSUC2* is the best-characterized member concerning its function in phloem loading. Mutation in *AtSUC2* results in severe stunting and delayed onset of reproductive growth and hyperaccumulation of soluble sugar, starch, and anthocyanin due to the impaired phloem transport (Srivastava *et al.*, 2009; Srivastava *et al.*, 2008). Strong expression of *AtSUC3* was detected in several sink cells and tissues, such as guard cells, trichomes, the developing seed coat, germinating pollen, root tips, and stipules, indicating a role for *AtSUC3* in the sucrose import into sink tissues. Moreover, *AtSUC3* expression is strongly enhanced upon wounding (Meyer *et al.*, 2004). *AtSUC4*, and *AtSUC2*, are significantly induced by abiotic stresses like salt, osmotic, low temperature and exogenous ABA treatment. Knock-out mutants of *AtSUC2* and *AtSUC4* showed a higher concentration of sugar in the shoots but lower in the roots, suggesting that they are required for plant sucrose distribution and balance (Gong *et al.*, 2015). Analysis of *AtSUC5* promoter and knock-out mutants of *AtSUC5* revealed that *AtSUC5* plays a vital role in early seed development (Baud *et al.*, 2005). The other four genes, *AtSUC6-9*, encode proteins that are 88-97% identical, making specific analyses more

complicated than the other family members. Recently, a protoplast assay with the sucrose analog esculin was successfully applied to analyze the transporter activities of AtSUC1, AtSUC2, AtSUC5, AtSUC6, AtSUC8, and AtSUC9. However, in contrast to all these AtSUCs, AtSUC7 did not enable the accumulation of esculin. Site-directed mutations of both AtSUC7 amino acids at site 67 and 436 restored the esculin and sucrose transporter activity of AtSUC7. Although AtSUC7<sub>Col-0</sub> has not been shown with any transporter activity to date, AtSUC7<sub>Col-0</sub> might transport other substrates (Rottmann *et al.*, 2018). Apart from transporter activity, AtSUC9 mutant accelerated plant flowering under short-day conditions probably because of a low concentration of extracellular sucrose caused by this mutation (Sivitz *et al.*, 2007).

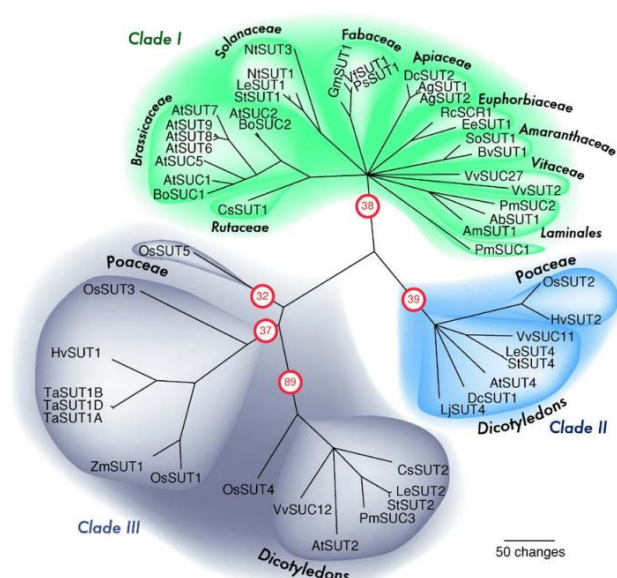


Fig. 5 Phylogenetic tree of known sucrose transporter sequences showing three distinct clades. (source: Lalonde *et al.*, 2004)

The establishment of utilizing *A. thaliana* as a model plant to carry out plant biological researches is a crucial step toward the unification in various aspects of plant biology (Bevan and Walsh, 2004). *A. thaliana*, with the advantages of its available small-sized complete genomic sequence, an extensive collection of mutants, and specialized experimental techniques, is a small, rapid cycling, self-fertilizing member of the Brassicaceae family (Kaul *et al.*, 2000). Creation of numerous “gain-of-function” and “loss-of-function” mutants via diverse forward (from phenotype to the gene) and reverse (from gene to phenotype) genetic tools allowed us to identify genes with specific functions. Activation-tagging was the first approach to generate gain-of-function mutants to discover novel candidate genes for particular traits (Kondou *et al.*, 2010). The system is based

on four copies of an enhancer element from the cauliflower mosaic virus (CaMV) 35S gene incorporated by T-DNA into the recipient plant genome to randomly activate plant genes (Chae *et al.*, 2020). Apart from these technical advantages, *A. thaliana* serves as an excellent reference source to guide studies of crop plants such as *B. napus* in particular since it also belongs to the Brassicaceae family (Schmidt *et al.*, 2001). Comparative mapping between *B. napus* and *A. thaliana* revealed that there are 21 conserved chromosomal blocks in the Arabidopsis genome which can be duplicated and reorganized to cover almost 90% of the present-day *B. napus* genome, suggesting that *A. thaliana* genome can be exploited to identify candidate genes within the larger and more complex genome of its close relative *B. napus* (Parkin *et al.*, 2005). *S. sclerotiorum* is a big threat to rapeseed production worldwide. In as early as 1992, Dickman *et al.* first started to utilize *A. thaliana* as a model plant to study plant-*S. sclerotiorum* interactions (Dickman and Mitra, 1992). In our study, *A. thaliana* was introduced to facilitate the functional analyses of candidate genes in plant defense response to *S. sclerotiorum* infection.

## **1.5 Objective and outline of the study**

So far, no effective resistance against *S. sclerotiorum* is available, and it remains a significant challenge to protect crops from the fungal infection. Dissecting and understanding molecular mechanisms between plant-*S. sclerotiorum* interactions may help us develop new strategies to control this disease. The prime aim of this thesis is the functional analysis of Arabidopsis *AtGLP5* and *AtSUC7* in the plant-*S. sclerotiorum* interaction and to elucidate their potential for resistance breeding. The germin-like protein *AtGLP5* is an ortholog of *BnGLP3* in *A. thaliana*. Since *BnGLP3* is a Superoxide Dismutase (SOD) mediating the reduction of superoxide anions to hydrogen peroxide ( $H_2O_2$ ) and proved to be involved in plant resistance against *S. sclerotiorum* (Rietz *et al.*, 2012), we thus assumed that *AtGLP5* might have a similar anti-fungal function as observed with *BnGLP3*. In addition to the disease susceptibility test and gene expression analysis of *AtGLP5* overexpression and knock out mutants, we further investigated the expression regulation of *AtGLP5* concerning different stress factors with the aid of a GUS reporter gene. We demonstrate that *AtGLP5* responds to several stimuli like pathogens (i.e., *S. sclerotiorum*, *V. longisporum*, *Pst* DC3000), flg22, wounding, and plant hormone SA. Furthermore, we analyzed another gene, encoding the plasma membrane-localized protein *AtSUC7* which had been



identified by screening an Arabidopsis T-DNA activation-tagging population (Weigel *et al.*, 2000). The Arabidopsis mutant, with an enhanced expression of *AtSUC7*, showed a higher level of resistance to *S. sclerotiorum*. This demonstrates that *AtSUC7* functionally regulates the expression of multiple defenses- and flowering-related genes, strongly suggesting its dual role in trade-off between plant development and defense response. The thesis consists of the following five chapters:

Chapter 1: This chapter gives a general introduction, including current knowledge about plant pathogen interactions with a focus on plant-Sclerotinia interactions as well as germin-like proteins and sucrose symporters.

Chapter 2: This chapter is a submitted manuscript and reports that, “AtGLP5, a germin-like protein of *Arabidopsis thaliana*, is a superoxide dismutase and plays an important role in plant development and innate immune response”.

Chapter 3: This chapter is a manuscript in preparation for submission. We report that “Enhancing expression of *AtSUC7* encoding a sucrose symporter confers plant defense against *Sclerotinia sclerotiorum* and promotes early flowering in *Arabidopsis thaliana*”.

Chapter 4: This chapter depicts the promoter analysis data, revealing a comprehensive regulation mechanism of *AtGLP5* in response to pathogen attacks.

Chapter 5: This chapter is a general discussion, where all data and their significance are reflected in the light of current knowledge and literature. Furthermore, a functional model is presented.

## References

Please see from page 120.

## Chapter 2

# **AtGLP5, a germin-like protein of *Arabidopsis thaliana*, is a superoxide dismutase and plays an important role in plant development and innate immune response**

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## 2.1 Abstract

The disease Sclerotinia stem rot, caused by *Sclerotinia sclerotiorum*, is one of the most destructive diseases of oilseed rape. Germin-like proteins play an essential role in plant defense against the *S. sclerotiorum* infection. The *Arabidopsis thaliana* genome consists of 32 genes encoding germin-like proteins. The biochemical nature and physiological function of AtGLPs remain largely unknown so far. We report that AtGLP5 is an ortholog of BnGLP3 of *Brassica napus*. Transcript profiling and promoter::GUS analysis revealed that *AtGLP5* was mainly expressed in the roots, shoot apical stem, and hydathode of the cotyledons, but not in the true leaves. Interestingly, in the true leaves, the expression of *AtGLP5* was activated by diverse stress factors, including plant pathogens, SA treatment, wounding, and by flg22 eliciting. We demonstrated that AtGLP5 is a superoxide dismutase localized in the plasma membrane and transcriptionally regulated by multiple signaling pathways, including the FLS2-mediated signaling route. We demonstrated that overexpression of *AtGLP5* in *A. thaliana* increased plant disease resistance to both *S. sclerotiorum* and *Pseudomonas syringae* pv. *tomato* DC3000, while its knockout mutants showed enhanced susceptibility, thus suggesting a functional role of *AtGLP5* in plant defense responses. Furthermore, our data provide the first evidence that *AtGLP5* represents a novel player involved in plant defense-development trade-offs.

## 2.2 Introduction

*Sclerotinia sclerotiorum* (Lib.) de Bary is one of the most destructive fungal plant pathogens worldwide and can infect over 400 plant species, including many important crops, such as rapeseed, soybean, peanut, sunflower, and nearly all vegetables (Saharan and Mehta, 2008). Most plants are relatively defenseless against *S. sclerotiorum* as the fungus has evolved effective strategies and tactics to achieve its infection process on the host. *S. sclerotiorum* exhibits distinct phases of its life cycle: in the initial stage, the fungus grows and distributes intracellularly without killing host cells, in a brief biotrophic phase, and then undergoes a transition to a necrotrophic phase and procures nutrients from the dead host tissue (Kabbage *et al.*, 2015). Oxalic acid (OA), secreted by *S. sclerotiorum*, is regarded as a critical virulence factor by interfering in numerous plant physiological processes, e.g., chelate calcium to weaken the host cell wall structure, form oxalate crystals to occlude xylem vessels, create a low pH environment to enhance hydrolytic enzyme activity, and induce programmed cell death (PCD) (Cessna *et al.*, 2000; Kabbage *et al.*, 2013; Kim *et al.*, 2008; Williams *et al.*, 2011). Besides, a wide array of cell wall degrading enzymes and secretory proteins or metabolites have been reported to have a function in facilitating pathogen penetration, suppressing host defense, and inducing host cell death, thus providing the fungus abundant nutrition as well as a preferable environment to maximize its aggressivity (Lumsden, 1979; Riou *et al.*, 1991; Zhu *et al.*, 2013). Another important strategy for the survival of fungus is to form a unique rounded structure, termed “sclerotia”, which can remain viable in the soil for many years (Merriman *et al.*, 1979). At certain times of the year and in suitable conditions, the sclerotia germinate to form apothecia and release ascospores, which are the main sources of inoculum for plants (Sharma *et al.*, 2016). Due to the limitation of traditional cultural management and the environmental damages caused by fungicide application, breeding for resistant cultivars and genetic engineering for plant resistance are more effective and promising approaches for sclerotia disease control. Recent quantitative trait loci (QTLs) mapping, genome-wide association (GWAS) analysis and RNA-seq studies in several crop species including rapeseed and soybean allowed for the identification of multiple loci being involved in plant partial resistance, greatly enhanced our understanding of the complex mechanisms underlying plant quantitative resistance to *S. sclerotiorum* (Arahana *et al.*, 2001; Wei *et al.*, 2016;

Wen *et al.*, 2018; Zhao and Meng, 2003). Wang *et al.* reported a series of plant mitogen-activated protein kinases (MAPKs or MPKs) and WRKY transcription factors including *BnMPK3*, *BnMPK4*, *BnMPK6*, *BnWRKY33* that are involved in regulating plant defense responses to *S. sclerotiorum* in oilseed rape (Wang *et al.*, 2019; Wang *et al.*, 2014; Wang *et al.*, 2009; Wang *et al.*, 2020b). Other than the previous oversimplified model that JA/ET signaling pathways are responsible for mediating the defense against necrotrophs, it is now widely accepted that the SA, JA, ET, and ABA signaling pathways, as well as their crosstalk, are all involved in *S. sclerotiorum*-*B. napus* interaction (Nováková *et al.*, 2014).

Germin-like proteins (GLPs) are a class of proteins that show high amino acid identities (average 50%) with wheat germins (Dunwell *et al.*, 2000). Germin, first discovered in wheat embryos, is well known as a protein marker of wheat development with different isoforms (Grzelczak and Lane, 1984; LANE *et al.*, 1992). Later, wheat germin was found to possess high sequence similarity with barley oxalate oxidase and also proved to be an oxalate oxidase (OxO), catalyzing the conversion of oxalates into hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and carbon dioxide (CO<sub>2</sub>) (Lane *et al.*, 1993). Owing to this enzymatic activity, germin proteins are beneficial in plant defense by clearing oxalate, a known pathogenicity factor of oxalate-producing pathogens like *S. sclerotiorum* (Magro *et al.*, 1984), thereby producing H<sub>2</sub>O<sub>2</sub>, which is well known as a critical messenger in plant signal transduction in response to both biotic and abiotic stresses (Stone and Yang, 2006). Germin proteins have been therefore widely investigated in plants. For example, overexpression of wheat *gf-2.8* gene in soybean and sunflower both successfully enhanced plant resistance against *S. sclerotiorum* (Donaldson *et al.*, 2001; Hu *et al.*, 2003).

Despite the high sequence similarity with germin proteins, most germin-like proteins lack oxidase activity. Instead, some of them exhibit a superoxide oxidase (SOD) activity that can generate H<sub>2</sub>O<sub>2</sub> by converting superoxide anions into H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>. In 1999, Yamahara *et al.* first reported the isolation of an extracellular Mn-SOD protein from the moss, which turned out to be a germin-like protein (Yamahara *et al.*, 1999). Carter *et al.* determined a tobacco nectar protein Nectarin I as a germin-like protein but could not observe its oxidase activity or other enzymatic function (Carter *et al.*, 1999). Later, they demonstrated that Nectarin 1 also possesses SOD activity (Carter and Thornburg, 2000). Until now, there are about 20 germin-like proteins

confirmed with SOD activity. The germin-like protein HvGLP1 was designated as an ADP-glucose pyrophosphatase/phosphodiesterase (AGPPase), which catalyzes the lysis of some NDP-monosaccharides (Rodríguez-López *et al.*, 2001). The transcription of *AtGLP4* gene is stimulated by auxin treatment, and *AtGLP4* can bind auxin *in vivo*, although the affinity is very low (Yin *et al.*, 2009). So far, Peruvianin-I, sharing a high identity with several GLPs, represents the only germin-like protein with proteolytic activity (Cruz *et al.*, 2020). Germin-like proteins are also reported to participate in plant defense against diseases. For instance, overexpression of *OsGLP2-1* resulted in a higher level of H<sub>2</sub>O<sub>2</sub> and quantitatively enhanced plant resistance to rice blast and bacterial blight (Liu *et al.*, 2016). GLPs in peanut are induced under different hormonal, biotic, and abiotic stresses (Wang *et al.*, 2013). The expression of a sugar beet germin-like protein *BvGLP1* in *A. thaliana* also increases H<sub>2</sub>O<sub>2</sub> level in the plant. It improves plant resistance to *Rhizoctonia solani* and *Verticillium longisporum* (Knecht *et al.*, 2010). GhABP10, a germin-like protein from *Gossypium hirsutum*, participate in plant defense against verticillium and fusarium wilt disease (Pei *et al.*, 2019). Germin-like proteins also participate in plant growth and development. For example, *A. thaliana* plasmodesmata germin-like protein 1 was primarily expressed in the root, and overexpression of *AtPDGLP1* disrupted root growth (Ham *et al.*, 2012). The rice *germin-like protein 2-1* (*OsGLP2-1*) regulated seed dormancy during seed development through ABA and GA signaling pathways (Wang *et al.*, 2020a). In addition to conferring anti-biotic and abiotic stresses, expression of the *Capsicum chinense CchGLP* gene in transgenic tobacco showed higher tolerance to heavy metals such as Cadmium and Aluminium (Al) (Cedillo-Jimenez *et al.*, 2020).

In our previous work, we investigated a family of 14 germin-like genes from *B. napus* (*BnGLPs*) and their potential functions in plant defense against *S. sclerotiorum*. One of them, *BnGLP3*, was upregulated in both susceptible ‘Falcon’ and tolerant ‘Zhongshuang 9’ *B. napus* varieties at 6 h after *S. sclerotiorum* infection. As *BnGLP3* is an H<sub>2</sub>O<sub>2</sub>-generating superoxide dismutase, early induction of *BnGLP3* is believed to participate in an oxidative burst that may play a vital role in defense of *B. napus* against *S. sclerotiorum* (Rietz *et al.*, 2012). However, the role of *BnGLP3* in the plant defense response remains unsolved.

The *A. thaliana* genome contains 32 genes encoding germin-like proteins (UniProt, <https://www.uniprot.org/>). So far, the biochemical nature and physiological function of *AtGLPs* remain largely unknown. Here, we report that Arabidopsis *AtGLP5* is an ortholog of BnGLP3. We demonstrate that in Arabidopsis, *AtGLP5* is expressed in a tissue-specific and development-dependent manner and responds to diverse stress factors. While overexpression of *AtGLP5* resulted in increased disease resistance to both *S. sclerotiorum* and *Pst* DC3000, knockout of *AtGLP5* enhanced plant susceptibility. Furthermore, we demonstrated that *AtGLP5* exhibits also SOD activity and is explicitly upregulated in response to flg22-induced PTI. Our data strongly suggest that *AtGLP5* is a crucial player in the plant immune system.

## 2.3 Materials and Methods

### 2.3.1 Plant materials and cultivation

*A. thaliana* ecotype Columbia-0 was used as the wild-type plant in this study. Plants were grown at 22 °C in a climate chamber under short-day conditions (8h light/ 16h dark). For the knockout mutant, two T-DNA insertion mutants in the Col-0 background were ordered from the Nottingham Arabidopsis Stock Center (NASC): *Atglp5-1* (N738879) and *Atglp5-2* (N863003). Homozygous mutants were verified with T-DNA- and gene-specific primer combinations by PCR. The absence of the *AtGLP5* transcript (knockout) was confirmed by qRT-PCR with gene-specific primers.

### 2.3.2 Vector construction and plant transformation

The full-length open reading frame of *AtGLP5* (At1g09560) was amplified from the cDNA of Col-0 plants with the primer pair *AtGLP5-F3* and *AtGLP5-R3* containing *att*-sites for the cloning into pDONR201 vector (Gateway® technology, Invitrogen) (**Supplementary Table S1**). From the pDONR201 entry clone, *AtGLP5* was transferred into the binary vector pGWB414 producing a 35S promoter-driven gene construct with a 3xHA-tag coding region at the 3' end (Nakagawa *et al.*, 2007). In analogy, *AtGLP5* was cloned into pGWB451, which adds a GFP-tag coding region in the frame to the 3' end to allow the expression of AtGLP5-GFP protein. Both constructs were transformed into *Agrobacterium tumefaciens* GV3101 strain to facilitate plant transformation. The transformation of *A. thaliana* plants was conducted by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on 1/2 MS medium (Murashige and Skoog medium, Duchefa) supplemented with 50 mg/L kanamycin and further confirmed by PCR.

### 2.3.3 Infection experiments with *S. sclerotiorum* and *Pst* DC3000

The *S. sclerotiorum* isolate used throughout this work was obtained from Professor W. Qian (Mei *et al.*, 2011). Infection of *A. thaliana* leaves with *S. sclerotiorum* was either performed by agar plug infection or by spray infection with mycelia suspension. In the first approach, *S. sclerotiorum* was grown on PDA (for transcript analysis) or a minimal medium (for resistance assay, 1 g NaOH, 2 g DL-malic acid, 1 g KH<sub>2</sub>PO<sub>4</sub>, 2 g NH<sub>4</sub>NO<sub>3</sub>, 0.1 g MgSO<sub>4</sub> x 7 H<sub>2</sub>O, 0.5 g yeast extract and 15 g Bacto-agar per liter) at 22 °C. After two or three days, agar-plugs were punched from the expanding margin of the mycelium using a 6 mm cork borer and placed on fully developed leaves



of 5-week-old plants with the mycelium facing the leaf surface. Leaves treated with plain agar-plugs served as control. After 2 days, a digital caliper was used to measure the major and minor diameters of the ellipse-shaped lesions. The area of lesions was estimated by multiplying half the length of the major axis by half the length of the minor axis, then multiplying by  $\pi$ . For the spray infection approach, 15 pieces of agar-plugs containing *S. sclerotiorum* mycelia were transferred into 70 ml 40% Czapek Dox medium (13,36 g Czapek Dox, 2 g yeast extract, 2 g malt extract per liter, pH 5.5) and shaken for 3 days at 22 °C. Subsequently, the mycelia were homogenized for ~35 sec and centrifuged at 6,000 rpm for 10 min. The sedimented mycelia were weighed and resuspended in 10 mM MgSO<sub>4</sub> at a concentration of 1 g/25 mL. Leaves of 5-week-old plants were sprayed with the mycelium suspension. Plants were covered with a transparent lid to keep high humidity. After two or three days, photos were taken to compare the severity of symptoms on each plant.

For bacterial infection, the strain *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) with an empty pVSP61 vector was used as described by Aarts (Aarts *et al.*, 1998). *Pst* DC3000 was cultivated on a Petri dish with NYGA medium (5 g bacto peptone, 3 g yeast extract, 30 ml glycerine, and 15 g Bacto agar per liter) plus appropriate antibiotics for 2 days at 28 °C. Bacteria were then collected by washing with 5 mL 10 mM MgCl<sub>2</sub> and adjusted to OD<sub>600</sub> = 0.2. After adding 0.04% (v/v) Silwet Gold, the bacteria suspension was sprayed on 5-week-old *A. thaliana* plants until complete wetness and then covered with a transparent lid. For control treatments, plants were sprayed with a solution containing 10 mM MgCl<sub>2</sub>, 0.04% Silwet Gold. To count the bacteria growth, leaf discs were excised at 3 dpi with a cork borer and shaken for 1 h in 500 µl of 10 mM MgCl<sub>2</sub> with 0.01% Silwet Gold. The bacteria number was calculated from serial dilutions grown on NYGA plates for two days.

### **2.3.4 DNA, RNA isolation and qRT-PCR analysis**

For DNA isolation, plant samples were collected in 1.5 ml microcentrifuge tubes and then ground to a fine powder using a plastic pestle in the tube itself with liquid nitrogen. DNA was isolated following the CTAB DNA Miniprep protocol as described (Clarke, 2009).

Total RNA was extracted from plant tissues with TRIzol® Reagent (Thermo Fisher Scientific) according to the manufacturer's instruction. The purity and concentration of RNA were measured with

GE Healthcare NanoVue Plus UV-Vis Spectrophotometer and evaluated by electrophoresis on an agarose gel. Subsequently, 1 µg of RNA was digested with DNase I (Thermo Scientific) for 30 min and reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The cDNA quality was checked by RT-PCR using the reference gene *Actin2*, and a twenty-fold dilution of the cDNA was made as a PCR template. qRT-PCR was conducted in 10 µl reactions with Maxima SYBR Green qPCR Master Mix (Thermo Scientific) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following conditions: 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Relative expression of mRNA was calculated by the delta-delta CT method (Livak and Schmittgen, 2001). And all values for the mRNA species were normalized to *Actin2*. qRT-PCR primers can be found in **Supplementary Table S2**.

### 2.3.5 Promoter::GUS analysis

The promoter region of *AtGLP5* ( $P_{AtGLP5}$ ) was amplified from genomic DNA with primer  $P_{AtGLP5}$ -F and  $P_{AtGLP5}$ -R comprising 1565 bp 5' upstream *AtGLP5* transcription start. The sequence was cloned into pGWB433 (Nakagawa *et al.*, 2007) in front of the β-glucuronidase (GUS) coding region. Stable transgenic *A. thaliana* plants harboring the  $P_{AtGLP5}$ ::GUS construct were generated through the floral dip. For histochemical GUS analysis, plant samples were incubated in GUS staining solution: 300 µl X-Gluc solution (30 mg X-glcA in 300 µl DMSO), 38 ml 0.2 M NaH<sub>2</sub>PO<sub>4</sub> solution, 62 ml 0.2 M Na<sub>2</sub>HPO<sub>4</sub> solution, 1 drop of Triton X-100, pH 7.0, for overnight at 37 °C. On the next day, the GUS staining buffer was carefully removed and replaced with 70% ethanol to bleach the green chlorophyll. Several changes of ethanol may be necessary until the tissues turned white. The stained samples were examined under a stereomicroscope (Zeiss).

For the different stresses, leaves of 4-week-old transgenic *A. thaliana* carrying  $P_{AtGLP5}$ ::GUS were collected at 24 hours after infection with *S. sclerotium* containing PDA agar plugs or *Pst* DC3000 bacteria suspension and after treatment of plant hormones (1 mM SA, 100 µM and 500 µM JA). For the wounding and flg22 treatment, leaves were collected at an earlier stage, at 5 hours after cutting with a pair of scissors or sprayed with 1 mM flg22, since the plant responded to these stimuli quite quickly. Control plants were either non-treated or sprayed with water. The detached leaves were then used for GUS staining and qRT-PCR analysis.

### 2.3.6 Subcellular GFP-localization

For subcellular protein localization of AtGLP5, AtGLP5-GFP transgenic plants were germinated on 1/2 MS medium with 50 mg/l kanamycin for one week. The positive seedling was then placed on a slide for observation of GFP under the Eclipse Ni-E Upright Motorized Microscope (Nikon) connected to the X-Cite 120 LED light source (Excelitas Technologies) for bright-field and fluorescence imaging aided with the NIS-Elements AR software (Nikon).

### 2.3.7 SOD activity assay

Transient expression of the recombinant proteins in *Nicotiana benthamiana* was carried out following the protocol of Witte (Witte *et al.*, 2004). In brief, an *Agrobacterium* suspension was infiltrated from the bottom side of the leaf, and the plants were left to grow continually in the climate chamber. At 5-6 days post infiltration, leaf material was harvested and stored at -80 °C until further processing.

Total protein was extracted from ~10 mg of transformed *N. benthamiana* leaf tissue by vigorously mixing with 110 µL extraction buffer (50 mM Tris/HCl pH 7.5, 2% SDS, 50 mM DTT). After centrifugation for 10 min at 13,000 rpm at 10 °C, 80 µL of the supernatant was supplemented with 20 µL 5 x gel loading buffer (0.25 M Tris-HCl, pH 6.8, 10% SDS, 30% glycerine, 0.02 % bromophenol blue) and loaded on a 10% SDS-PAGE without boiling. An in-gel SOD activity assay was performed following the protocol of Beauchamp and Fridovich (Beauchamp and Fridovich, 1971). The germin-like protein 12 from *B. napus* (BnGLP12) served as a positive control (Rietz *et al.*, 2012). For immunodetection, proteins were blotted on a PVDF membrane and visualized with an anti-HA antibody combined with a Lumi-Light<sup>PLUS</sup> Western Blotting Kit (Rocher *et al.*) following the manufacturer's instructions.

### 2.3.8 DAB staining to detect H<sub>2</sub>O<sub>2</sub> production in Arabidopsis plants

*S. sclerotiorum* was grown on PDA medium at 22 °C. After two or three days, agar-plugs were punched from the expanding margin of the mycelium using a 6 mm cork borer and placed on leaves of 5-week-old plants with the mycelium facing the leaf surface. One day after inoculation, the leaves were detached from the plant and immersed in fresh prepared 1 mg/ml DAB staining solution at RT for overnight. On the next day, the DAB staining buffer was carefully removed and

replaced with 70% ethanol to bleach the green chlorophyll. Several changes of ethanol may be necessary until the tissues turned white. The stained samples were examined under a stereomicroscope (Zeiss). The integrated density of DAB staining was then quantified with the ImageJ Colour Deconvolution plugin.

### **2.3.9 Statistical Analysis**

GraphPad Prism 8 computer software was used to perform all statistical analyses (GraphPad Prism, San Diego, CA, USA). The results were represented as means  $\pm$  S.E., and different numbers of asterisks indicated significantly different values. The significant difference between the two groups was performed using the student's *t*-test. And multiple comparisons of means were analyzed by one-way ANOVA, followed by Dunnett's multiple comparisons test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

## 2.4 Results

### 2.4.1 AtGLP5 is the ortholog of BnGLP3 and is induced upon *S. sclerotiorum* and *Pst* DC3000 infection

The *A. thaliana* genome contains 32 genes that are translated into germin-like proteins (UniProt, <https://www.uniprot.org/>). To identify the ortholog of BnGLP3 in *A. thaliana*, we aligned these sequences with the germin-like protein family (BnGLPs) of *B. napus* (Rietz *et al.*, 2012) and constructed a phylogenetic tree using the MEGA6 software (Tamura *et al.*, 2013). As shown in **Fig. 1**, only the protein sequence coded by *At1g09560* was closely related to BnGLP3. The corresponding protein had been named as germin-like protein 5 (GLP5) by Carter *et al.* (Carter *et al.*, 1998). Due to their high sequence similarity, we proposed that AtGLP5 represents the ortholog of BnGLP3 and thus possesses conserved functions between *A. thaliana* and *B. napus* and started to investigate the role of *AtGLP5* in regulating plant-pathogen interactions.

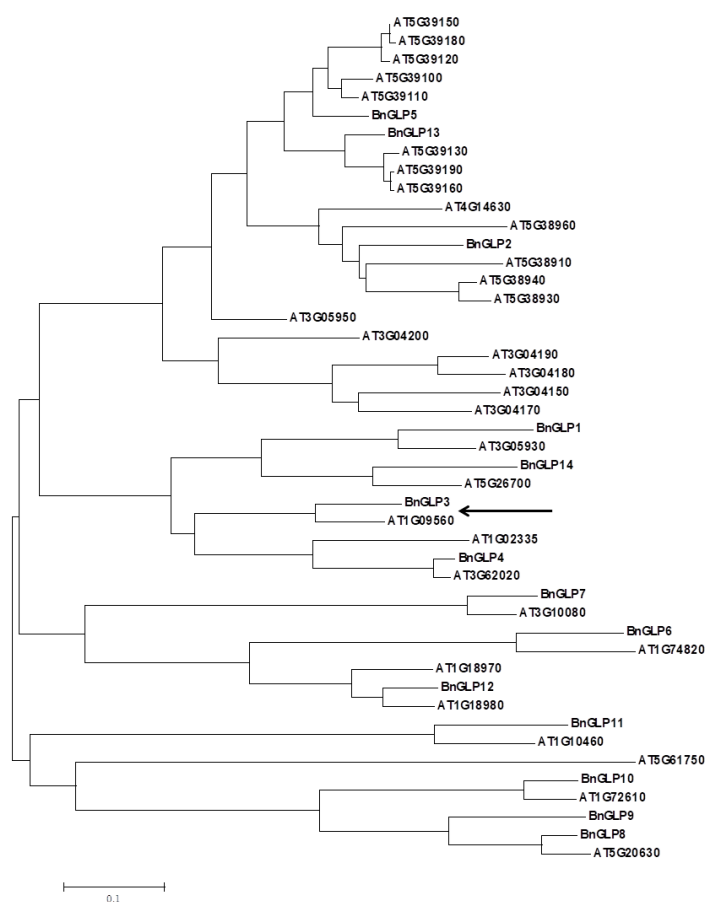


Fig. 1 Phylogenetic tree of germin-like proteins from *B. napus* and *A. thaliana*. Sequences were aligned with Dialign2.1, and the tree was calculated using Neighbor-joining analysis and Bootstrap method with 500 replications (MEGA6). The black arrow indicates the position of AtGLP5 (AT1G09560) and BnGLP3.

Usually, genes that are participated in plant defense are transcriptionally induced upon pathogen attack. To check whether *AtGLP5* might be involved in plant defense, we infected 5-week-old *A. thaliana* leaves with the necrotrophic fungi *S. sclerotiorum* and the bacterium *Pst* DC3000 and compared their transcript levels with control plants (**Fig. 2**). Upon *S. sclerotiorum* infection, *AtGLP5* transcription increased at a very early stage (3hpi), peaked at 6 hpi by about 5 fold, and then decreased gradually (**Fig. 2A**). This result suggested that *AtGLP5* might function in plant early-stage defense response. The induction of *AtGLP5* can also be observed when the plants were infected with *Pst* DC3000, although at a relatively slower pace (**Fig. 2B**). Taken together, we conclude that *AtGLP5* is involved in general plant responses to fungal and bacterial infection.

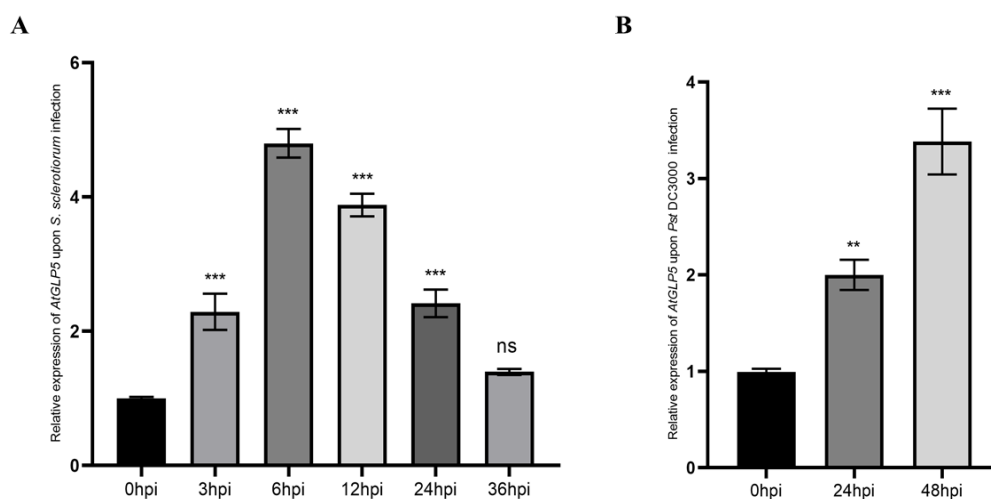


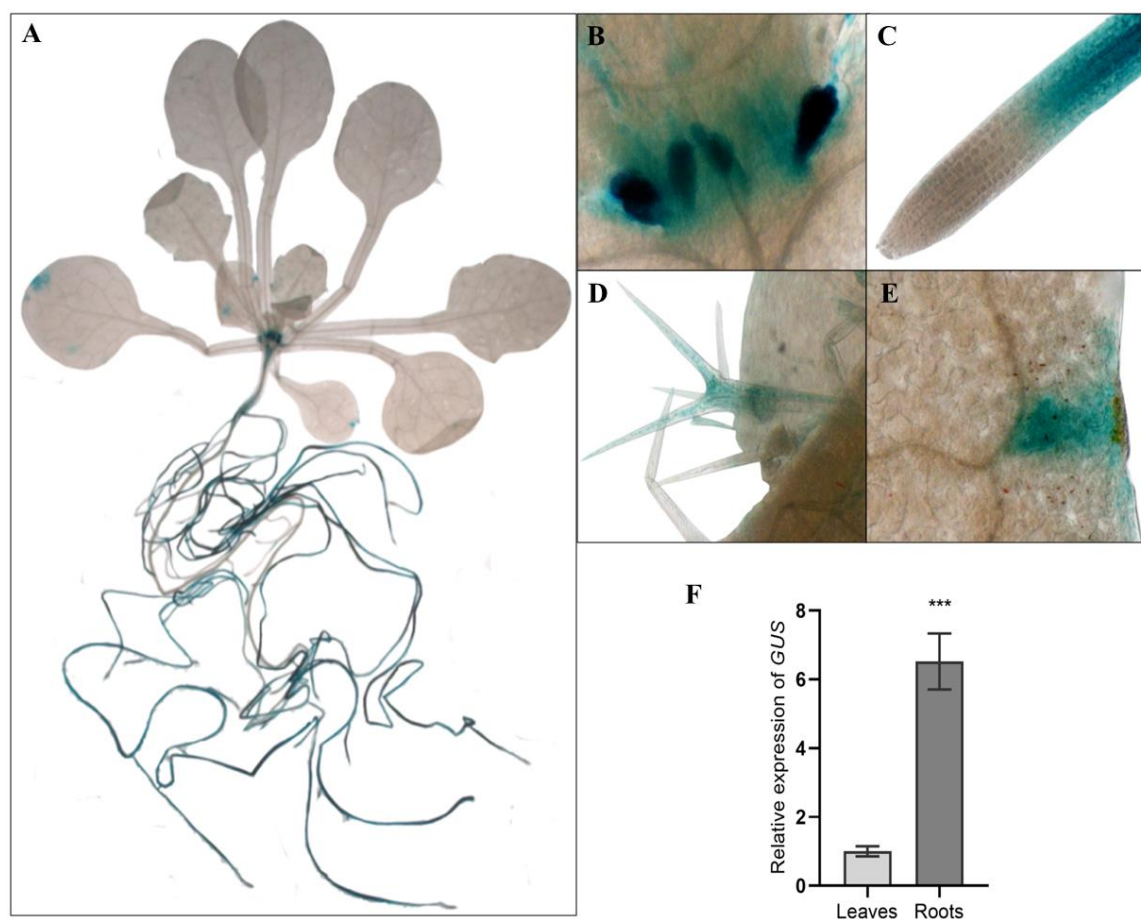
Fig. 2 Transcriptional regulation of *AtGLP5* upon *S. sclerotiorum* and *Pst* DC3000 infection was analyzed in wild-type Col-0 leaves. (A) *AtGLP5* was immediately up-regulated upon *S. sclerotiorum* infection, reached the highest level at 6 hpi, and then went down gradually until 36hpi. (B) The expression of *AtGLP5* was checked at two time points after the *Pst* DC3000 inoculation. *AtGLP5* was also induced, though at a slower pace compared with *S. sclerotiorum*. The relative expression levels were measured by qRT-PCR. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with one-way ANOVA, followed by Dunnett's multiple comparisons test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

#### 2.4.2 *AtGLP5* expression is tissue-specific and varies at different developmental stages

To investigate the expression pattern of *AtGLP5*, we then cloned its predicted promoter sequence and fused it to the  $\beta$ -glucuronidase (GUS) reporter gene and finally transformed the  $P_{AtGLP5}::GUS$  construct into *A. thaliana*.

GUS staining in the transgenic *A. thaliana* seedlings revealed that GUS activity was detected mainly in the root (**Fig. 3A**), slightly in the shoot apical meristem (**Fig. 3B**), and epithem, the

loosely arranged cells within hydathode close to the vascular ending of cotyledons (**Fig. 3E**), but was absent at the root apex, where cell division occurs (**Fig. 3C**). A weak GUS activity could be observed in the star-shaped trichomes on the first true leaves (**Fig. 3D**). Surprisingly, no signal could be detected in the true leaves (**Fig. 3A**). This observation is consistent with the expression data from the *Arabidopsis* eFP browser, which shows an abundant expression of *AtGLP5* in the roots at the vegetative stage (**Fig. S1**) (<http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>). We also evaluated the transcript level of the GUS gene in the roots and leaves in 10-day-old transgenic seedlings by qRT-PCR. A higher *GUS* transcription level in the roots as compared with its low expression in the leaves showed a correlation with the distinct intensities of GUS activities observed in different tissues, as illustrated by histochemical staining (**Fig. 3A and 3F**).



**Fig. 3** Histochemical GUS staining and GUS expression analysis in transgenic plants containing the *P<sub>AtGLP5</sub>::GUS* construct. (A-E) Histochemical GUS staining of 20-day-old seedlings. GUS activity can be detected mainly in the root (A), shoot apical meristem (B), trichome (D), hydathode (E), but not in the root tip (C). (F) Similarly, the expression of the *GUS* gene in the roots was significantly higher than in the leaves. The relative expression levels were measured by qRT-PCR. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

Additionally, after the reproductive phase transition, GUS activity was detected in the floral organs. The GUS activity was also observed in the nectaries at the base of stamens throughout the maturing process (**Fig. S2**). A weak GUS staining was given in the style and the narrow replum structure of the gynoecium (**Fig. S2A, B**). However, following flower opening, the signal shrank rapidly to the limited style region of the developing fruit (**Fig. S2C**) and finally disappeared as the siliques were ripening (**Fig. S2D, E**). These observations suggested that *AtGLP5* is involved in multiple plant physiological and developmental processes.

#### **2.4.3 The expression of *AtGLP5* is induced by pathogens and plant hormone salicylic acid as well as by wounding**

To understand the transcriptional regulation of *AtGLP5*, we then tested its responsiveness to various biotic stimuli. Since the expression of *AtGLP5* was upregulated after the inoculation of plant pathogens *S. sclerotiorum* and *Pst* DC3000 in plants (**Fig. 2**), we first checked the effect of these two pathogens on GUS expression. As shown in **Fig. 4A** and **Fig. S4**, a pronounced induction of GUS activity at the infection sites was observed after the inoculation with both pathogens. Strikingly, an intense GUS activity was observed in mature leaves at the infection sites. In contrast, no GUS staining appeared in the control leaves, thus, strongly suggesting a pathogen-responsive expression of *AtGLP5*.

Next, we tested the effect of the exogenic application of plant hormones, salicylic acid (SA) and jasmonic acid (JA) on *AtGLP5* expression, respectively. We observed that only SA could induce GUS expression (**Fig. 4A**). In support of this, even with a higher concentration of 500  $\mu$ M JA, no GUS expression was induced in the leaves (data not shown). We also performed a qRT-PCR analysis to determine the regulation of *AtGLP5* expression in response to SA and JA treatment in wild-type *A. thaliana* plants. Consistently, the expression of *AtGLP5* was induced by SA but not by JA (**Fig. 4B**).

In a next step, we examined whether mechanical wounding could activate the GUS expression as there are several predicted wound-responsive elements in the promoter sequence. As shown in **Fig. 5A**, an intense GUS activity was detected at both sides of the cutting edges, suggesting a wounding inducing nature of the *AtGLP5* expression.



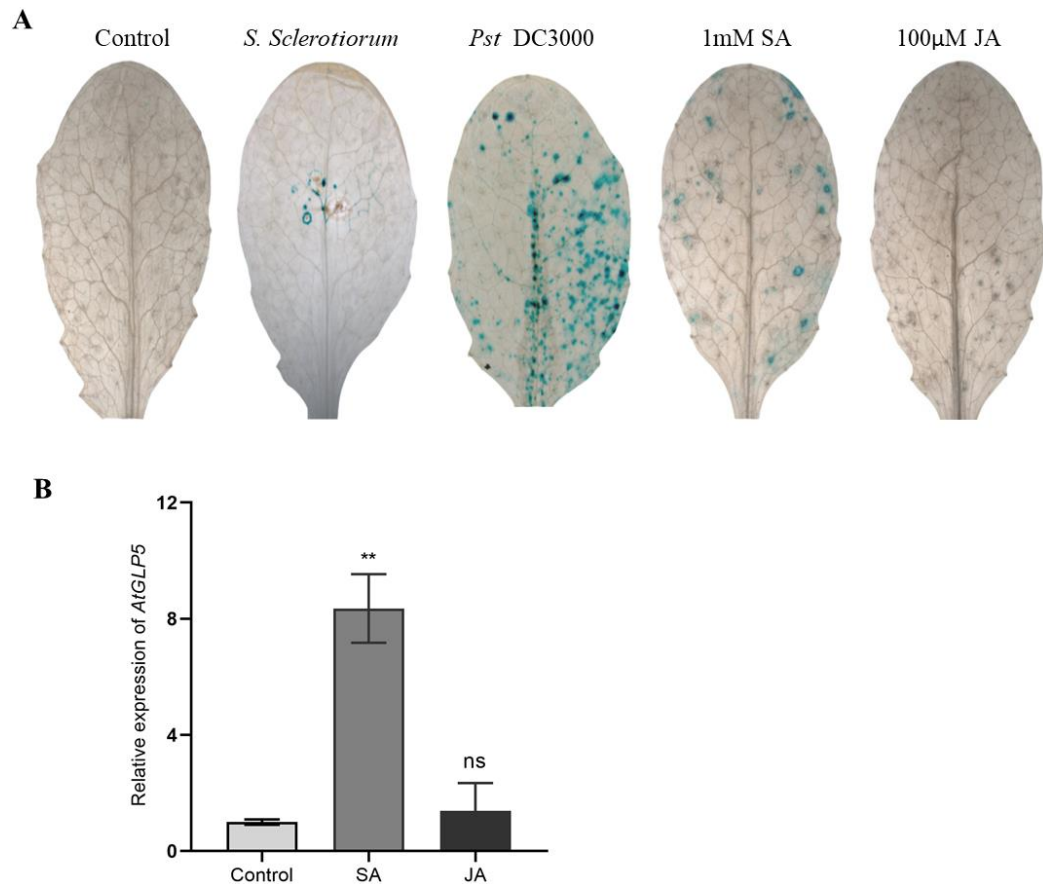


Fig. 4 Expression of *AtGLP5* in response to pathogen attacks and plant hormone treatments. (A) GUS expression was activated in transgenic plant leaves treated with *S. sclerotiorum*, *Pst* DC3000, and SA, but not JA. (B) The transcript level of *AtGLP5* was also significantly up-regulated upon SA treatment but not JA treatment. The relative expression levels were measured by qRT-PCR. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

#### 2.4.4 The expression of *AtGLP5* is activated by treatment with flg22

The GUS expression activated by wounding provoked us to address whether the observed induction of *AtGLP5* expression by pathogens might also be a wounding effect due to cellular damage caused by pathogen invasion. To distinguish between these two effects, we treated the transgenic plants carrying the  $P_{AtGLP5}::GUS$  construct with flg22, a synthetic bacterial flagellin peptide (Misra *et al.*, 2019), which can trigger plant immune response without causing mechanical damage to the plant tissue. As a result, the GUS expression was induced also by treatment with flg22 (Fig. 5A), providing evidence that *AtGLP5* responds to plant pathogens and wounding through two separate signaling pathways. In accordance, the expression of *AtGLP5* was significantly upregulated in Col-0 plants after injuring or flg22 treatment (Fig. 5B).

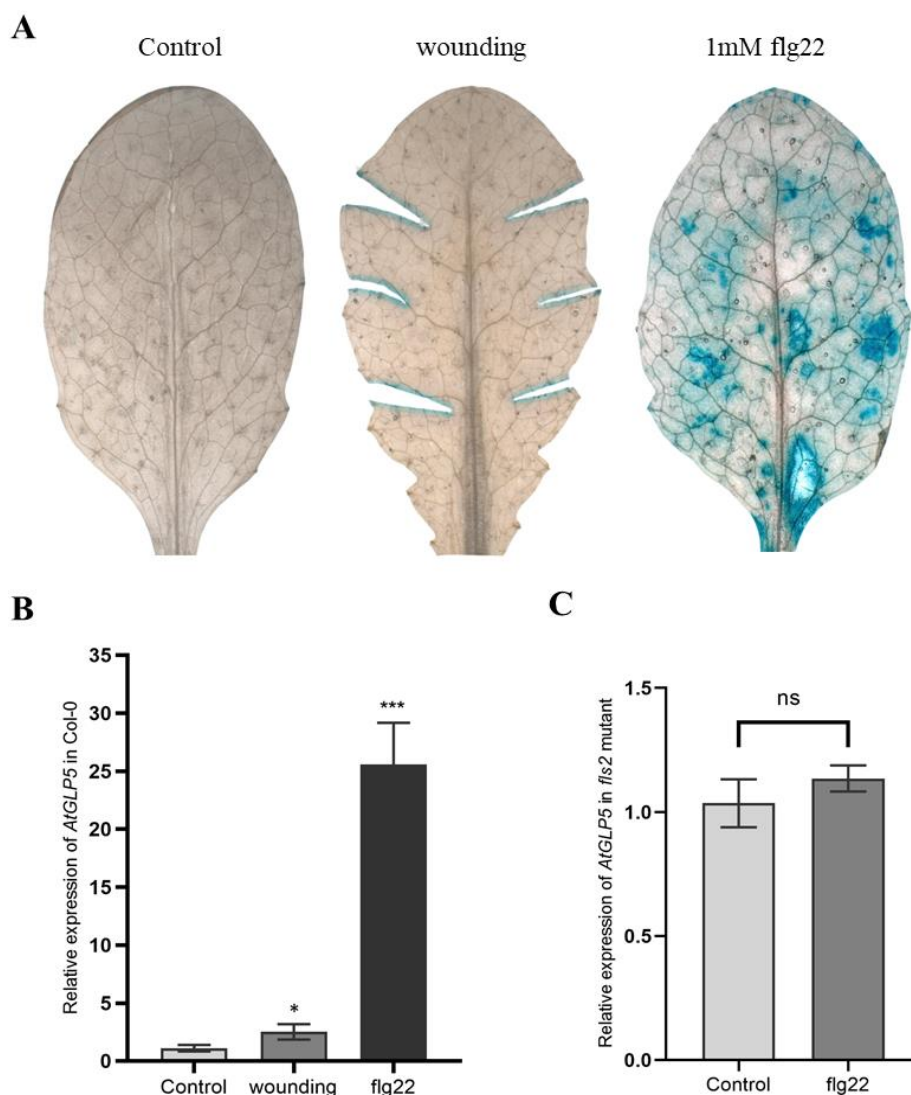


Fig. 5 The expression of *AtGLP5* in response to wounding and flg22 treatments. (A) GUS staining can be detected in transgenic plant leaves treated by wounding or flg22. (B) An accordant higher transcription of *AtGLP5* was observed in wounding and flg22 treated leaves compared with that of control leaves. (C) The up-regulation of *AtGLP5* by flg22 was abolished in the *fls2* mutant. The relative expression levels were measured by qRT-PCR. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

Flagellin perception in *Arabidopsis* is sensed by the flagellin-sensitive 2 (FLS2) receptor. *fls2* mutants that lack the FLS2 receptor are expected to be insensitive to flg22 treatment (Gómez-Gómez and Boller, 2000). To confirm the involvement of *AtGLP5* in the FLS2-mediated plant defense response, we further analyzed the responsive expression of *AtGLP5* in *Arabidopsis fls2* mutant upon flg22 treatment. Results shown in **Fig. 5C** demonstrated that the transcription of *AtGLP5* was not affected after flg22 treatment in *fls2* mutant compared with that observed in the wild-type Col-0 plants. These data prove that *AtGLP5* was induced during PTI.

### 2.4.5 AtGLP5 is localized at the plasma membrane and exhibits SOD activity

Protein localization in the cell is generally essential for its biological function and can hint about its active mode. To determine the subcellular location of AtGLP5, we fused its coding region with a green fluorescent protein (GFP). We expressed this fusion protein AtGLP5-GFP under the control of the CaMV 35S promoter stably in *A. thaliana*. The 7-day-old seedlings of transgenic *A. thaliana* were used for subcellular localization observation. As shown in **Fig. 6**, in the leaf epidermal cells, the GFP fluorescent signal is tightly associated with the cell wall, suggesting its localization at the plasma membrane.

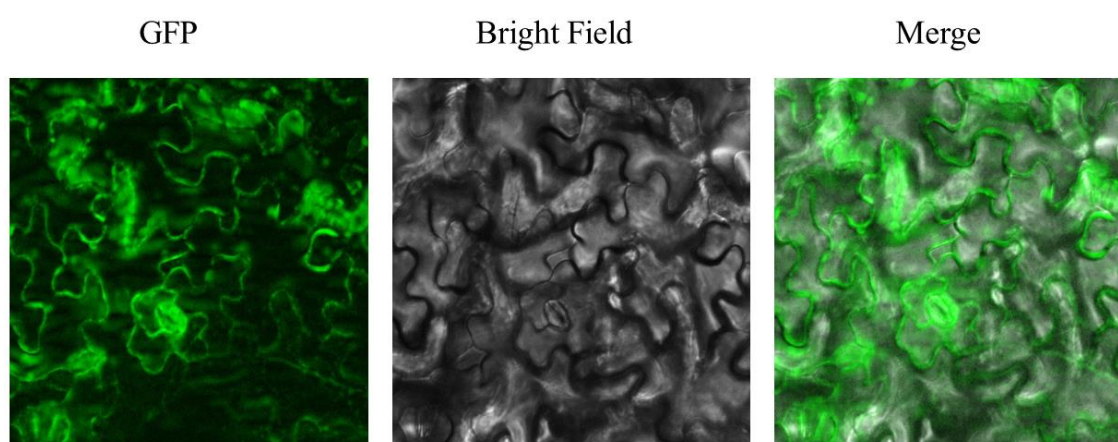


Fig. 6 Subcellular localization of AtGLP5 under a fluorescence microscope. The AtGLP5-GFP construct was transformed into *A. thaliana* to observe the protein localization. Leaves of 7-day-old seedlings were examined under a Nikon Upright Motorized Microscope. AtGLP5 is localized at the plasma membrane.

Up to now, there is no report that AtGLPs is associated with enzyme activity. Since BnGLP3 and BnGLP12 are proved to possess superoxide dismutase (SOD) activity and participate in plant early defense responses (Rietz *et al.*, 2012), we assumed that AtGLP5 might have similar biological activity as its homolog BnGLP3. To test this, AtGLP5 protein with a C-terminal triple HA-tag (AtGLP5:HA) was transiently expressed in *N. benthamiana* leaves and extracted in the presence of 2% SDS and 50 mM DTT. Extraction without SDS or lower concentration did not yield detectable amounts of AtGLP5:HA (data not shown). Total protein was separated in an SDS-PAGE without prior boiling of the samples and blotted onto PVDF membrane for HA-specific immunodetection or developed for SOD activity in the gel. We also included the recombinant BnGLP12:HA protein, shown previously to possess SOD activity as a positive control. As depicted in **Fig. 7B**, BnGLP12:HA and AtGLP5:HA migrated in the gel at higher and

lower molecular weight sizes. The latter conformed to the calculated molecular weights of both GLPs of ~29 kDa, and the higher molecular weight bands probably represented complex formations. While most of BnGLP12:HA protein migrated at a size above 150 kDa, most AtGLP5:HA was present between 30~60 kDa under this extraction condition. Since the protein samples were not fully denatured by heating prior loading, protein migration in the gel may differ from the molecular weight standards and allow only limited interpretation of the exact molecule sizes. When the protein extracts were tested for SOD activity, clear signals appeared in samples with BnGLP12:HA and AtGLP5:HA, demonstrating that AtGLP5 also has SOD activity. As expected, most SOD activity was associated with higher molecular weight complexes (**Fig. 7C**).

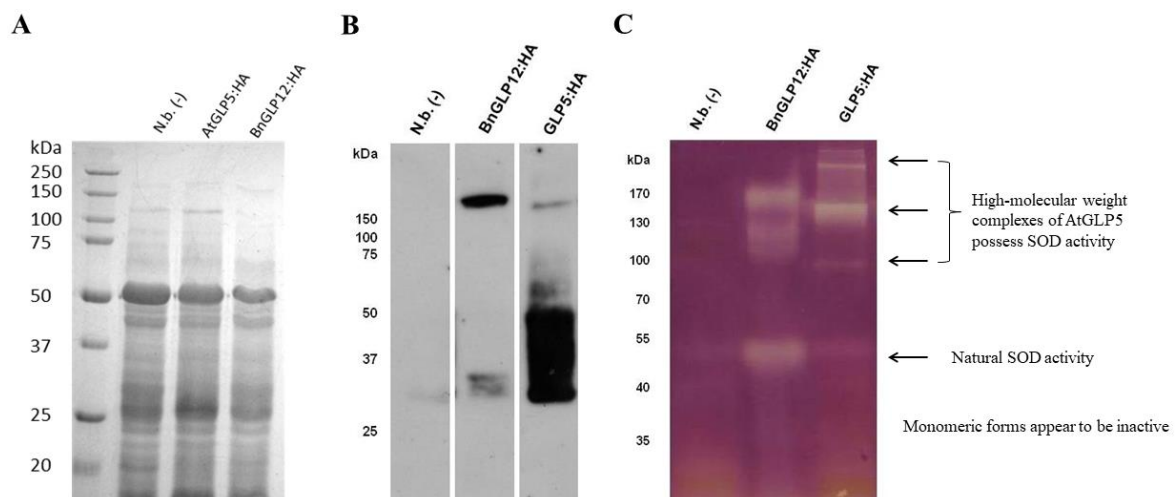


Fig. 7 SOD in-gel assay with total protein extracts from *N. benthamiana* leaves transiently expressing proteins. (A) Coomassie staining of total proteins of *N. benthamiana* leaves. (B) Immunodetection of recombinant proteins with HA-specific antibody. (C) Protein extracts were tested for SOD activity. N.b.(-): non-infiltrated tobacco leaves served as a negative control; BnGLP12:HA: tobacco leaves transiently expressing BnGLP12:HA served as a positive control; AtGLP5:HA: tobacco leaves transiently expressing AtGLP5:HA.

#### 2.4.6 *AtGLP5* is involved in regulating plant root growth and development

To observe the role of *AtGLP5* in plant root development, we first analyzed *Atglp5* knockout mutants and transgenic plants overexpressing *AtGLP5* compared with wild-type Col-0 plants. The *Atglp5* mutants (**Fig. S3**) showed no significant difference in root length when compared with wild-type. However, overexpression of *AtGLP5* resulted in shortened plant primary roots (**Fig. 8A**), suggesting a functional role of *AtGLP5* in root development. Auxin is an essential hormone

regulating root growth and development. To check if *AtGLP5* is regulated by auxin, we analyzed the GUS expression in 7-day-old transgenic seedlings treated with exogenous auxin application by incubating the roots in IAA and IBA solution. No noticeable change in GUS activity was observed compared with control plants (**Fig. S5**), suggesting that *AtGLP5* possibly affects root development through regulation of the auxin-mediated downstream pathway.

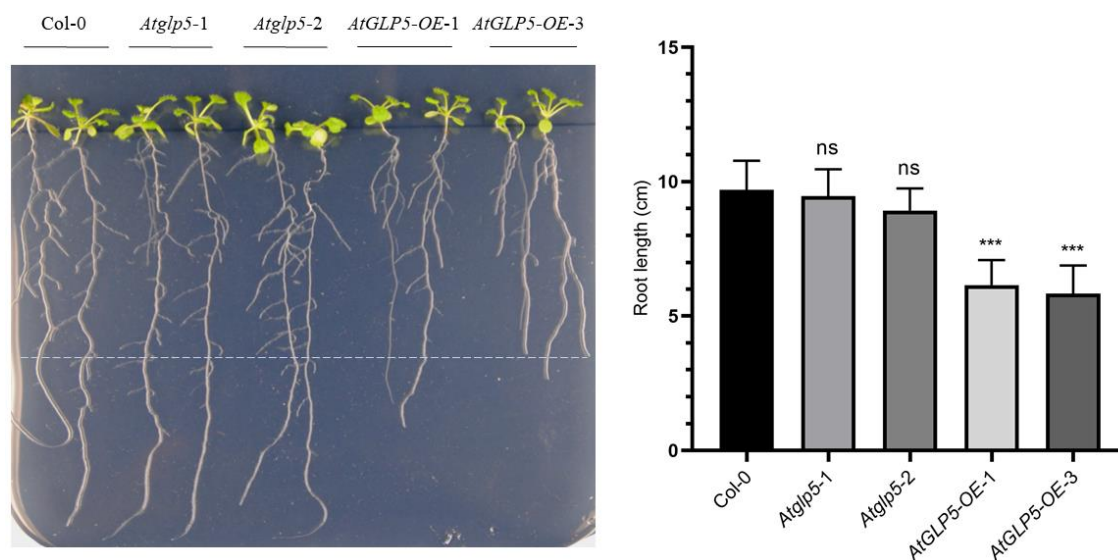


Fig. 8 *AtGLP5* affects *A. thaliana* root growth and development. Overexpression of *AtGLP5* reduced the length of plant primary root, while the knockout mutants displayed no obvious phenotype. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments, with at least 8 plants for every independent experiment. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

#### 2.4.7 Knock out of *AtGLP5* reduces plant resistance to *S. sclerotiorum* and *Pst* DC3000

To test whether the loss of *AtGLP5* function compromises plant resistance, we identified two independent T-DNA insertion mutants in the coding region of *AtGLP5* that lack *AtGLP5* transcripts (**Fig. S3**) and then challenged the mutant plants with *S. sclerotiorum* and *Pst* DC3000. At two days post-inoculation with *S. sclerotiorum*, the lesion sizes on *Atglp5-1* and *Atglp5-2* leaves were significantly larger than wild-type Col-0 (**Fig. 9A**). Similarly, the disease symptoms on the mutants after challenge with *Pst* DC3000 were also more severe, and the bacteria growth was enormously increased in *Atglp5-1* and *Atglp5-2* leaves compared with the growth in Col-0 (**Fig. 9B**). These data add more evidence that *AtGLP5* plays a role in plant general disease resistance.

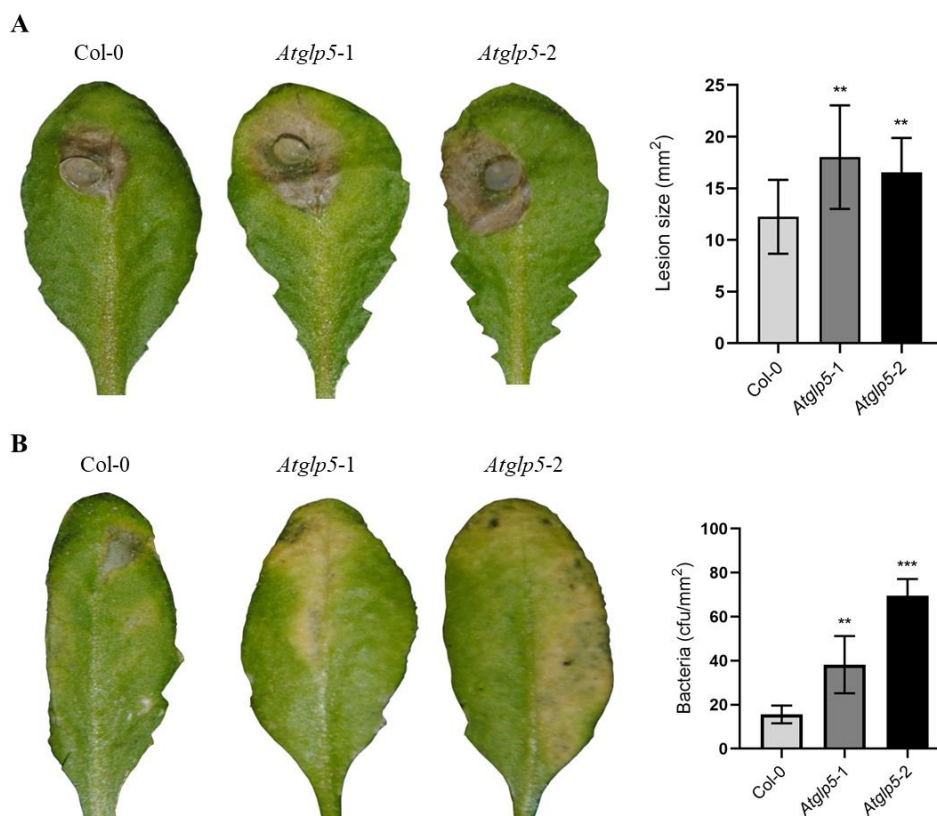


Fig. 9 Loss of function of *AtGLP5* in response to pathogen infection. (A) The knockout mutant developed larger leaf lesions after *S. sclerotiorum* infection than Col-0 plants. (B) Three days after infection, both knockout mutants showed more pronounced disease symptoms and had a higher number of bacteria growth than Col-0 plants. Standard errors are denoted as black lines. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

#### 2.4.8 Overexpression of *AtGLP5* enhances plant resistance to *S. sclerotiorum* and *Pst* DC3000

To confirm the putative function of *AtGLP5* in plant-pathogen interaction, we also generated transgenic *A. thaliana* plants overexpressing *AtGLP5*. The transgenic plants were screened by growing on 1/2 MS medium containing 50 mg/l kanamycin, and the elevated expression of *AtGLP5* in the positive plants was confirmed by qRT-PCR. Two independent overexpression lines were used for further analysis (Fig. 11A). We then carried out infection experiments to determine whether overexpression of *AtGLP5* improves plant resistance to pathogens.

For the Sclerotinia disease test, two infection methods were carried out (agar plugs and *S. sclerotiorum* mycelial suspension). At two days post-inoculation with agar plugs, the average lesion sizes on the leaves of transgenic overexpression plants were about 30% smaller than

wild-type plants (**Fig. 10A**). Likewise, three days after spraying mycelial suspension, the disease symptoms on the Col-0 plants were more severe than the transgenic plants (**Fig. 10B**). Since *AtGLP5* was demonstrated to exhibit SOD activity, we further detected  $H_2O_2$  in the transgenic and wild-type plants at one day after *S. sclerotiorum* infection using 3-3'-Diaminobenzidine (DAB) staining. DAB can be oxidized by  $H_2O_2$  and form a brown precipitate. This precipitate is used as a stain to observe the distribution of  $H_2O_2$  in plant cells (Daudi and O'Brien, 2012). As shown in **Fig. 10C**, both OE lines accumulated higher level of  $H_2O_2$  in response to *S. sclerotiorum*. As for the *Pst* DC3000 infection, more pronounced disease symptoms and a higher level of bacteria growth were observed in the Col-0 plants compared to transgenic plants at 2dpi (**Fig. 10D**). These results demonstrated that overexpression of *AtGLP5* increased plant basal resistance against both *S. sclerotiorum* and *Pst* DC3000.

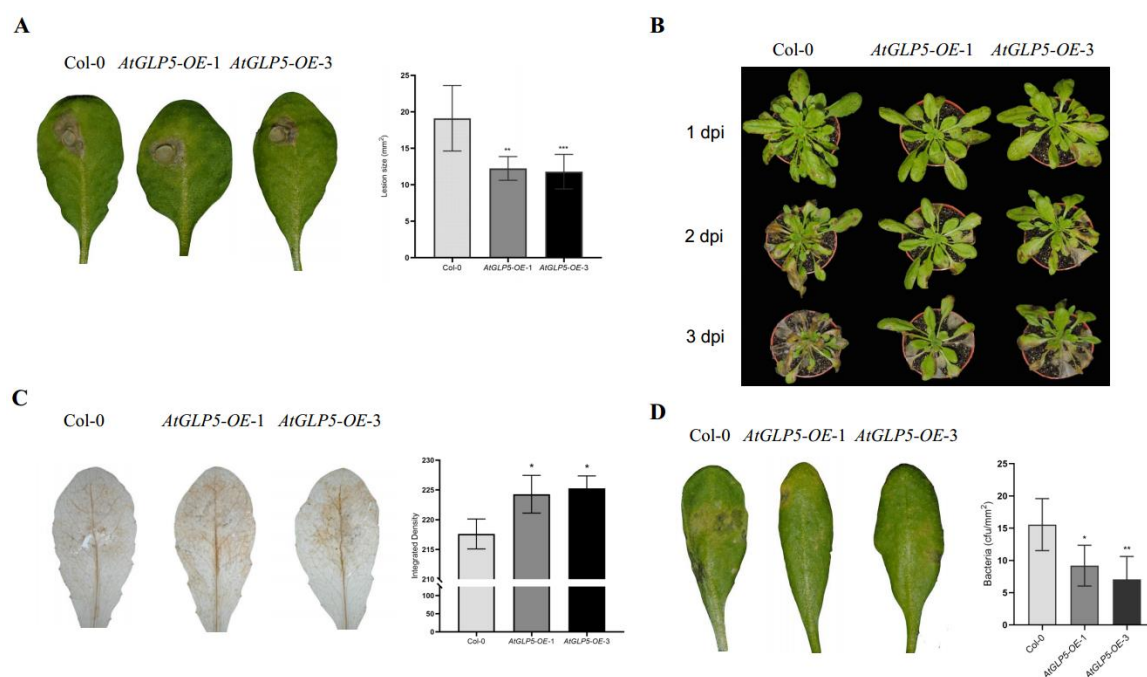


Fig. 10 Overexpression of *AtGLP5* enhanced plant disease resistance in *A. thaliana*. (A) Arabidopsis leaves were inoculated with agar plugs containing actively growing cultures of *S. sclerotiorum*. Disease lesions were measured at 2dpi. (B) Whole plants were sprayed with *S. sclerotiorum* mycelial suspension. A and B suggested that overexpression of *AtGLP5* enhanced plant resistance to *S. sclerotiorum*. (C) *AtGLP5-OE* transgenic plants produced more  $H_2O_2$  upon *S. sclerotiorum* infection compared with wild-type Col-0 plants. (D) Col-0 plants exhibited more pronounced chlorosis and water-soaking phenotype after *Pst* DC3000 infection. And a lower level of bacteria growth was also observed in the transgenic overexpressing plants. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

### 2.4.9 *AtGLP5* constitutively activates the expression of defense-related genes

To characterize the role of *AtGLP5* in plant defense response, we then assessed the transcript levels of a list of plant defense marker genes in the non-inoculated overexpression and wild-type plants by qRT-PCR. As shown in **Fig. 11A**, transgenic plants overexpressing *AtGLP5* accumulated higher transcripts of *PDF1.2* and *NCED3* associated with the plant JA and ABA signaling pathways. In *A. thaliana*, *flg22* signaling can activate a MAP kinase cascade to regulate the expression of early-defense response genes (Asai *et al.*, 2002). We found that the expression of *MPK3*, *MPK4*, and *MPK6* in transgenic plants were also significantly elevated (**Fig. 11B**). As *WRKY* transcription factors are essential regulators of numerous defense responses, and 27 of them were reported to be induced following *flg22* treatment (Birkenbihl *et al.*, 2018), we further investigated the expression of *WRKY* genes including *WRKY18*, *WRKY22*, *WRKY33*, *WRKY40*, and *WRKY53*. As shown in **Fig. 11B**, all these genes were significantly upregulated in the *AtGLP5* overexpression plants. Taken together, we conclude that *AtGLP5* activates the expression of a multiple of defense-related genes, implying its vital role in the plant immune system, in general.

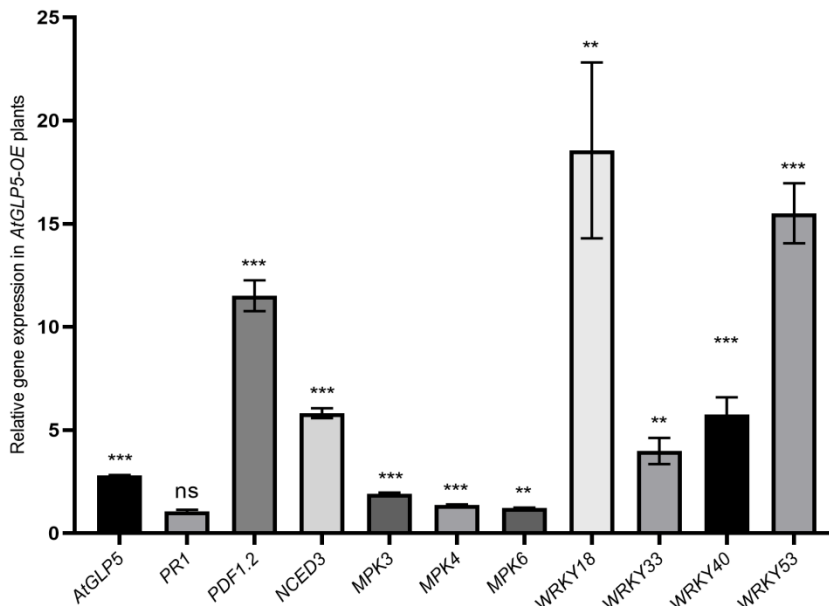


Fig. 11 Transcript profiles of defense-related genes in *AtGLP5* overexpression plants compared with wild-type Col-0 plants. *AtGLP5* expression levels were higher in the transgenic plants. Expression of *PDF1.2* and *NCED3* associated with JA and ABA signaling pathways was elevated in the transgenic overexpression plants. The *MPK* and *WRKY* family genes were also up-regulated. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).



## 2.5 Discussion

In our previous work, we demonstrated that *BnGLP3* is transcriptionally upregulated after *S. sclerotiorum* infection and had SOD activity (Rietz *et al.*, 2012). Here, we report that *AtGLP5*, a homolog gene of *BnGLP3* in *A. thaliana*, responds not only to *S. sclerotiorum* but also to *Pst* DC3000 and other stress factors such as wounding. Additionally, our data suggest that *AtGLP5* might fulfill multiple functions in regulating plant physiological, developmental and defense processes.

An important finding of this study is the organ-specific expression of *AtGLP5*. In the young seedlings (vegetative stage), *AtGLP5* was mainly expressed in the root tissue, consistent with the data from its developmental map on the Arabidopsis eFP browser *AtGLP5* is highly accumulated in the root. Nevertheless, transgenic plants expressing the  $P_{AtGLP5}::GUS$  showed that the *AtGLP5* expression was not observed in the root apical meristem. Also, a slight GUS activity was detected at the shoot apical meristem and hydathode in the margins of seedling cotyledons. The shoot apical meristem consists of undifferentiated, dividing stem cells located at the tip of the shoot. It keeps initiating new organs, including leaves, stems, and flowers throughout the entire postembryonic phase of plant life (Gaillochet *et al.*, 2015). Hydathode is a highly specialized structure positioned at the leaf margin which is responsible for mediating the excretion of plant liquid droplets, a process also called guttation, involving the transport of water, nutrients, metabolites, hormones, and pathogenesis-related proteins (Pilot *et al.*, 2004; Singh, 2014). Some of the trichomes on the young leaves also showed a weak GUS expression. Trichomes are specialized unicellular epidermal cells and exist as a kind of defense mechanism of the plant. They function primarily to block, e.g., harmful UV radiation from reaching the plant leaves (Yan *et al.*, 2012). In some species, trichomes contain chemicals that contribute to plant resistance against herbivores (Handley *et al.*, 2005; Tian *et al.*, 2012). No GUS activity was detected in the mature leaves. After the flowering transition (reproductive stage), the shoot apical meristem ceases to produce vegetative leafy shoots and converts to an inflorescence meristem, producing the sepals, petals, stamens, and carpels of the flower instead (Irish, 2010).

Interestingly, we observed that the expression of GUS in the meristematic dome also continued through this floral conversion and was observed in floral organs like nectaries and some parts of

the gynoecium. The nectary is known to be accountable for the synthesis and secretion of plant nectar. Nectar comprises sugars and various other components, including amino acids, alkaloids, flavonoids, free fatty acids, etc. (Heil, 2011). These secreted compounds' functions are distinct, ranging from rendering nectar unpalatable to nectar thieves to ward off microbial infection and providing additional nutrition to pollinators to achieve efficient pollination (González-Teuber and Heil, 2009; Kram *et al.*, 2009). The gynoecium, the female reproductive organ of angiosperms, is a highly complex organ. Morphologically, it consists of three distinct regions: the bottom region gynophore, the middle section ovary, and the apical-most region, consisting of style and stigma (Hawkins and Liu, 2014; Trigueros *et al.*, 2009). The style connects the apical stigma with the sizeable central ovary. The expression of *AtGLP5* observed in nectary and gynoecium suggested that it might regulate nectar secretion and fruit development. Based on these data, it is reasonable to speculate that *AtGLP5* may be involved in plant growth and development. Secretory tissues occur in most vascular plants (Fahn, 1988). These secretory structures' primary function is mostly related to the defense response against both pathogens and herbivores (Lange, 2015). *AtGLP5* expression was tightly associated with hydathodes, trichomes, and nectaries, which were all external secretory organs, provides a strong hint at its function in regulating the plant secretory system.

Noticeably, the expression of *AtGLP5* can be highly induced in the leaves in response to various stress factors. Both the necrotrophic fungi *S. sclerotiorum* and the hemibiotrophic bacterium *Pst* DC3000 can induce GUS expression in the leaves. We believe that the gene *AtGLP5* might be functional in the plant immune system against pathogenic attacks in general. Plant hormones also play a vital role in plant defense and developmental regulation (Shigenaga *et al.*, 2017). It is well known that JA and ethylene (ET) signaling pathways are induced in *A. thaliana* after the infection with necrotrophic pathogens, such as *Botrytis cinerea* (Govrin and Levine, 2002). Differing from this, in this study, the *AtGLP5* expression responded to the necrotrophic fungus *S. sclerotiorum* and the plant hormone SA, but not to JA. This result may exemplify a complex interaction between different signaling pathways and the underlying crosstalk between these regulations during plant defense response (Schenke and Cai, 2020).

Considering that there are several predicted wound-responsive elements in the *AtGLP5* promoter region, we also demonstrate experimentally that the expression of *AtGLP5* can be induced by wounding. This finding prompted us to address whether the induced expression of *AtGLP5* by *S. sclerotiorum* and *Pst DC3000* is attributed to pathogen-specific activation or a mechanical wounding caused by the pathogen invasion process. Evidently, the treatment by flg22, a synthetic bacterial flagellin peptide alone, also leads to the GUS expression in leaves, thus demonstrating that *AtGLP5* responds to wounding and plant pathogen attacks independently.

FLS2/flg22 is one of the best-studied receptor-ligand models in *A. thaliana*. The molecule flg22 can initiate a complicated signaling cascade to trigger plant immune response, as demonstrated in many cases (Bentham *et al.*, 2020). This process relies on the receptor FLS2 and FLS2-mediated signaling pathway (Jelenska *et al.*, 2017). The plant receptor kinase BAK1 acts as a partner of FLS2 to induce an array of immune responses (Chinchilla *et al.*, 2009). To verify if *AtGLP5* participates in this flg22-responsive cascade, we tested the regulation of *AtGLP5* upon flg22 treatment in the *fls2* mutant, which lacks the FLS2 receptor and compared with the wild-type Col-0. As expected, the induction of *AtGLP5* in response to flg22 was impaired in the *fls2* mutant compared with the wild-type. Even though it is not clear by what mechanism is *AtGLP5* functional in this pathway, the participation of *AtGLP5* in the FLS2-mediated signaling pathway provides a highly interesting aspect to understand the FLS2-mediated plant immune system and underlying mechanism deeply. In the light of its plasma membrane-localization, as revealed by GFP detection, we propose that *AtGLP5* may function like BAK1 as a co-receptor or efficiently activating the FLS2-downstream signalling.

So far, there is no report about germin-like proteins in *A. thaliana* exhibiting SOD activity. It has been demonstrated that germin proteins or germin-like proteins are of great potential for genetic engineering for improving plant resistance, partially because of their enzymatic function as O<sub>x</sub>O or SOD, producing H<sub>2</sub>O<sub>2</sub>, which can modulate cell signaling. Following this, it is tempting to believe that *AtGLP5* might harbor SOD activity due to its homolog BnGLP3. In this study, to our knowledge, we demonstrate for the first time that *AtGLP5* is a superoxide dismutase (SOD). Further analysis of the role of its SOD activity in regulating plant immune system against pathogen attacks is needed to shed more light on the underlying mechanisms.

We comparatively characterized the root developments of *AtGLP5* overexpression and knockout transgenic plants compared with wild-type plants. While *AtGLP5* overexpressing transgenic plants showed shortened primary root length, knockout of *Atglp5* exhibited average root growth. A similar observation had been reported by Ham et al. (Ham *et al.*, 2012) that germin-like proteins PDGLP1 and PDGLP2 interfered with plant root growth and development. The transgenic plants overexpressing PDGLP1/2 proteins displayed shortened primary roots. However, neither *pdglp1* or *pdglp2* single mutant nor *pdglp1 pdglp2* double mutant showed any visible interrupted root growth phenotype. The expression of PDGLP1 and PDGLP2 seemed to be insensitive auxin (Ham *et al.*, 2012).

Lastly, the *AtGLP5* gain and loss of function analysis in transgenic plants provide evidence for the involvement of *AtGLP5* in plant immune response to pathogen attacks. Our results showed that overexpression of *AtGLP5* enhanced plant resistance, while its knockout mutants displayed enhanced susceptibility to both pathogens tested. Besides, we identified several defense-related genes that are affected in transgenic plants overexpressing *AtGLP5*, including *PDF1.2* and *NCED3*. As expected, several *MAPK* and *WRKY* genes that participate in the FLS2-mediated signaling pathway were found to be significantly upregulated. Plant *WRKY* transcription factors are critical regulators in regulating the expression of defense-related genes and the crosstalk between SA and JA pathways. For example, *AtWRKY53* acts in a complex signaling network regulating plant senescence and biotic/abiotic stresses (Miao *et al.*, 2004). *AtWRKY33* was reported to confer plant disease resistance to necrotrophic pathogens (Zheng *et al.*, 2006). It is reasonable to assume that *AtGLP5* probably contributes to plant defense by activating these *WRKY* transcription factors, finally leading to plant defense activation.

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## Supplementary data

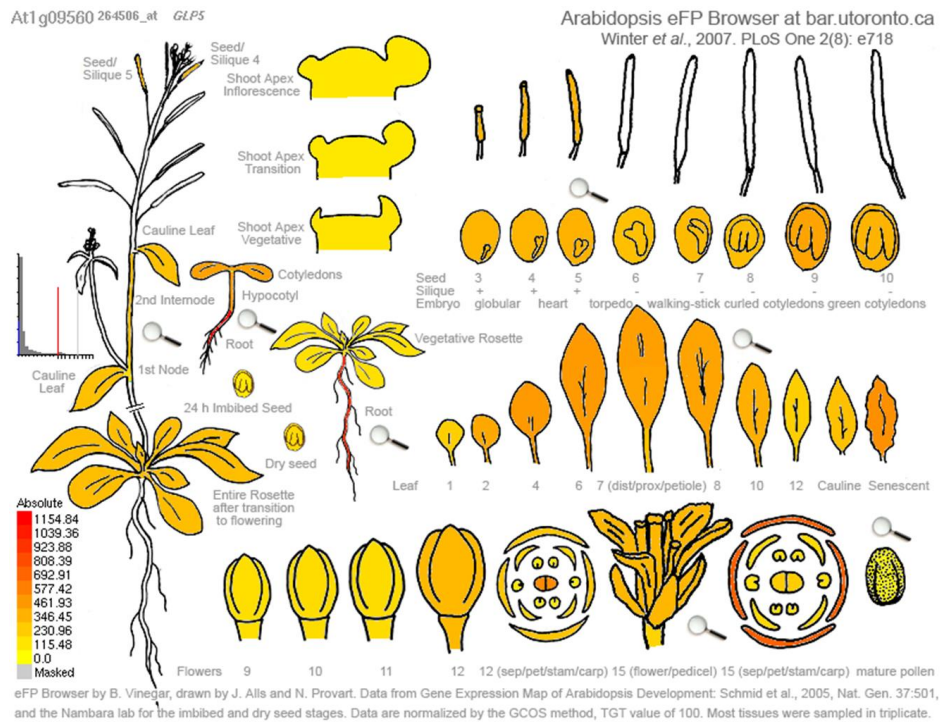


Fig. S1 Expression patterns of *AtGLP5* during Arabidopsis development based on eFP browser. The expression of *AtGLP5* is highest in the roots. The relative intensity of the color indicates mRNA abundance in different tissues at different developmental stages (yellow = absent or low, red = high).

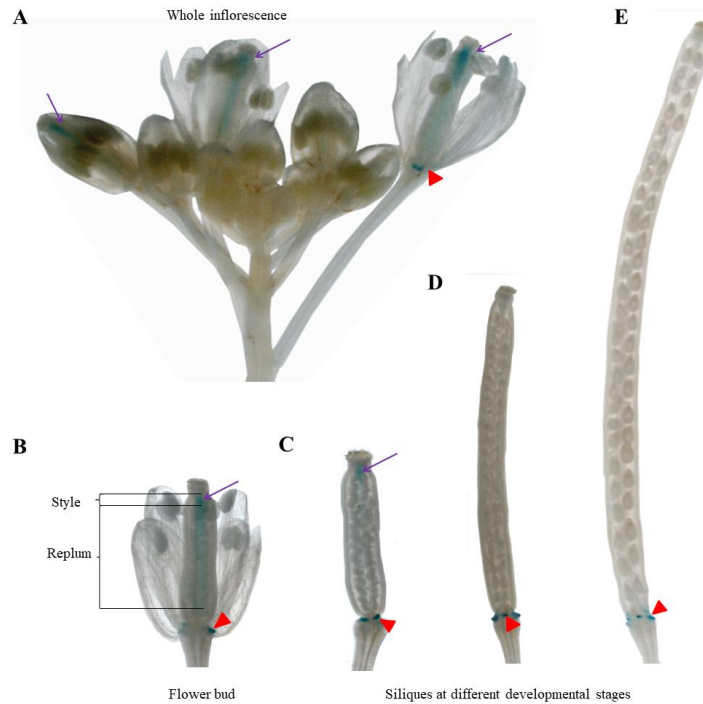


Fig. S2 GUS staining assays in floral organs. GUS expression was observed in the nectaries, style, and the replum. However, following the flower opening, the signal shrank rapidly to the limited style region of the developing fruit and finally disappeared as the siliques are ripening.

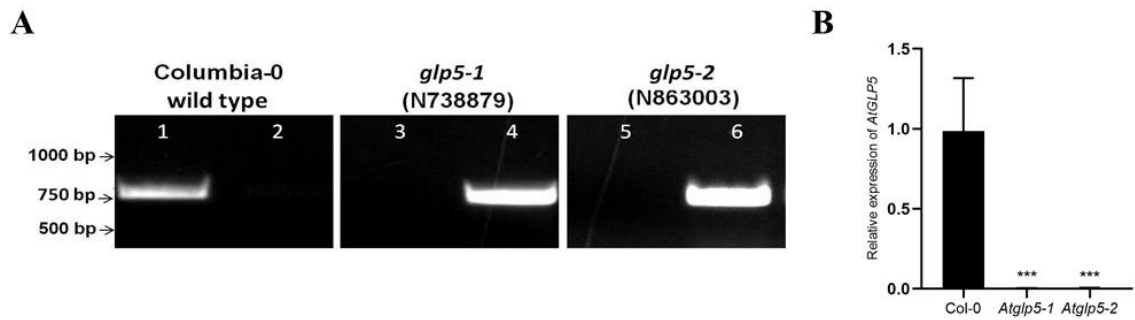


Fig. S3 Confirmation of *Atglp5* T-DNA mutants. (A) Homozygosity test of knockout mutants *Atglp5-1* (N738879) and *Atglp5-2* (N863003). Lane 1, 3, and 5 showed the PCR amplification with gene-specific primers. Lane 2, 4, and 6 depicted the PCR amplification with the corresponding LB primer and the reverse genomic primer. (B) Confirmation of *AtGLP5* expression in *Atglp5-1* and *Atglp5-2* by qRT-PCR. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

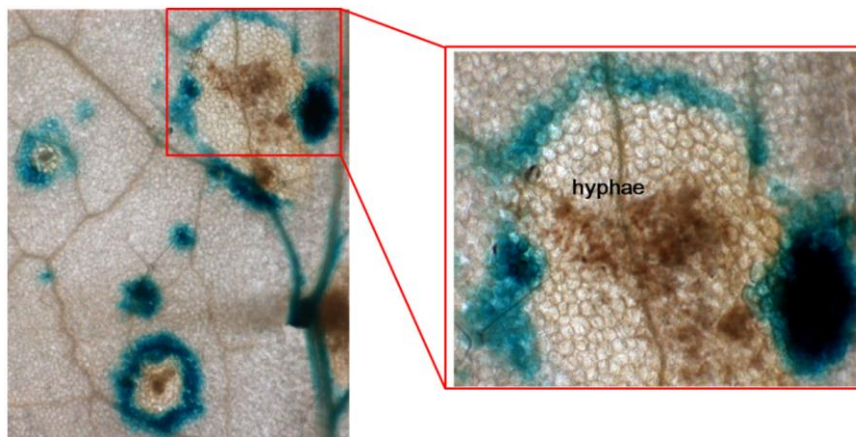


Fig. S4 GUS staining assays after infection of *S. sclerotiorum*. The clustering of hyphae was also microscopically observed inside the darkly stained regions, indicating that GUS expression was locally activated at the advancing margins of lesions.

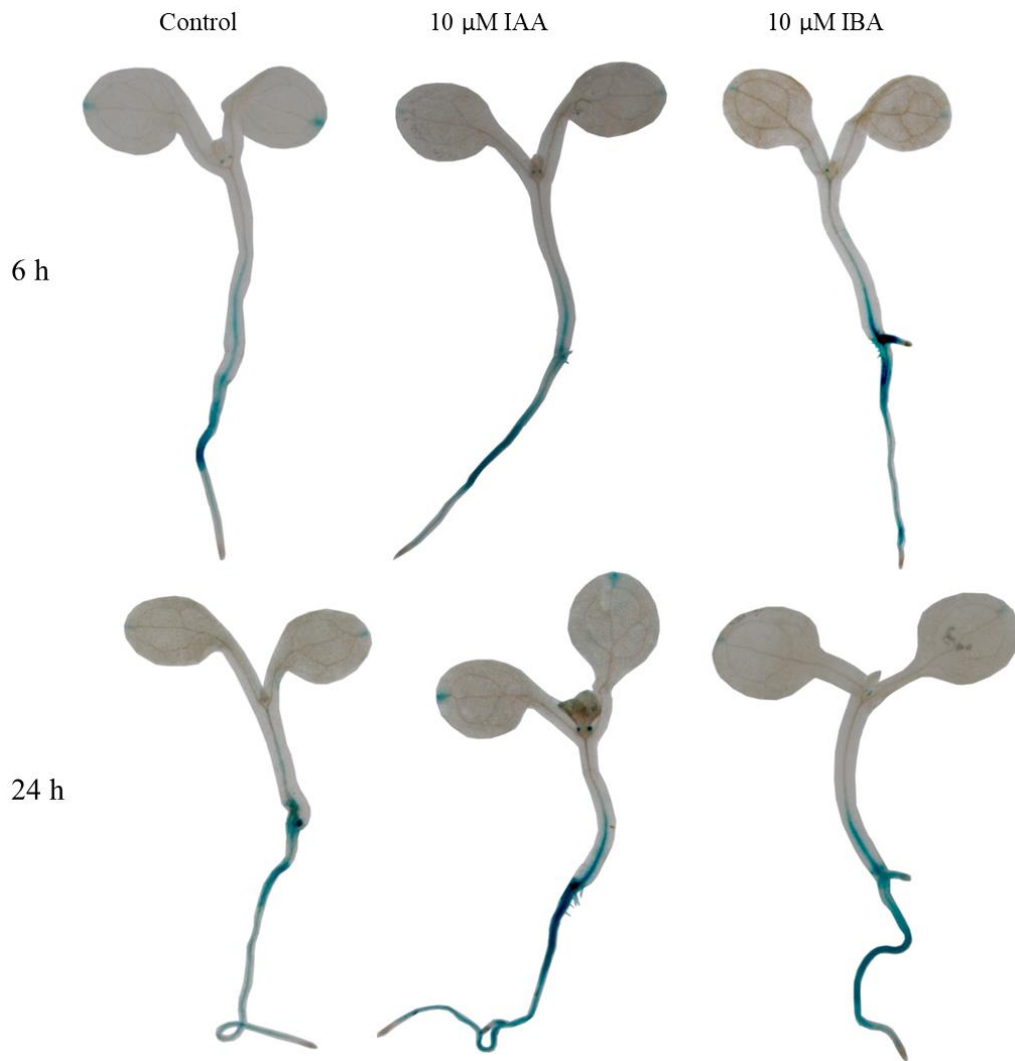


Fig. S5 GUS activity in  $P_{AtGLP5}::GUS$  transgenic *A. thaliana* seedlings in response to auxin treatment. 7-day-old seedlings were incubated in 10  $\mu$ M IAA or 10  $\mu$ M IBA solutions. After 6 hours and 24 hours, the plants were stained with GUS staining solution. Seedlings incubated in 0.1% ethanol for the same time, served as control. The results indicated that *AtGLP5* didn't respond to auxin.

Table S1 Primers for cloning of various constructs and genotyping of T-DNA insertion mutants.

Primers	Sequence (5'→3')
<i>AtGLP5-F1</i>	ATGGCTTCACCAACTCTTAC
<i>AtGLP5-R1</i>	TTTCTTGGGTTGGAACCTGGAT
<i>AtGLP5-F2</i>	CAAAATCAATGGCTTCCCTTGC
<i>AtGLP5-R2</i>	CGAGGACGAAAACGACCTCAGT
<i>AtGLP5-F3</i>	GGGGACAAGTTTGTACAAAAAAGCAGGCT ATGGCTTCACCAACTCTTAC
<i>AtGLP5-R3</i>	GGGGACCACTTTGTACAAGAAAGCTGGGTC TCTTGGGTTGGAACCTGGAT
<i>P<sub>AtGLP5</sub>-F</i>	GGGGACAAGTTTGTACAAAAAAGCAGGCTATAAAGCATCCGTCTTGGTC
<i>P<sub>AtGLP5</sub>-R</i>	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGGGATTGTGGTAGTGGTG
<i>Atglp5-1</i>	CCCATTTGGACGTGAATGTAGACAC
<i>Atglp5-2</i>	TAGTCTGAATTTTCATAACC AATCTCGATACAC
<b>LB3</b>	TAGCATCTGAATTTTCATAACCAATCTCGATACAC

Table S2 Primers for qRT-PCR analysis.

Primers	Forward	Reverse
<i>ACT2</i>	ACCTTGCTGGACGTGACCTTACTGAT	GTTGTCTCGTGGATTCCAGCAGCTT
<i>AtGLP5</i>	CAAAATCAATGGCTTCCCTTGC	CGAGGACGAAAACGACCTCAGT
<i>PDF1.2</i>	CGCTGCTCTTGTCTCTTTGC	TCCATGTTTGGCTCCTTCAA
<i>PR2</i>	CTACAGAGATGGTGTCA	AGCTGAAGTAAGGGTAG
<i>GST1</i>	TCGAGCTCAAAGATGGTGAACACA	TCCTTGCCAGTTGAGAGAAGGTTG
<i>NCED3</i>	CGCTTGGGAAGAGCCAGAAA	GAGATGATCGGACGGCGAGT
<i>EDS1</i>	ACCAGATCATGGTCAGCC	TGTCCTGTGAACACTATCTGTTTTCTACT
<i>MPK3</i>	CCAAGAAGCCATAGCACTCA	AGCCATTCGGATGGTTATTG
<i>MPK4</i>	CGTTGTGCCACCCATATTT	AAAATTGAACGGCCTCACAC
<i>MPK6</i>	ACCACCACCAACCTCAAAAG	CCTCCAGGAGCTTCTGTCAT
<i>WRKY18</i>	GCTCTAGGTGACGGGTTGTC	GAAGCACACGTCAGAAACCATC
<i>WRKY22</i>	GAAGCACACGTCAGAAACCATC	ATCATCGCTAACCACCGTATCC
<i>WRKY33</i>	GCAAAGGAAAGGAGAGGATGGT	GAGGTTTAGGATGGTTGTGGCT
<i>WRKY40</i>	AAATCAGCCCTCCCAAGAAACG	CTTCACGACAGTCTTCTCTCTG
<i>WRKY53</i>	CAGAGATCAGACGGGGATGC	TTTTGGGTAATGGCTGGTTTGAC

## Chapter 3

# Enhancing expression of *AtSUC7* encoding a sucrose symporter confers plant defense against *Sclerotinia sclerotiorum* and promotes early flowering in *Arabidopsis thaliana*

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Keywords: *Arabidopsis thaliana*, sucrose symporter 7 (*AtSUC7*), *Sclerotinia sclerotiorum*, early flowering, defense and growth tradeoffs, activation-tagging mutants.

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### 3.1 Abstract

Plants respond to environmental stresses often by redirecting resources between the immune response and plant development. In this work, we report the identification and characterization of an *Arabidopsis* mutant, referred to as IRS30, obtained from screening a large collection of *Arabidopsis thaliana* activation-tagged mutants. The IRS30 mutant plants showed increased disease resistance along with an early flowering phenotype. The IRS30 mutant contains a T-DNA insertion in the last intron of *AtSAG24*, resulting in an upregulated expression of a sucrose symporter gene *AtSUC7*. Functional characterization of *AtSUC7* revealed that it encodes a plant cell membrane-localized protein. The gene was activated in response to pathogen infection. With both the IRS30 mutant and transgenic *AtSUC7*-overexpressing plants, we demonstrated a dual role of *AtSUC7* in enhancing plant resistance and accelerating flowering time. We showed that an enhanced expression of *AtSUC7* led to the upregulation of plant defense-related genes like *PRI*, *PDF1.2*, and *AtWRKY53*, a vital transcription factor involved in plant defense and senescence control. In contrast, a SA signaling pathway-associated gene *EDS1* was drastically repressed. We demonstrated that an enhanced *AtSUC7* expression affects flowering time probably through fine-tuning of the autonomous pathway leading to the repression of the negative regulator *FLC* causing activation of *SOCI* transcription. Taken together, our results highlight a crucial role of plant sucrose symporters in balancing plant defense and growth trade-offs.

### 3.2 Introduction

Plants are continuously challenged by microbes (bacteria, fungi, and oomycetes) and have developed multilayered defense strategies and sophisticated regulatory pathways that enable the perception, signal transduction, and further downstream responses to defend themselves against these microbial pathogens (Conrath *et al.*, 2015; Han, 2019). In addition to preformed physical and chemical barriers (Anderson, 1982), cell surface-localized pattern recognition receptors (PRRs) can sense pathogen-associated molecular patterns (PAMPs) and host-derived damage-associated molecular patterns (DAMPs) to stimulate defense reactions, named PAMP-triggered immunity (PTI). To counteract this, pathogens have successfully evolved particular effector proteins to interfere with PTI. However, some plants can detect such effectors and activate stronger effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI is triggered when the cytosolic resistance proteins encoded by *R* genes directly or indirectly interact with avirulence factors, activating plant defense (Thordal-Christensen, 2020). Genetic engineering of disease-resistant plants expressing *R* genes is one of the major strategies to confer plant resistance against pathogens (Poltronieri *et al.*, 2020).

*Sclerotinia sclerotiorum* is a necrotrophic, non-host specific phytopathogenic fungus that infects a wide range of economically important plants and causes sclerotinia stem rot (SSR) disease (Dickman and Mitra, 1992). In nature, infection of a plant by *S. sclerotiorum* occurs mostly through ascospore colonization of senescent flower petals that land on the leaves, petioles, or stems during the flowering time (Bolton *et al.*, 2006; Garg *et al.*, 2010). Necrotic lesions develop rapidly on the stems and leaves, leading to premature wilting, necrosis, stem breakage, yield losses, and decreased seed quality (Zhao *et al.*, 2009). Sclerotia are the primary long-term survival structures and can survive in the soil for many years (Bolton *et al.*, 2006). Currently, the disease management relies largely on integrating partially resistant cultivars with cropping practices, such as the alteration of row spacing and varieties, together with chemical control. Although much more effective than cropping system management, the application of fungicides is limited by its negative impacts on the environment, the decreased effectiveness of chemicals, and insufficiency in determining the proper application time (Willbur *et al.*, 2019). Breeding of resistant cultivars is therefore a promising option to control this disease. Over the last years, considerable attempts



have been made to illustrate plant resistance mechanisms and improve plant resistance against *S. sclerotiorum* in various plant species. For instance, plant WRKY transcription factors like *BnWRKY33*, *AtWRKY28*, and *AtWRKY75* are reported to positively regulate resistance to *S. sclerotiorum* (Chen *et al.*, 2013; Wang *et al.*, 2014). Plant germin and germin-like proteins, with their particular OxO (oxalate oxidase) or SOD (superoxide dismutase) enzymatic activities, are also potent candidates for engineering plant resistance against *S. sclerotiorum* disease (Donaldson *et al.*, 2001; Rietz *et al.*, 2012).

Plant flowering is a well-characterized developmental process modulated by several environmental factors and endogenous signals (Valverde, 2011). In the model plant *Arabidopsis thaliana*, the transition from vegetative to reproductive growth is a complex trait regulated by an intricate network of genetic pathways, including the vernalization, autonomous, gibberellin (GA), thermosensory, photoperiod (day length, light intensity), and aging (typically miR156 and miR172-dependent) pathways (Amasino, 2005; Bao *et al.*, 2020; Blázquez *et al.*, 2003; Chowdhury *et al.*, 2020; Jung *et al.*, 2011; Khan *et al.*, 2014; Valverde, 2011). These pathways converge to regulate a set of floral integrators such as FLOWERING LOCUS T (*FT*), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (*SOC1*), and LEAFY (*LFY*) that regulate floral initiation genes (Lee and Lee, 2010; Moon *et al.*, 2005; Simpson and Dean, 2002). *FLC*, encoding a MADS-domain transcription factor, is a dominant repressor of flowering via the vernalization and autonomous pathways by directly inhibiting the expression of floral activators (Michaels and Amasino, 1999; Salathia *et al.*, 2006; Yan *et al.*, 2010). Various activators and repressors regulate *FLC* expression. For example, FRIGIDA (*FRI*) positively activates *FLC* expression that inhibits flowering (Johanson *et al.*, 2000). In contrast, another group of genes, such as FLOWERING CONTROL LOCUS A (*FCA*), *FY*, *FPA*, and FLOWERING LOCUS K (*FLK*), repress *FLC* expression to accelerate flowering (Lim *et al.*, 2004; Liu *et al.*, 2010; Marquardt *et al.*, 2006). Also, various chromatin modifications, including nucleosome remodeling, DNA methylation, and various histone modifications have been shown to mediate *FLC* regulation and thus control flowering (Crevillen and Dean, 2011; He, 2012; Jean Finnegan *et al.*, 2005). Other factors, such as drought and heat can also influence flowering time (Barnabás *et al.*, 2008; Cho *et al.*, 2017). Apart from GA, salicylic acid (SA) is also incriminated in the regulation of

floral transition in that SA-deficient mutants are late flowering, although SA alone is not sufficient to promote flowering (Martínez *et al.*, 2004; Yamada and Takeno, 2014). In addition to their roles of energy resources, sugars also act as signals regulating flowering time and immune responses in plants (Bolouri Moghaddam and Van den Ende, 2013; Yu *et al.*, 2013). The transport of photosynthetically fixed carbon (predominantly as sucrose) from source leaves to sink tissues such as roots, fruits, flowers, and young leaves is mediated via active, energy-dependent sugar transporters. In *A. thaliana*, two families of transporters are involved in sucrose transport: Sucrose transporters (SUTs) (Andrés *et al.*, 2020) or sucrose carriers (SUCs) (Tsuchiya and Eulgem, 2010), and Sugars Will Eventually be Exported Transporters (SWEETs) (Zakhartsev *et al.*, 2016). Sucrose efflux is facilitated by the SWEETs (Chen *et al.*, 2012), and is subsequently accumulated in the phloem sieve element-companion cell (SE/CC) complex catalyzed by membrane-localized H<sup>+</sup>/sucrose symporters (Gottwald *et al.*, 2000). RNAi inactivation of the *StSUT4* gene causes the transgenic potato to flower earlier and accumulates fewer leaves than wild-type plants (Chincinska *et al.*, 2008). Moreover, *A. thaliana* mutants defective in *AtSUC9*, which encodes an ultrahigh-affinity sucrose transporter, flower earlier under short-day conditions but not under long-day conditions (Sivitz *et al.*, 2007). It has been recently demonstrated that overexpression of a SWEET family gene, *AtSWEET10*, accelerates flowering through the photoperiodic pathway involving the activities of *FT* and *SOC1* (Andrés *et al.*, 2020). So far, the mechanisms that underpin how sucrose transporters regulate flowering time are still poorly understood.

Activation-tagging is a useful tool for genome-wide functional studies in plants (Weigel *et al.*, 2000). Specifically, this system relies on tetramer copies of cauliflower mosaic virus (CaMV) 35S transcription enhancers incorporated by T-DNA into the recipient plant genome, thereby improving the transcription of neighboring genes leading to particular phenotypes. Screening of the developed mutants for the phenotypic trait of interest and molecular characterization of the insertion site provides valuable resources to identify genes responsible for biochemical or developmental phenotypes.

In this study, we conducted a large-scale screening of approximately 35,000 *A. thaliana* activation-tagged mutants for altered susceptibility to *S. sclerotiorum* and successfully identified a mutant, referred to as IRS30, which displayed elevated plant resistance against *S. sclerotiorum*

and earlier flowering phenotype. Thus, the disease resistance trait and early bolting were possibly linked in the IRS30 mutant.

We focused on the functional analysis of *AtSUC7*, which expression is activated in the T-DNA insertion mutant and involved in modulating plant defense and flowering time. Our results suggest that the elevated *AtSUC7* expression results in early flowering time mainly by repressing *FLC* through the autonomous pathway. And, the enhanced *S. sclerotiorum* resistance might be attributed to the upregulation of plant defensive genes, including *PRI*, *PDF1.2*, and *WRKY53* in IRS30. Intriguingly, we observed a strong inhibition of *EDS1* gene expression in both IRS30 and *AtSUC7*-overexpressing transgenic plants. Furthermore, *AtSUC6*, another transporter gene, was highly expressed in the *AtSUC7-OE* transgenic plant, suggesting a co-regulation of both genes. Finally, we proposed a functional model of *AtSUC7* in regulating plant defense mechanisms and flowering time.

### 3.3 Materials and Methods

#### 3.3.1 Plant materials and cultivation

*A. thaliana* ecotype Columbia-0 (Col-0) and Columbia-2 (Col-2) were used as the wild-type plants in this study. Plants were grown at 22 °C in a climate chamber under short-day conditions (8h light/ 16h dark cycle) or long-day conditions (16h light/ 8h dark cycle). The activation-tagged mutants were bought from NASC.

#### 3.3.2 Determination of the T-DNA insertion site in activation-tagged mutants

Thermo asymmetric interlaced PCR (TAIL-PCR) was performed as described by Singer et al. and Robinson et al. with a combination of nested T-DNA-specific primers (LB) and arbitrary degenerate primers (AD) (**Supplementary Table 1**) (Robinson *et al.*, 2009; Singer and Burke, 2003). Specific PCR product with the expected size was excised and purified using the GeneJET PCR Purification Kit (Thermo Scientific), cloned into the pGEM-T Easy vector (Invitrogen), and fully sequenced.

After removing the sequences of pGEM-T and pSKI015 vectors, the insertion site of the T-DNA activation-tagged mutant was determined using an online BlastN search of the retrieved TAIL-PCR sequence against the TAIR Arabidopsis database.

#### 3.3.3 Selection of homozygous T-DNA activation-tagged lines

DNA is extracted from a single leaf of the plants and PCR amplified with two primer pairs. The first pair contains two gene-specific primers that span the predicted T-DNA insertion site and can be used to detect the presence of a wild-type copy of the gene. The second pair consists of one T-DNA-specific primer and one of the gene-specific primers that amplify the T-DNA/genomic DNA junction sequence. A homozygous plant will give no band with the first pair of primers but will produce a T-DNA band with the second pair of primers. The wild-type plant will only produce one band with the first pair of primers. And heterozygotes will be positive for both PCR reactions, producing a band of the gene and also a band of the T-DNA.

#### 3.3.4 *S. sclerotiorum* infection experiments

The *S. sclerotiorum* isolate used throughout this work was obtained from Professor W. Qian (Mei

*et al.*, 2011). Infection of *A. thaliana* leaves with *S. sclerotiorum* was either performed by agar plug infection or by spray infection with mycelia suspension. In the first approach, *S. sclerotiorum* was grown on PDA (for transcript analysis) or a minimal medium (for resistance assay, 1 g NaOH, 2 g DL-malic acid, 1 g KH<sub>2</sub>PO<sub>4</sub>, 2 g NH<sub>4</sub>NO<sub>3</sub>, 0.1 g MgSO<sub>4</sub> x 7 H<sub>2</sub>O, 0.5 g yeast extract and 15 g Bacto-agar per liter) at 22 °C. After two or three days, agar-plugs were punched from the expanding margin of the mycelium using a 6 mm cork borer and placed on fully developed leaves of 5-week-old plants with the mycelium facing the leaf surface. Leaves treated with plain agar-plugs served as control. After 2 days, a digital caliper was used to measure the sizes of lesion areas. For the spray infection method, 15 pieces of agar-plugs containing mycelia were transferred into 70 ml of 40% Czapek Dox medium and shaken for 3 days at 22 °C. Subsequently, the mycelium was homogenized for ~35 sec and centrifuged at 6,000 rpm for 10 min. The sedimented mycelium was weighed and resuspended in 10 mM MgSO<sub>4</sub> to a concentration of 1 g/25 mL. Leaves of 5-week-old plants were sprayed evenly with the mycelium suspension. Plants were covered with a transparent lid to keep high humidity. Photos were taken every day to record the disease development of the infection process.

### **3.3.5 Flowering time measurement**

Flowering time was measured by counting the number of days, and the total number of rosette leaves taken from germination until the emergence of a 1cm bolt in healthy, uninfected plants. At least ten plants were assessed for the counting.

### **3.3.6 Isolation of DNA and RNA and qRT-PCR analysis**

For DNA isolation, plant samples were collected in 1.5 mL microcentrifuge tubes and immediately frozen in liquid nitrogen and then ground using a plastic pestle in the tube itself. Genomic DNA was extracted following the cetyltrimethylammonium bromide (CTAB) DNA Miniprep protocol as described (Clarke, 2009).

Total RNA was isolated from plant tissues using TRIzol<sup>®</sup> Reagent (Thermo Fisher Scientific) according to the manufacturer's instruction. The purity and concentration of RNA were measured with GE Healthcare NanoVue Plus UV-Vis Spectrophotometer and evaluated by electrophoresis on an agarose gel. Subsequently, 1 µg of RNA was digested by treatment with DNase I (Thermo

Scientific) for 30 min. First-strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The cDNA quality was checked by RT-PCR using the reference gene *Actin2*, and a ten-fold dilution of the cDNA was made as a PCR template. The qRT-PCR was performed in 20  $\mu$ L reactions with Maxima SYBR Green qPCR Master Mix (Thermo Scientific) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following conditions: predenaturation at 95  $^{\circ}$ C for 3 min; then 40 cycles of denaturation at 95  $^{\circ}$ C for 10 s, annealing at 60  $^{\circ}$ C for 10 s and extension at 72  $^{\circ}$ C for 10 s. Each reaction had three technical repeats. Relative expression levels of mRNA were calculated using the delta-delta CT method based on the threshold cycles of interested genes and the internal control gene *Actin2* (Livak and Schmittgen, 2001). qRT-PCR primers can be found in **Supplementary Table 3**.

### **3.3.7 Construction of plant expression vectors**

The full-length open reading frame of *AtSUC7* was amplified from infected leaves of IRS30 plants with the primers (**Supplementary Table 2**) containing *att*-sites for the cloning into the pDONR201 vector. From the pDONR201 entry clone, *AtSUC7* was transferred into binary vector pGWB402 producing a 35S promoter-driven gene construct (Nakagawa *et al.*, 2007). In analogy, *AtSUC7* was cloned into pGWB405, which adds a GFP-tag coding region in the frame to the 3' end to allow the expression of AtSUC7-GFP protein. Both constructs were transformed into *Agrobacterium tumefaciens* GV3101 strain to facilitate plant transformation.

### **3.3.8 Subcellular localization of GFP-tagged AtSUC7**

For subcellular protein localization of *AtSUC7*, *AtSUC7*-GFP construct was first transiently expressed in tobacco epidermal cells was performed as described by Sparkes *et al.* (Sparkes *et al.*, 2006). Briefly, the *Agrobacterium* culture with the *AtSUC7*-GFP fusion protein vector was prepared and grown in the presence of 50 mg/L kanamycin, pelleted at 4,000 rpm for 10 min and diluted to an OD600 of 1.0 in 10 mM MES pH 5.6, 10 mM  $MgCl_2$ , 150  $\mu$ M acetosyringone and then infiltrated into the abaxial side of the leaves using a needleless 1ml syringe with gentle pressure to reduce damage to the leaf. After 2~3 days, leaf tissue within the infiltrated region was excised, and GFP was visualized under the Eclipse Ni-E Upright Motorized Microscope (Nikon) connected to the X-Cite 120 LED light source (Excelitas Technologies) for bright-field and fluorescence imaging aided with the NIS-Elements AR software (Nikon).

For the stable expression, the AtSUC7-GFP construct was introduced into *A. thaliana* through the floral dip method. Transgenic plants were germinated on half MS medium with 50 mg/L kanamycin for one week, and the positive seedling was then placed on a slide for observation of GFP.

### **3.3.9 The floral dip-mediated plant transformation**

Primary bolts of healthy *A. thaliana* plants were clipped to produce more secondary bolts, and flowers were then dipped using the floral dip method twice at 7-day intervals to allow late flowers to be transformed (Clough and Bent, 1998). Transgenic plants were selected on half MS medium supplemented with 50 mg/L kanamycin and further confirmed by PCR.

### **3.3.10 Phylogenetic analysis and multiple sequence alignment**

The sequences of *A. thaliana* sucrose symporter proteins were obtained from NCBI and aligned with ClustalW. And the phylogenetic tree was constructed using MegaX software (Kumar *et al.*, 2018). The neighbor-joining method was used to produce a phylogenetic tree with the following parameters: bootstrap analysis (500 replicates), Poisson correction, and pairwise deletion. DNAMAN Software was used to carry out amino acid sequence alignments among the sequences of AtSUC7 from different ecotypes.

### **3.3.11 Statistical Analysis**

GraphPad Prism 8 computer software was used to perform all statistical analyses (GraphPad Prism, San Diego, CA, USA). The results were represented as means  $\pm$  S.E., and different numbers of asterisks indicated significantly different values. The significant difference between the two groups was performed using the student's *t*-test. And multiple comparisons of means were analyzed by one-way ANOVA, followed by Dunnett's multiple comparisons test ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ).

### 3.4 Results

#### 3.4.1 Identification of the IRS30 mutant with improved *S. sclerotiorum* tolerance

Initially, in an attempt to screen plants with increased *S. sclerotiorum* tolerance and identify novel genetic regulators that mediate this disease resistance, a population of around 35,000 activation-tagged *A. thaliana* mutant lines was sprayed with fresh *S. sclerotiorum* mycelia suspension. In this way, we obtained 32 plants that survived at 7 days post-inoculation (dpi), showing somehow elevated resistance to *S. sclerotiorum* (**Fig. 1A**). One of the isolated mutants, designated as IRS30 (Increased Resistance to S. *sclerotiorum*), was selected for further investigation in this report.

To determine the gene responsible for the phenotype of the IRS30 mutant, we carried out thermal asymmetric interlaced (TAIL)-PCR (Singer and Burke, 2003). We identified a T-DNA insertion in the last intron of *ATIG66580* encoding a senescence-associated gene 24 (**Fig. 1B**). According to Weigel et al., overexpressed genes in the activation-tagged mutants are almost always found directly next to the inserted CaMV 35S enhancers (Weigel *et al.*, 2000). The gene upstream of *AtSAG24* is *AtSUC7* (sucrose-proton symporter 7) at a distance of 1.9 kb, and the gene downstream is *AtCOX19-1* (cytochrome c oxidase 19-1) at a distance of 1.2 kb. So we next examined the expression of both *AtSUC7* and *AtCOX19-1* in the IRS30 mutant via qRT-PCR. **Fig. 1C** showed that the transcript abundance of *AtSUC7* was elevated 35-fold in the IRS30 mutant, whereas that of *AtCOX19-1* displayed no significant difference. Therefore, our hypothesis was that the *S. sclerotiorum* resistant phenotype of the IRS30 mutant is probably caused by the overexpression of the *AtSUC7* gene.



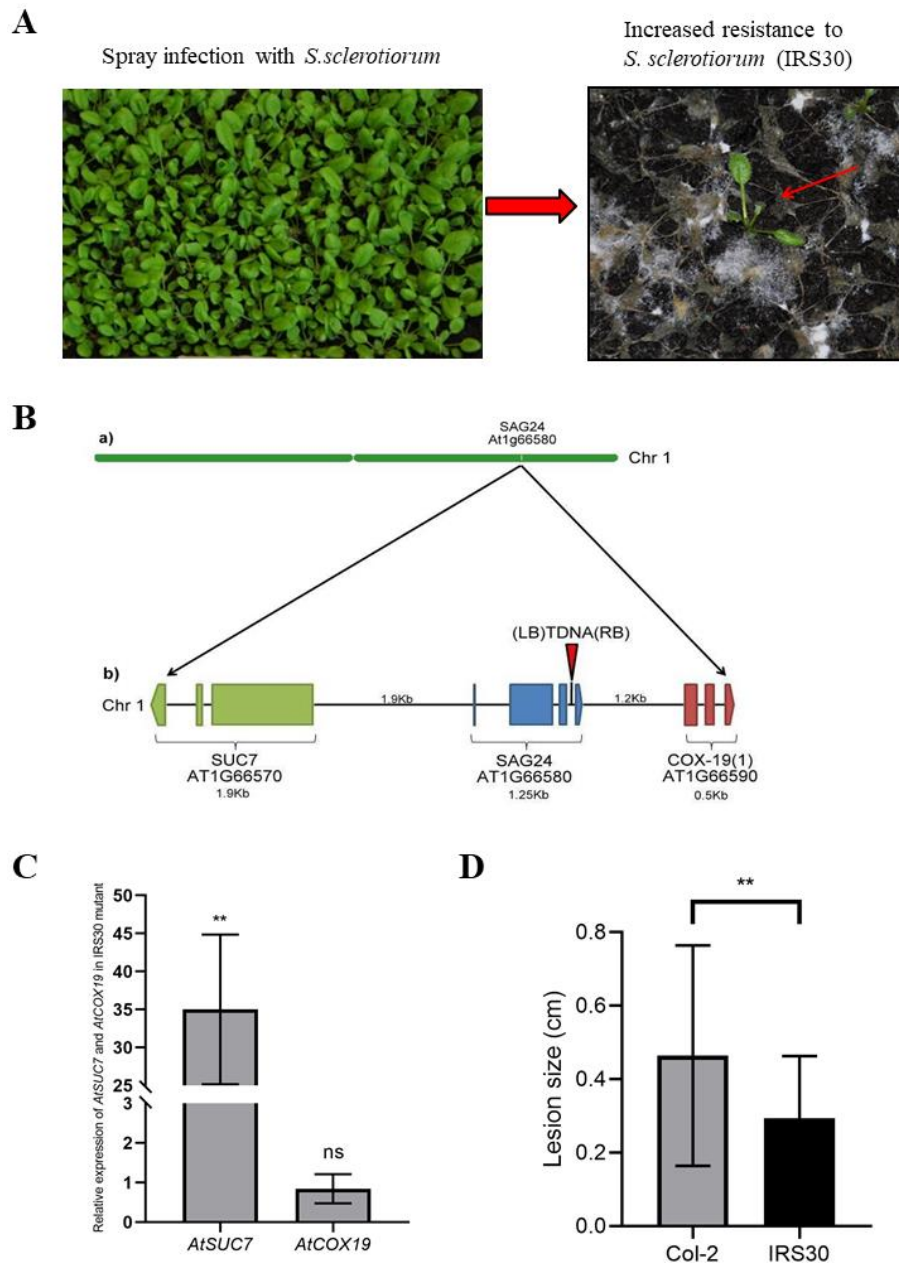


Fig. 1 Isolation and molecular characterization of the gain-of-function IRS30 mutant with increased disease resistance. (A) A pool of approximately 35,000 activation-tagged *A. thaliana* mutant seeds were germinated and the seedlings were then sprayed with fresh *S. sclerotiorum* mycelium suspension. Individual plants that survived at 7dpi regarded as plants with higher resistance were isolated and transplanted into another pot so that the fungus will not kill the plant finally. (B) Diagram illustrating the T-DNA insertion site at the last intron of *AtSAG24* in the IRS30 mutant. The genes upstream and downstream of *AtSAG24* was *AtSUC7* and *AtCOX19*, respectively. (C) Transcript levels of *AtSUC7* and *AtCOX19* in Col-2 wild-type and IRS30 mutant plants were determined by qRT-PCR. The expression of *AtSUC7* was activated in the activation-tagged mutant, but *AtCOX19* expression remained unaltered. (D) The average lesion size of the IRS30 mutant was significantly smaller compared with wild-type Col-2 plants. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

### 3.4.2 The IRS30 mutant enhances disease resistance mainly by modulating the expression of plant defense-related genes

After harvest, seeds from the IRS30 mutant were germinated and genotyped to identify homozygous individuals (data not shown). Progenies of the homozygotes were used to confirm the heritability of disease resistance and for further analysis. As shown in **Fig. 1D**, the average lesion size of the IRS30 mutant was significantly smaller compared with wild-type Col-2 plants at 2dpi. Thus, the IRS30 mutant exhibits an inherited increased disease resistance to *S. sclerotiorum*.

To unravel the molecular mechanisms by which IRS30 mutant regulated plant resistance against *S. sclerotiorum*, we compared the expression levels of several plant defense-related genes in IRS30 mutant and wild-type plants. The relative expression of genes like *PR1* and *WRKY53* about two times higher in IRS30 compared with the wild-type plants. Interestingly, another gene, *EDS1*, displayed an inhibited expression in the IRS30 mutant as compared with the wild-type plants (**Fig. 2**). This result indicates that the elevated expression of *PR1* and *WRKY53* might be crucial for the enhanced disease resistance in the IRS30 mutant. On the other hand, higher expression of *AtSUC7* resulted in suppression of *EDS1*.

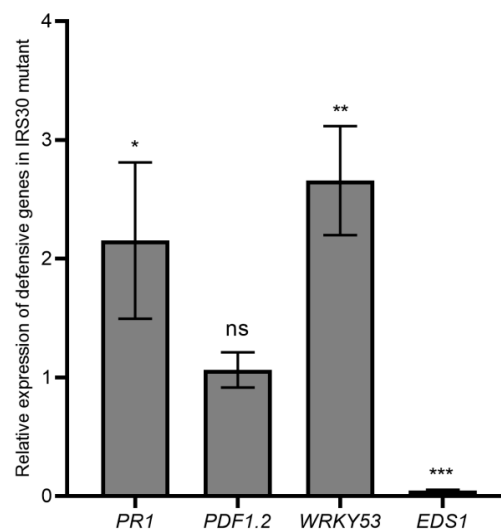


Fig. 2 Expression of defense-related genes in the IRS30 mutant and wild-type Col-2 plants by qRT-PCR. Plant defense-related genes *PR1* and *WRKY53* were upregulated, while *EDS1* was repressed in the IRS30 mutant. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

### 3.4.3 The IRS30 mutant flowered earlier and altered the transcription levels of major flower-related genes

The apparent difference of flowering times between Col-2 and IRS30 mutant plants was observed under both short-day (data not shown) and long-day (Fig. 3A) conditions, suggesting that *AtSUC7* positively accelerated plant transition to flowering independently of day length. The average flowering time of IRS30 mutants was about five days shorter than that of the wild-type Col-2 plants (Fig. 3B). And the number of rosette leaves at bolting was 8 in the IRS30 mutants, which were only about half of that observed in Col-2 plants (Fig. 3C).

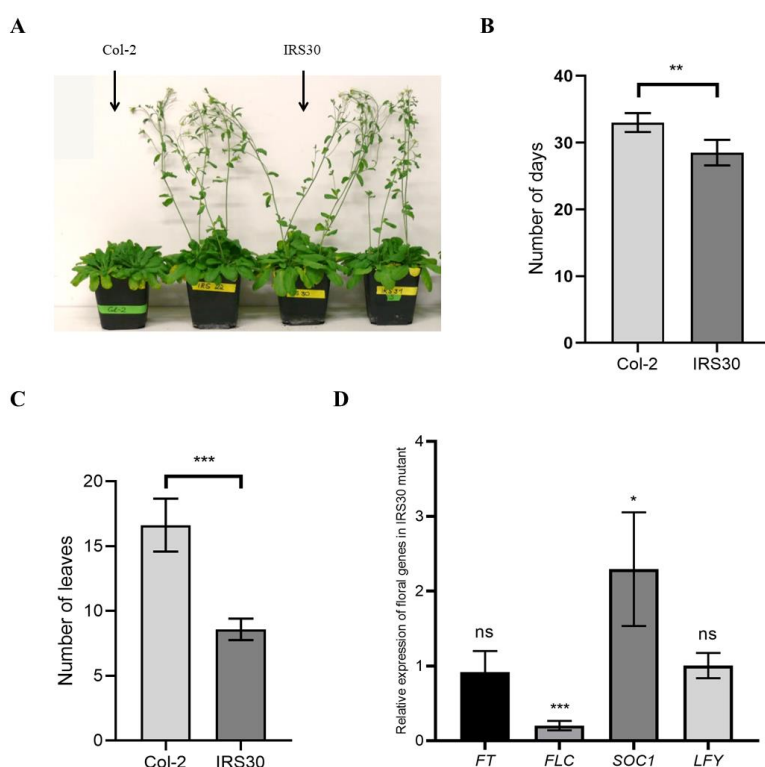


Fig. 3 IRS30 is an early flowering mutant in *A. thaliana*. (A-C) The flowering phenotype of the IRS30 mutant. The IRS30 mutant flowered several days earlier and accumulated fewer rosette leaves at bolting under long-day conditions. (D) The expression of *FLC* was downregulated, while *SOC1* was upregulated in the IRS30 mutant. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

To clarify the nature of the IRS30 mutant on flowering time, the expression levels of several major flowering regulators, including *FT*, *FLC*, *SOC1*, and *LFY*, were evaluated. As shown in Fig. 3D, the mRNA level of *FLC* was significantly repressed, whereas *SOC1* expression was slightly upregulated in the IRS30 mutant compared with wild-type Col-2 plants. Since *FLC* repressed

*SOC1* by direct binding to its promoter (Lee and Lee, 2010), the upregulation of *SOC1* in *ISR30* mutants might be due to the reduced *FLC* levels. The expression of two other floral genes, *FT* and *LFY*, however, remained unaffected. These results suggest that *AtSUC7* promoted flowering by repressing *FLC* and activating *SOC1* expression.

In *A. thaliana*, *FLC* expression is regulated by several genes, including various *FLC* activators and repressors. To determine the upstream factors that downregulate *FLC* expression, we checked the relative expression of ten *FLC* activators and nine *FLC* repressors in the *IRS30* mutant. Throughout all of the 19 genes, only two *FLC* repressors, *FCA* and *REF6*, showed a significant change in their transcript abundance in the *IRS30* mutant, thus suggesting that the upregulation of *FCA* and *REF6* expression might result in the repression of *FLC* (Fig. 4). Since both *FCA* and *REF6* belong to the autonomous pathway, it is possible that enhanced expression of *AtSUC7* promotes plant flowering by interfering with the autonomous pathway.

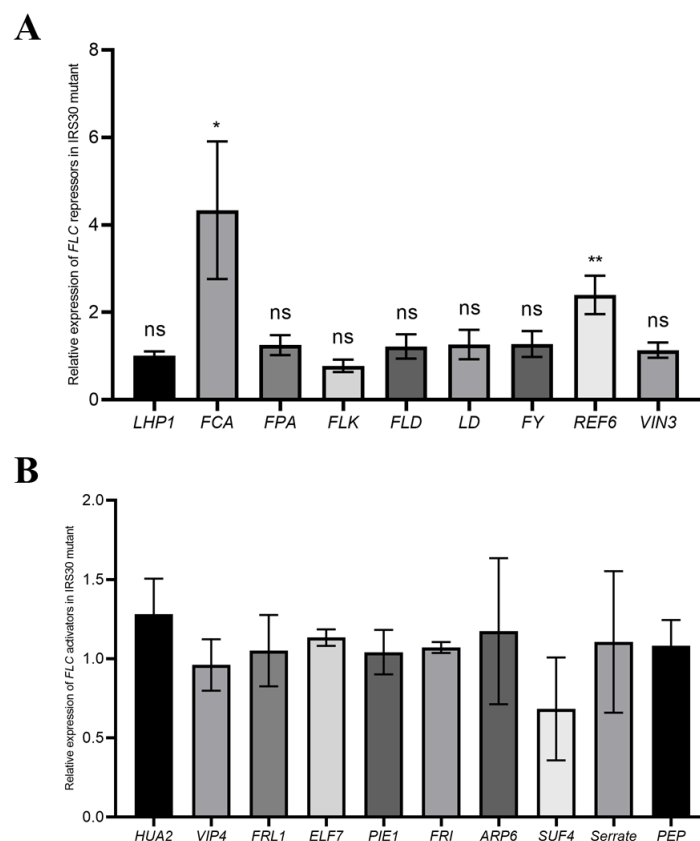


Fig. 4 Transcript levels of *FLC* activation and repression genes. (A) Two *FLC* repressors, *FCA* and *REF6*, were upregulated in the *IRS30* mutant. (B) The expression of ten *FLC* activators remained unaffected. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

#### **3.4.4 *AtSUC7* is induced by *S. sclerotiorum* infection, and the protein is localized at the plasma membrane**

The mechanism of the increased disease resistance and earlier flowering phenotype of the IRS30 mutant was further investigated by functional analysis of the gene *AtSUC7*. Firstly, to determine whether *AtSUC7* functions in defense responses to pathogen attack, we infect the wild-type plants with *S. sclerotiorum*. The inoculated leaves were collected at local tissues with a cork borer at 3 hpi (hours post-inoculation), 6 hpi, 12 hpi, 24 hpi, and 36 hpi, separately, while non-infected leaf tissues served as a control. We found that at the early infection stage, the expression of *AtSUC7* was significantly upregulated at three hpi, as determined by qRT-PCR. After reaching a peak at 12 hpi, the transcript level of *AtSUC7* was drastically downregulated (**Fig. 5**).

To assess the subcellular localization of *AtSUC7* protein, the green fluorescent protein (GFP) tag was fused to the *AtSUC7* coding region. The construct was transiently expressed in *N. benthamiana* leaf epidermal cells. Leaf discs collected at 2 days post infiltration were observed under a fluorescence microscope. As shown in **Fig. 6**, the fluorescence signal of the *AtSUC7*-GFP construct appeared in the plasma membrane. Besides, the *AtSUC7*-GFP construct was also transformed into *A. thaliana* by the agrobacterium mediated transformation. Again, we observed that *AtSUC7* was predominantly localized at the plasma membrane in both leaves and roots (**Fig. 6**).

#### **3.4.5 Sclerotinia rot disease tolerance phenotype can be recapitulated by overexpressing *AtSUC7* in wild-type *A. thaliana***

Sequencing of the cloned *AtSUC7*<sub>IRS30</sub> gave a full-length sequence encoding for a protein consisting of 491 amino acids. When compared with the sequence described in the Tair10 (<https://www.arabidopsis.org/index.jsp>), we found several variations in the nucleotide sequence resulting in eight different amino acid residues. More interestingly, the *AtSUC7*<sub>IRS30</sub> sequence shared a high level of sequence identity with *AtSUC7*<sub>Ws</sub> of the ecotype Wassilewskija (Ws-0) as described by Sauer et al. (Sauer *et al.*, 2004), in which *AtSUC7*<sub>IRS30</sub> carries an isoleucine residue (I) whereas *AtSUC7*<sub>Ws</sub> contains a valine (V) instead (**Fig. S1**).

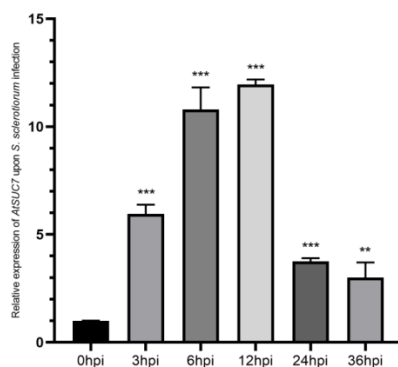


Fig. 5 Responsive expression of *AtSUC7* to *S. sclerotiorum* infection. Relative expression of *AtSUC7* was determined by qRT-PCR at 0, 3, 6, 12, 24, and 36 h after inoculation. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

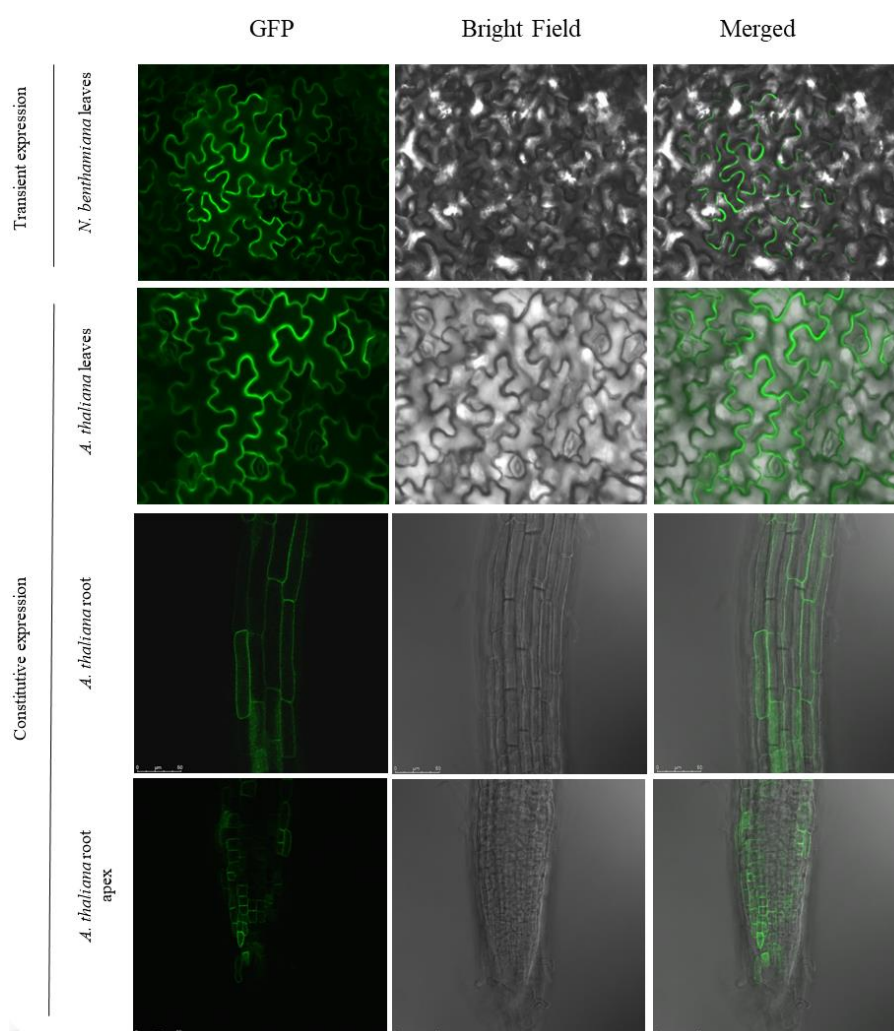


Fig. 6 Plasma membrane localization of *AtSUC7*-GFP in tobacco and transgenic *A. thaliana* plants. The coding sequence of *AtSUC7* was fused to a GFP tag and was either transiently expressed in the tobacco leaves or stably transformed into *A. thaliana*. GFP signal can be observed in both leaf and root tissues in the transgenic *AtSUC7*-GFP plants.

To discern whether the overexpression of *AtSUC7* can cause the same phenotype as IRS30, we generated transgenic plants carrying the *AtSUC7* protein-coding sequence under the transcriptional control by the CaMV 35S promoter in the Col-0 background. Two independent lines overexpressing *AtSUC7*, referred to as *AtSUC7-OE-3* and *AtSUC7-OE-4*, were selected for further analysis. In an *in-vitro* infection experiment, leaves from the transgenic plants and untransformed wild-type plants were detached and subsequently inoculated with *S. sclerotiorum* mycelial plugs. The results showed that the average lesion size of *AtSUC7-OE* plants was about 30% smaller than wild-type control plants (**Fig. 7A**). And, in an *in-vivo* experiment, plants were infected by spraying *S. sclerotiorum* mycelial suspension. As shown in **Fig. 7B**, all the infected plants showed disease symptoms and displayed less difference concerning disease progression at 2 dpi. However, at 3 dpi, on the control of Col-0 plants, disease symptoms were becoming more severe, while, in contrast, the transgenic plants showed much less tissue damage than the wild-type control. These results provide evidence that the overexpression of *AtSUC7* triggers plant resistance to *S. sclerotiorum*, supporting that the resistant phenotype of the IRS30 mutant is attributed to the activation of the *AtSUC7* gene in IRS30.

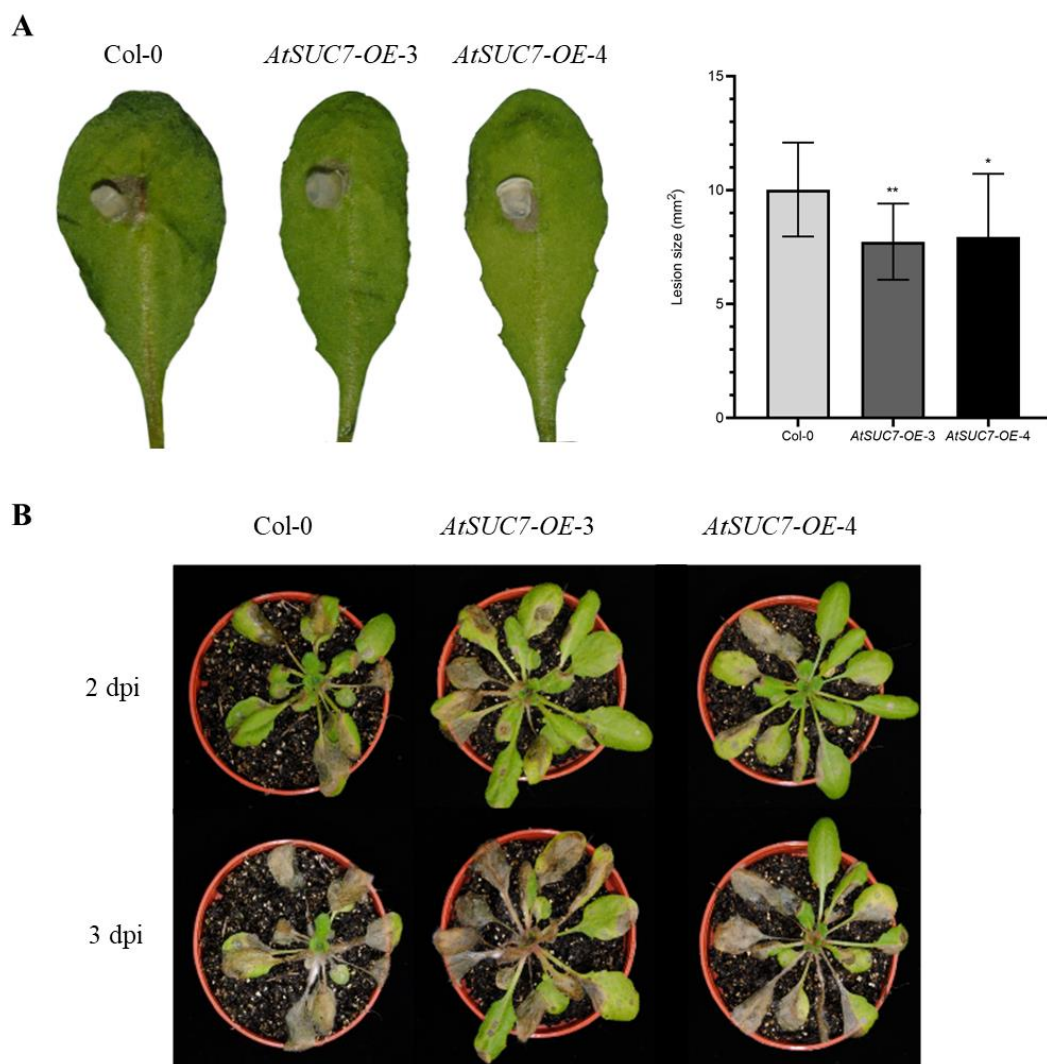


Fig. 7 Overexpression of *AtSUC7* recapitulated the disease-resistant phenotype of the IRS30 mutant. (A) Leaves of *AtSUC7-OE* and Col-0 plants were detached and inoculated with *S. sclerotiorum* mycelium plugs. The average lesion sizes of transgenic plants were smaller than wild-type plants. (B) 5-week-old *AtSUC7-OE* and Col-0 plants were evenly sprayed with *S. sclerotiorum* mycelial suspension. The disease symptom was more severe in the Col-0 plants compared with transgenic *AtSUC7-OE* plants. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

To explore the molecular mechanisms underlying the *AtSUC7*-triggered *S. sclerotiorum* disease resistance, we then compared the expression levels of several plant defense-related genes between the *AtSUC7-OE* transgenic and wild-type Col-0 plants. As shown in **Fig. 8**, the level of *AtSUC7* transcript abundance was significantly higher in the transgenic plants than wild-type Col-0 plants as measured by qRT-PCR. The relative expression of *PDF1.2* was about 9-fold higher in the *AtSUC7-OE* plants than in Col-0, and *WRKY53* was also upregulated in the *AtSUC7-OE* plants,



whereas *PRI* remained unaffected. Consistent with the inhibited expression of *EDS1* in the IRS30 mutant, *AtSUC7-OE* plants also showed a downregulated expression of *EDS1* (**Fig. 8**). Thus, we conclude that the enhanced expression of *AtSUC7* in plants results in the activation of defense-related genes, conferring plant resistance, while a high expression level of *AtSUC7* represses the *EDS1* expression.

#### **3.4.6 Overexpression of *AtSUC7* promotes flowering through regulating *FLC* and *SOCI* expression**

To further validate the function of *AtSUC7* in accelerating plant flowering, we recorded the flowering time of both *AtSUC7-OE* and the wild-type Col-0 plants. Strikingly, the flowering time of *AtSUC7-OE* plants was similar to that recorded on the IRS30 mutant, showing an early flowering phenotype (**Fig. 9**). Under long-day conditions, two independent lines of *AtSUC7-OE* plants flowered about 3 days earlier than Col-0 plants. The average leaf number was 10 for Col-0 at bolting, but 8 for *AtSUC7-OE* plants.

In the next step, we examined the expression levels of major flowering genes, including *FT*, *FLC*, *SOCI*, and *LFY* in *AtSUC7-OE* plants, and compared them with the wild-type control plants. Coincidentally, the expression level of *FLC* was lower in *AtSUC7-OE* plants compared with Col-0 plants. In contrast, *SOCI* was upregulated in *AtSUC7-OE* plants (**Fig. 9D**). Therefore, we conclude that overexpression of *AtSUC7* can promote plant flowering via the regulation of *FLC* and *SOCI*, as observed in IRS30.

Next, we performed an expression analysis of *FLC* activators and repressors in *AtSUC7-OE* plants. As expected, transcriptional levels of *FCA* and *REF6* were also elevated in transgenic *AtSUC7-OE* plants. Additionally, we found another repressor gene *FY*, which regulates the flowering process in the autonomous pathway, expressed at a higher level (**Fig. 10**). The *FLC* repressor genes *FLK* and *LD* and several activator genes were all slightly activated in the *AtSUC7-OE* plants. These results provide additional evidence that an enhanced expression of *AtSUC7* accelerates plant vegetative-to-reproductive transition via the regulation of the autonomous pathway to repress *FLC* while elevating *SOCI* expression in plants.

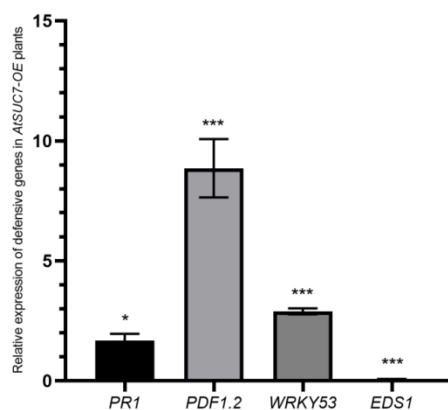


Fig. 8 Expression of defense-related genes in *AtSUC7-OE* plants. Overexpression of *AtSUC7* led to an upregulation of *PRI*, *PDF1.2* and *WRKY53*. Consistent with the *IRS30* mutant, *EDS1* was also repressed in *AtSUC7-OE* plants. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

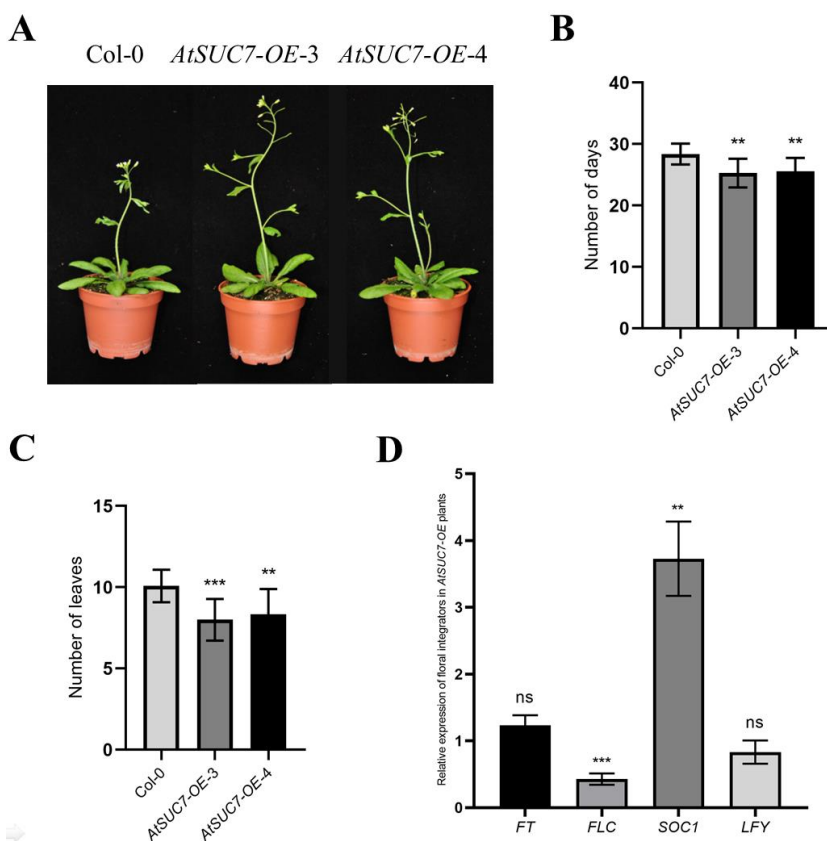


Fig. 9 Early flowering of *AtSUC7-OE* plants. (A-C) Both independent *AtSUC7-OE* lines showed an early flowering phenotype with a shorter number of days and fewer rosette leaves at bolting. (D) The expression of four floral integrators was analyzed. The floral repressor gene *FLC* was repressed, whereas the floral integrator gene *SOC1*, which positively regulates plant flowering, was activated in the *AtSUC7-OE* plants. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

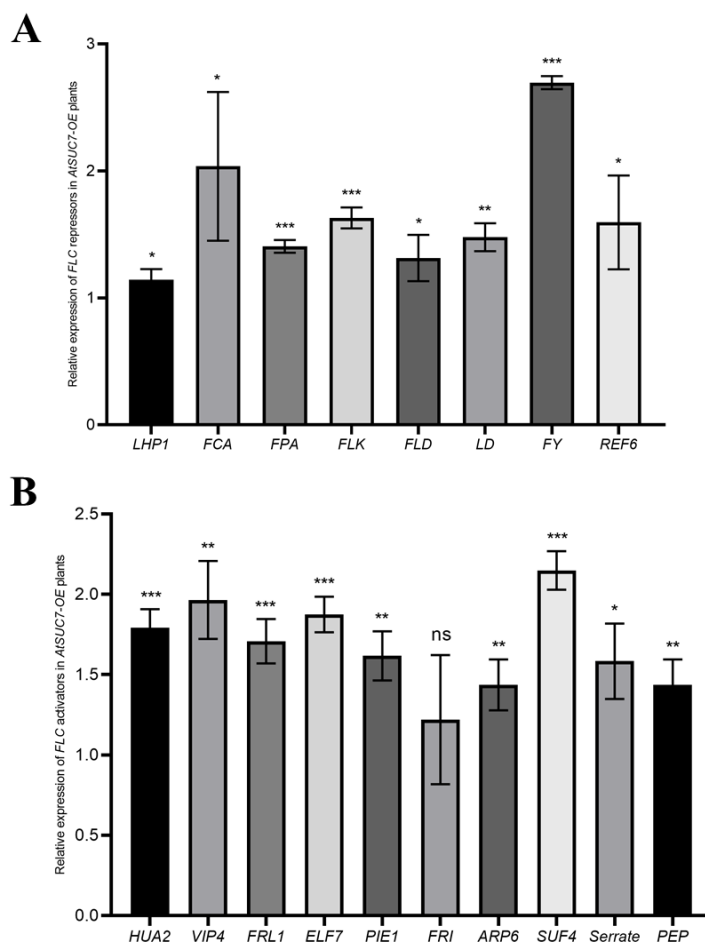


Fig. 10 Transcript analysis of *FLC* regulators in *AtSUC7*-OE plants. (A) Several *FLC* repressors, including *FCA*, *FLK*, *LD*, *FY*, and *REF6*, were upregulated. (B) Except for *FRI*, all the other *FLC* activators tested were also upregulated. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

### 3.4.7 Real-time qRT-PCR analysis of *A. thaliana* sucrose symporter family members

*AtSUC7* is a member of the sucrose symporter gene family composed of nine members in *A. thaliana*, and several genes have been functionally characterized (Lalonde *et al.*, 1999). Sequence comparisons suggested that the nine sucrose transporters share between 45.3 and 97.1% identical amino acids. Among them, *AtSUC6*, *AtSUC7*, *AtSUC8*, and *AtSUC9* are most closely related (Sauer *et al.*, 2004). The phylogenetic tree constructed through neighbor-joining analysis indicates that *AtSUC7* exhibits a close genetic relationship to *AtSUC6* (Fig. S2). We also analyzed the expression of all the nine sucrose symporter genes in both transgenic *AtSUC7*-OE and wild-type Col-0 plants by qRT-PCR. Gene-specific primers were designed and optimized to ensure specificity. As a result, we found that *AtSUC1*, *AtSUC2*, *AtSUC3*, *AtSUC5* were co-upregulated in

the *AtSUC7-OE* plants, from which, to our surprise, the expression of *AtSUC6* was upregulated up to about 300 folds as compared with the wild-type control. Genes *AtSUC4*, *AtSUC8*, and *AtSUC9* remained transcriptionally unaltered (**Fig. 11**). These results suggest a functional interaction between *AtSUC7* and the other sucrose symporter genes, especially *AtSUC6*.

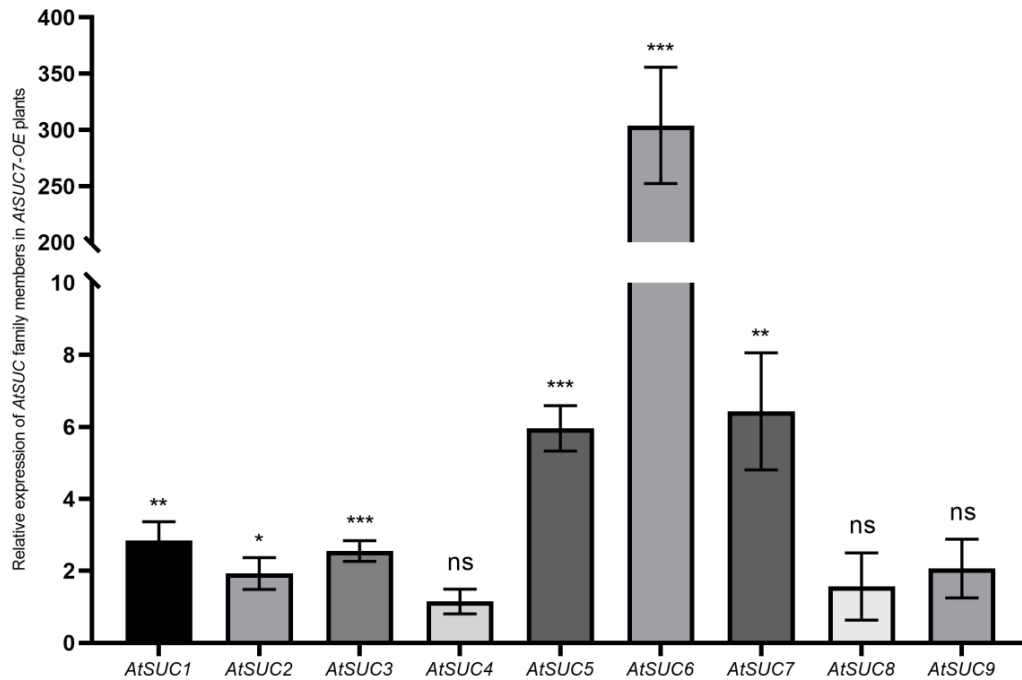


Fig. 11 Transcript analysis of Arabidopsis sucrose symporter family members in *AtSUC7-OE* plants. Among the eight *AtSUCs*, *AtSUC1*, *AtSUC2*, *AtSUC3*, *AtSUC5*, and *AtSUC6* were activated by the overexpression of *AtSUC7*. *AtSUC6*, which is the closest to *AtSUC7*, was extremely upregulated. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

### 3.5 Discussion

In this work, we isolated the IRS30 mutant from an activation-tagged *A. thaliana* seed pool (Weigel *et al.*, 2000) and uncovered a genetic integrator *AtSUC7* associated with plant defense and early flowering. After *S. sclerotiorum* challenge, we obtained 32 plants, which showed weaker disease susceptibility. One of them, IRS30, also displayed an earlier flowering phenotype compared to the wild-type plants. Through sequencing, we identified a T-DNA insertion in the last intron of *AtSAG24*, leading to activation of the adjacent *AtSUC7* gene. *AtSUC7* has been previously described as a member of the *A. thaliana* sucrose symporter family, which shows neither sucrose nor esculin transport function (Rottmann *et al.*, 2018; Sauer *et al.*, 2004). Firstly, the progenies of the T1 plant were used to confirm the heritable mutant phenotype. Then full-length *AtSUC7* cDNA was cloned and overexpressed in *A. thaliana* plants for further molecular and functional characterization of *AtSUC7*.

Consistent with the IRS30 mutant, an enhanced disease-resistant phenotype against *S. sclerotiorum* was observed in the *AtSUC7-OE* transgenic plants, supporting the enhanced expression of *AtSUC7* was responsible for the *S. sclerotiorum* resistance observed in IRS30. The plant defense mechanism is regulated by a complex network and the crosstalk between multiple signal transduction pathways, which allow plants to activate the most efficient and optimum defense response against specific attackers (Schenke and Cai, 2020). It is well known that salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are critical players in the regulation of signaling pathways in plants. To understand the molecular mechanism underlying *AtSUC7*-mediated plant defense, we analyzed the expression levels of several plant defense-related marker genes, including *PRI*, *PDF1.2*, *WRKY53*, and *EDS1*. In the IRS30 mutant, transcripts of *PRI* and *WRKY53* were slightly upregulated, while in the *AtSUC7-OE* transgenic plants, the expression of *PDF1.2* and *WRKY53* was highly upregulated. It is reasonable to speculate that *AtSUC7* might positively affect the *WRKY53* expression. Besides, the upregulation of *PDF1.2* and *PRI* indicates their involvement in both SA and JA signaling pathways. Intriguingly, the expression of *EDS1* was almost abolished by activation or overexpression of *AtSUC7* in plants, suggesting a specific adverse effect of *AtSUC7* on *EDS1*. Enhanced disease susceptibility 1 (*EDS1*) is a nucleo-cytoplasmic protein which forms several molecularly and

spatially distinct complexes with Phytoalexin deficient 4 (PAD4) and a Senescence associated gene 101 (SAG101) acting downstream of R protein activation to regulate basal plant defense (Cui *et al.*, 2017; Lapin *et al.*, 2020; Wagner *et al.*, 2013). We assume that repression of *EDS1* by *AtSUC7* may reflect a result of the cross-talk between plant hormones SA and JA, benefiting the plant JA-dependent resistance response to necrotrophic pathogens such as *S. sclerotiorum*. Further experiments are needed to get more insight into the underlying mechanism.

Under long-day conditions, the early flowering phenotype of IRS30 mutant was also observed in the transgenic *AtSUC7-OE* plants, supporting that the early flowering phenotype of IRS30 was resulted by an elevated expression of *AtSUC7* in plants. The early flowering phenotype was even more conspicuous under short-day conditions as the IRS30 mutant flowered with about 25 rosette leaves. In contrast, the wild-type plants flowered only after producing more than 40 leaves (data not shown). This observation suggests that the acceleration of flowering time by *AtSUC7* is independent of the photoperiod pathway. In either activation-tagged or stably transgenic *AtSUC7-OE* plants, the floral integrator gene *SOCI*, which positively regulates plant flowering time, was significantly upregulated. *SOCI* is activated by the photoperiod pathway and repressed by *FLC*, a central repressor of flowering time in which expression is regulated via both the autonomous and vernalization pathways. As in our case, *FLC* was dramatically downregulated. We believe that *AtSUC7* might regulate flowering by repressing *FLC* expression. To gain more insight into which pathway is *AtSUC7* involved in regulating *FLC*, we analyzed the expression of 19 related genes, representing *FLC* activators or repressors through either the autonomous or vernalization pathway. We found that in the IRS30 mutant, two *FLC* repressors, *FCA* and *REF6*, which belong to the autonomous pathway, were upregulated. Thus, it is most likely that the activation of *AtSUC7* in the IRS30 mutant results in an increase in the expression of *FCA* and *REF6*, and the upregulation of these *FLC* repressors, finally leading to the repression of *FLC*. While the *FLC* expression is depressed, the expression of the positive flowering regulator *SOCI* is increased, consequently resulting in early flowering. It is interesting to observe that the transcription of all the *FLC* activators remained unaffected in this study. In consistency, in the *AtSUC7-OE* plants, both *FCA* and *REF6* were upregulated, and other *FLC* repressors, including *FY*, *LD*, and *FLK*, were found to be also upregulated.

Nevertheless, a slight activation of almost all the *FLC* activators except for *FRI* was observed in *AtSUC7-OE* plants. In plants, flowering time control involves the integration of multiple signals. The *FCA*-mediated repression of *FLC* might be the best-understood mechanism of the autonomous components (Wu *et al.*, 2020). *FCA* encodes an RNA-binding protein containing two RNA-binding domains and a WW protein interaction domain. Jung *et al.* (Jung *et al.*, 2011) reported that *FCA* could promote the processing of primary transcripts of miR172 in response to changes in ambient temperature, suggesting a more complicated role of *FCA* in flowering time regulation. *REF6* belongs to another group of genes that encode histone demethylases that alter epigenetic marks at the *FLC* locus to repress *FLC* expression and promote flowering (Chowdhury *et al.*, 2020; Lu *et al.*, 2011). Therefore, the repression of *FLC* by an enhanced expression *AtSUC7* in the *IRS30* and *AtSUC7-OE* plants might be not only simply regulated by these *FLC* activators or repressors (i.e., *FCA* and *REF6*), but also be the result of, e.g., RNA processing or histone methylation. It is interesting to determine whether a sucrose transporter plays a role in *FLC* chromatin modifications in the future.

The observation of the recapitulated phenotype of *AtSUC7-OE* transgenic plants as that in *IRS30* mutant supports that the disease-resistant and early flowering phenotypes were attributed to the activation of a single *AtSUC7* gene expression. Two distinct signaling pathways regulate plant defense and flowering time. However, under adverse environmental conditions, plants do not only initiate defense reactions but also take advantage of an elaborate signaling network to coordinate their growth and defense for survival and to maximize their fitness (van Loon, 2016). There have been several investigations into the crosstalk between plant defense and flowering time control. A couple of studies showed that the two pathways are linked via the SA pathway. For example, HOPW-1-INTERACTING3 (*WIN3*) was reported to act with SA regulators to affect SA synthesis to control broad-spectrum disease resistance to biotrophic and necrotrophic pathogens and contribute to flg22-induced PTI, and simultaneously affect the expression of *FLC* and *FT* to repress floral transition (Wang *et al.*, 2011). Overexpression of another zinc-binding nuclear protein, *HIPP3*, upregulated the expression of many genes in the salicylate pathway and delayed flowering (Zschiesche *et al.*, 2015). *PUB13*, a U-box/ARM E3 ligase, negatively regulates plant defense to biotrophic pathogens but positively regulates resistance to necrotrophic pathogens.

Besides, it negatively regulates flowering time under middle-day and long-day conditions, in a photoperiod-dependent manner (Li *et al.*, 2012a; Li *et al.*, 2012b). Histone methylation could also be a joint in regulating crosstalk between diverse developmental and physiological processes. Dutta *et al.* found that an Arabidopsis JmjC DOMAIN-CONTAINING PROTEIN 27 (JMJ27) regulates pathogen defense and flowering time by controlling methylation levels of H3K9 histones of plant defense-related genes such as *PRI* and *WRKY25* as well as major flowering integrators like *CO* and *FLC* (Dutta *et al.*, 2017). In addition to *WRKY25*, several other WRKY transcription factors were also demonstrated to affect flowering. For example, both AtWRKY12 and AtWRKY13 can interact with SPL10 and bind to the promoter of *miR172b* to regulate age-mediated flowering (Ma *et al.*, 2020). *AtWRKY53*, a senescence-related factor, is a positive regulator of plant flowering initiation (Miao *et al.*, 2004). Here, we showed that *AtSUC7* accelerated plant flowering by repressing *FLC* expression. The SA signaling pathway marker gene *PRI* was slightly upregulated in the IRS30 mutant, and the JA signaling pathway marker gene *PDF1.2* was also upregulated in the *AtSUC7-OE* transgenic plants. *WRKY53* was upregulated in both IRS30 mutant and *AtSUC7-OE* transgenic plants. Interestingly, another gene *EDS1*, knock-out of which lead to SA-deficient mutant, was inhibited in both mutants expressing a higher level of *AtSUC7*. Thus, it seems likely that *AtSUC7* might regulate flowering time independent of the *EDS1*-mediated SA signaling pathway.

Increasing evidence suggests the signal crosstalk between flowering and defense response, but the mechanisms underlying this process are poorly understood. Here, we report a sucrose symporter gene *AtSUC7* acting as a positive regulator of both plant flowering and disease resistance by modulating the expression of several defenses- and floral-related genes, as summarized in the proposed model (**Fig. 12**). In summary, *AtSUC7* is a plasma membrane protein containing 12 transmembrane domains induced by *S. sclerotiorum* infection. Activation of *AtSUC7* regulates plant defense against *S. sclerotiorum* mediated by elevating *PRI*, *PDF1.2*, and *WRKY53* gene expression, while inhibiting the SA signaling pathway-related gene *EDS1*. This regulation consequently accelerates plant flowering by repressing *FLC* expression. Furthermore, our data suggest that *AtSUC7* may affect *FLC* expression through the autonomous pathway by, e.g., upregulating *FLC* repressors like *FCA* and *REF6*. Whether chromatin modification is involved in



the repression of *FLC* in this study remains unexplored and needs to be investigated in the future. Further characterization of the sucrose symporter genes, *AtSUC6*, and *AtSUC1*, *AtSUC3*, and *AtSUC5* are of great interest. It may shed more light on the molecular mechanism underlying these sucrose transporters and their contribution to the defense and growth tradeoffs of plants.

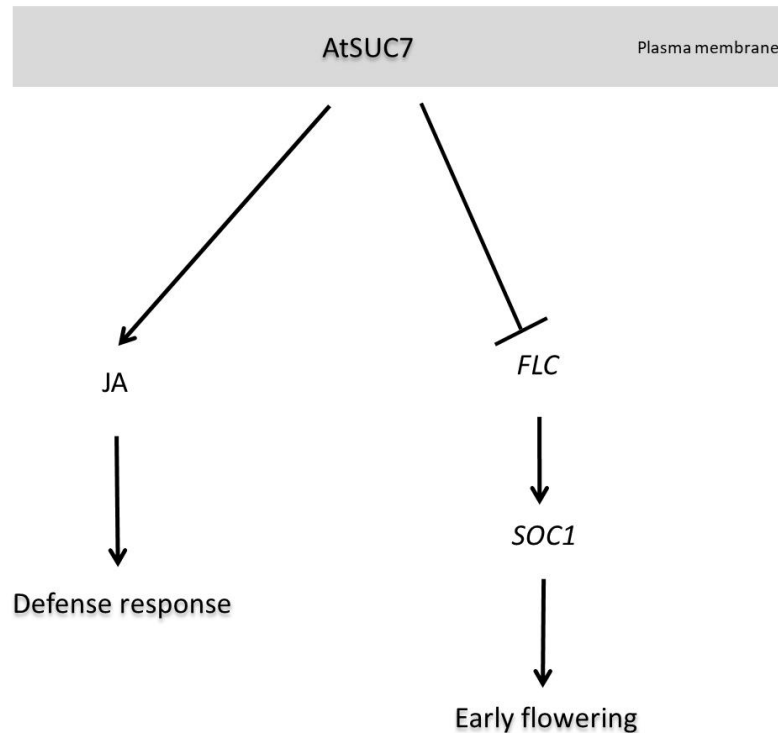


Fig. 12 A proposed functional model depicting the role of *AtSUC7* in modulating plant defense response and flowering time. Overexpression of the sucrose symporter gene *AtSUC7* can trigger the upregulation of *PDF1.2* involving JA signaling pathway. *AtSUC7* accelerated plant floral transition mainly by repressing the expression of the critical floral repressor gene, *FLC*.

### Acknowledgements

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## Supplementary data

Col-0	MSDLQANKDETTVDRQSSSSVLDLGGPSPLRKMISVASIAAGIQFGWALQSLTTPYVQLLGVPHKWSFIWLCGPVSGLLVQPSVGYFSDRCTSRFGRRR	100
IRS30 mutant	MSDLQANKDETTVDRQSSSSVLDLGGPSPLRKMISVASIAAGIQFGWALQSLTTPYVQLLGVPHKWSFIWLCGPVSGLLVQPSVGYFSDRCTSRFGRRR	100
<i>Ws</i>	MSDLQANKDETTVDRQSSSSVLDLGGPSPLRKMISVASIAAGIQFGWALQSLTTPYVQLLGVPHKWSFIWLCGPVSGLLVQPSVGYFSDRCTSRFGRRR	100
Col-0	FFIATGALLVAVSVVLIGYAADFGHSMGDKIDKPKVRAVVIFALGFWILDVANNTLQGPCRAFLGDLAGDAQKTRTANAFSSFFMAVGNVLGYAAGSY	200
IRS30 mutant	FFIATGALLVAVSVVLIGYAADFGHSMGDKIDKPKVRAVVIFALGFWILDVANNTLQGPCRAFLGDLAGDAQKTRTANAFSSFFMAVGNVLGYAAGSY	200
<i>Ws</i>	FFIATGALLVAVSVVLIGYAADFGHSMGDKIDKPKVRAVVIFALGFWILDVANNTLQGPCRAFLGDLAGDAQKTRTANAFSSFFMAVGNVLGYAAGSY	200
Col-0	TNLYKIFPFTMTKACDIYCANLKSCFFLSITLLLVVITIALWYVEDKQWSPKADSDNEKTPFFGEIFGAFKVMKREMWMLLIVTALNWIWVFPFLYDTD	300
IRS30 mutant	TNLYKIFPFTMTKACDIYCANLKSCFFLSITLLLVVITIALWYVEDKQWSPKADSDNEKTPFFGEIFGAFKVMKREMWMLLIVTALNWIWVFPFLYDTD	300
<i>Ws</i>	TNLYKIFPFTMTKACDIYCANLKSCFFLSITLLLVVITIALWYVEDKQWSPKADSDNEKTPFFGEIFGAFKVMKREMWMLLIVTALNWIWVFPFLYDTD	300
Col-0	WMGREVYGGDSKGGDKMKKLYNQGIHVVALGLMLNSIVLGVMSLGIIEGSRKMGAKRLWGA VNIILAVCLAMTVLVTKKAEHRRRIAGPMALPTDGIRA	400
IRS30 mutant	WMGREVYGGDSKGGDKMKKLYNQGIHVVALGLMLNSIVLGVMSLGIIEGSRKMGAKRLWGA VNIILAVCLAMTVLVTKKAEHRRRIAGPMALPTDGIRA	400
<i>Ws</i>	WMGREVYGGDSKGGDKMKKLYNQGIHVVALGLMLNSIVLGVMSLGIIEGSRKMGAKRLWGA VNIILAVCLAMTVLVTKKAEHRRRIAGPMALPTDGIRA	400
Col-0	GALLTFALLGIPFAITFNIPFALASIISSSSGAGGRLSLGVLNMAIVIPQMIVSFGVGPIDALFGGNLPGFVVGAIAAAVSSIVAFTVL	490
IRS30 mutant	GALLTFALLGIPFAITFNIPFALASIISSSSGAGGRLSLGVLNMAIVIPQMIVSFGVGPIDALFGGNLPGFVVGAIAAAVSSIVAFTVL	490
<i>Ws</i>	GALLTFALLGIPFAITFNIPFALASIISSSSGAGGRLSLGVLNMAIVIPQMIVSFGVGPIDALFGGNLPGFVVGAIAAAVSSIVAFTVL	490

Fig. S1 Amino acid sequence alignment of *AtSUC7* from different ecotypes, including Col-0 (Tair 10), *Ws* (Sauer et al., 2004), and the IRS30 mutant. *AtSUC7*<sub>IRS30</sub> sequence matched almost perfectly with *AtSUC7*<sub>Ws</sub>, with only one mutation at position 481. There were eight amino acid exchanges between *AtSUC7*<sub>IRS30</sub> and *AtSUC7*<sub>Col-0</sub>. The sequences were aligned with the DNAMAN Software. Similar residues are shaded gray and identical residues are shaded red.

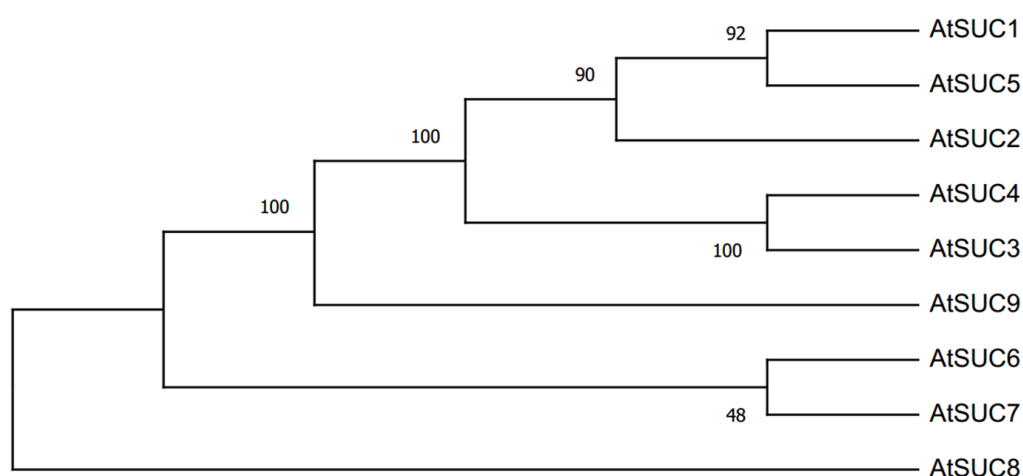


Fig. S2 Phylogenetic tree of *A. thaliana* sucrose symporters. Full-length proteins of the nine sucrose symporter members were retrieved from the NCBI nucleotide database and aligned using ClustalW. The phylogenetic tree was constructed using MegaX software. The neighbor-joining method was used to produce a phylogenetic tree with the following parameters: bootstrap analysis (1000 replicates), Poisson correction, and pairwise deletion.

Table S1 Primers used for mutant characterization.

<b>Primer</b>	<b>Sequence</b>	<b>Purpose</b>
<b>AD1</b>	NGTCGASWGANAWGAA	AD primer for TAIL-PCR
<b>AD2</b>	TGWGNAGSANCASAGA	AD primer for TAIL-PCR
<b>AD3</b>	AGWGNAGWANCAWAGG	AD primer for TAIL-PCR
<b>AD6</b>	WGTGNAGWANCANAGA	AD primer for TAIL-PCR
<b>SK_LB1</b>	TTCTCATCTAAGCCCCCATTTGG	T-DNA-specific primer for TAIL-PCR
<b>SK_LB2</b>	TGGACGTGAATGTAGACACGTCG	T-DNA-specific primer for TAIL-PCR
<b>SK_LB3</b>	ATACGACGGATCGTAATTTGTCTG	T-DNA-specific primer for TAIL-PCR
<b>GABI-Kat LB</b>	ATAATAACGCTGCGGACATCTACATTTT	T-DNA-specific primer for TAIL-PCR
<b><i>AtSAG24-R</i></b>	TGACGGTTAGCCAATGGACC	Gene-specific primer for genotyping

Table S2 Primers used for vector construction.

<b>Primer</b>	<b>Sequence</b>	<b>Purpose</b>
<b><i>AtSUC7-F</i></b>	GGGACAAGTTTGTACAAAAAAGCAGGC TTCGTGAAGAAGATGAGTGACCTCC	Forward primer for the amplification of <i>AtSUC7</i> coding region
<b><i>AtSUC7-R1</i></b>	GGGACCACTTTGTACAAGAAAGCTGGG TCCTATTTTAAAGGTAAAACGGTAAATGC	Reverse primer for the amplification of <i>AtSUC7</i> coding region (for OE)
<b><i>AtSUC7-R2</i></b>	GGGACCACTTTGTACAAGAAAGCTGGG TCAGGTAAAACGGTAAATGCCAC	Reverse primer for the amplification of <i>AtSUC7</i> coding region (for GFP)

Table S3 Gene-specific primers used in qRT-PCR.

<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>
<i>AtCOX19</i>	GGCAGGTGGAGCATTTGGAG	GCCATATCCTGCTTTGCCATC
<i>AtSAG24</i>	TGGTGTGCCAGATCCCCAAA	AGCTCCAGCACACGAAAGCA
<i>AtSUC7</i>	CCTGTCTCCGGCTTGCTTGT	CATGCTGTGGCCGAAATCAG
<i>ACT2</i>	ACCTTGCTGGACGTGACCTTACTGAT	GTTGTCTCGTGGATTCCAGCAGCTT
<i>PRI</i>	TTCTTCCCTCGAAAGCTCAA	AAGGCCACCAGAGTGTATG
<i>PDF1.2</i>	CGCTGCTCTTGTCTCTTTGC	TCCATGTTTGGCTCCTTCAA
<i>EDS1</i>	ACCAGATCATGGTCAGCC	TGTCCTGTGAACACTATCTGTTTTCTACT
<i>WRKY53</i>	CAGAGATCAGACGGGGATGC	TTTTGGGTAATGGCTGGTTTTGAC
<i>FT</i>	TTGTCAGAGGGAGAGTGGCTG	GGCATCATCACCGTTCGTTAC
<i>SOCI</i>	TCTCATGAAAGCGAAGTTTGGTC	AGAACAAGGTAACCCAATGAACAAT
<i>FLC</i>	CCGTGACTAGAGCCAAGAAGAC	CTGCTCCCACATGATGATTATTCT
<i>LFY</i>	ACAGAGGGAGCATCCGTTTATC	CTGAATACTTGGTTCGTCACCTTG
<i>LHP1</i>	ACAGCATCTGTATCAGACAATGT	GAACACAACGAACCCGAGG
<i>FRI</i>	TTTAGGAGTACAGATTTGCTGGAT	GCCAAAGGTATAAACCATTTCAGG
<i>FCA</i>	GAAGTGGACAGCAGCAAGGCTGTTG	AGGGTGCCTATGCGTTCTCTCTCC
<i>FPA</i>	TGGGTGTCGATGAGAGGTCAT	CAATTGACGACCTCAGGCAGT
<i>FLK</i>	CCACCAATGGTTCGCTCAGCAAG	ATCCGTAGCGTATCCTCCAGGCG
<i>FRL1</i>	TCACAAGTTCCAGTTCGAGTCAGCA	TTGAGGCGGCAACACATATCCAGTTT
<i>HUA2</i>	CTTACCCTTTCCCACCTCAACGTGAT	TCTGGGGAGCAGTATGACCTGGAAT
<i>PIE1</i>	TGCTGCAGAGAACCGTTACAGGAATG	GAGATTCGGATCATTTGGCCTAACCC
<i>VIP4</i>	GCCAGACCGTCAAGACGTCAAATGGAG	ATCCTTCTCTGCCCTACCTCCCAGCA
<i>ELF7</i>	CCTGGCACATACTGGTATCATTTGAC	ACCAACCCTAGAAGAGTAAACCCCTGA
<i>FLD</i>	CTCTCTTGATCGCGTTGGTT	GGACATTTATTCTTGAGGTTCA
<i>LD</i>	CAACTAATCCTGGAATGAGTGG	GGTTGTTGAGATTGGTTGTTGT
<i>FY</i>	GATGCCTGGATCAATGGGAATG	TGCTGCTGTTGGAAAGGGTGT
<i>REF6</i>	CGGAATACCGTGTTCAGGTTAG	CCGGATAGCAGCATCTTTAGCCA
<i>ARP6</i>	GTACCTGAGACGTTATTCCAGCCTGC	CTCCATACACCTAGTATGGGGTCTCC
<i>SERRATE</i>	CGGATATAAACCCACCACCAATGCTG	AGCATCTAGGTCTTGGTAGCTGCGCA
<i>SUF4</i>	ATTCTTATGCCTCTGGCCCAAACACT	CTGCATTTATCGAGTTCATCTGGCTGG
<i>PEP</i>	TCTGGTGCATCAGTTCGTATTT	CTCATCCTGTGCAGCATAAAAAC
<i>AtSUC1</i>	CTCTCCTCCTCATCGTCACC	CGATCCAGTTTAAGGCCGTG
<i>AtSUC2</i>	TTCGCCATCCTCGGTATCC	CGCCAATACACCACTTACCG
<i>AtSUC3</i>	TTGAACCAATGTGTCAGCGG	CTGCGGTTCTTGTTGTTTCG
<i>AtSUC4</i>	TGGGTGCACTTGGTTTGTATG	ATTAACACAGCAGCAAACACG
<i>AtSUC5</i>	CTCAAGTGGACCATTTGGACG	ATCCCATAGCCCCTGACATG
<i>AtSUC6</i>	AATATCATCCTCGCCGTGTG	CGGAGCTGCTAGAGATTATGG
<i>AtSUC8</i>	TGAATATCATCCTTGCTGTGTG	CCGGAACACTTGAGATTATGG
<i>AtSUC9</i>	CTTCTTTGGCGAGATCTTTGG	CCCCAACGAACCAACTTGG



## Chapter 4

### **Promoter analysis reveals a comprehensive regulatory mechanism of *AtGLP5* expression in plant defense response**

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Keywords: *AtGLP5*, *Verticillium longisporum*, flg22, promoter analysis, pathogen-responsive expression, GUS staining assays.

(For submission to Plant Stress)

## 4.1 Abstract

Our previous study demonstrated that *AtGLP5* is predominantly expressed in the root but activated in leaves by diverse stress factors, including pathogen attacks. However, the regulatory mechanism underlying this expression pattern remains unsolved. To address the transcriptional regulation of *AtGLP5*, we performed the *AtGLP5* promoter analysis in transgenic Arabidopsis expressing various *P<sub>AtGLP5</sub>::GUS*-deletion constructs and challenged them with different stress factors, including infection with *Verticillium longisporum* and treatment by flg22, respectively. GUS staining assays revealed a constitutive root-specific GUS expression under the transcriptional control of the *AtGLP5* promoter, which is consistent with our previous data. Much to our surprise, we found that the GUS expression in roots was repressed, along with the infection progression of *V. longisporum*. But, the GUS activity in leaves was detected at the late infection stage in response to *V. longisporum* and after spraying of flg22, supporting its pathogen-responsive expression. Also, the flg22-induced GUS expression was found to be significantly mitigated on Arabidopsis leaves in the *fls2* background, thus providing evidence for the involvement of *AtGLP5* in the FLS2/flg22-mediated signaling pathway and immunity. Furthermore, the promoter deletion analysis unveiled several *cis*-element candidates within a 240bp region of the *AtGLP5* promoter, which is mainly responsible for the regulation of *AtGLP5* expression.

## 4.2 Introduction

Plants have developed a set of fine-tuned mechanisms to react to pathogen attacks, including the transcript accumulation of many defense-related genes (Neukermans *et al.*, 2015). In *Brassica napus*, sclerotinia stem rot caused by the necrotrophic fungus *Sclerotinia sclerotiorum* is one of the most important diseases leading to severe crop yield losses (Del R ó *et al.*, 2007). Genetic engineering offers a promising strategy to control the disease (Punja, 2001). Numerous studies have contributed to the improvement of host resistance against *S. sclerotiorum*. For instance, the *WRKY* transcription factors, the second largest group of *Arabidopsis thaliana* transcription factors, are involved in diverse physiological processes and plant response to biotic and abiotic stresses (Ülker and Somssich, 2004). Overexpression of *AtWRKY75* in *A. thaliana* elevated plant resistance to *S. sclerotiorum* by activating the JA/ET pathway (Chen *et al.*, 2013). Transgenic oilseed rape plants expressing a wheat oxalate oxidase (OxO) displayed an increase in plant OxO activity and improved plant resistance to both oxalate acid (OA, the main pathogenicity factor of *S. sclerotiorum*) and the pathogen *S. sclerotiorum* (Dong *et al.*, 2008). In soybean, the transcript level of a germin-like protein, GmGLP10, was induced by plant hormones SA, MeJA, ET, and plant-pathogen *S. sclerotiorum*. Overexpression of *GmGLP10* in tobacco plants remarkably increased plant resistance against *S. sclerotiorum* (Zhang *et al.*, 2018).

Germin protein was first discovered as a molecular marker during wheat embryo germination (Grzelczak and Lane, 1984; LANE *et al.*, 1992). Both germin and germin-like proteins are glycosylated and resistant to high temperature, sodium dodecyl sulfate (SDS), and extreme pH (Dunwell *et al.*, 2008). Unlike the “true” germains, which have only been found in cereals, germin-like proteins are encoded by multigene families in almost all angiosperms (Bernier and Berna, 2001). Most of the analyzed germin-like proteins possess an N-terminal secretory signal, indicating a role associated with cell wall function or defense response to plant pathogens (Carter *et al.*, 1998). A key mechanism for their antimicrobial capability is the superoxide dismutase (SOD) activity, which can generate hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), a multi-functional signaling molecule that catalyzes cross-linking of cell wall components and triggers signal transduction pathways in plant defense responses and cell death (Mehdy *et al.*, 1996; Yamahara *et al.*, 1999). For example, two germin-like proteins BnGLP3 and BnGLP12 in *B. napus* had been

demonstrated to possess SOD activity and contribute to plant resistance against *S. sclerotiorum* (Rietz *et al.*, 2012). Pei *et al.* reported that GhABP19, a germin-like protein from *Gossypium hirsutum*, exhibits SOD activity and enhances plant defense against verticillium and fusarium wilt (Pei *et al.*, 2019). Nevertheless, the functions of GLPs in regulating plant development and responses to stress are poorly understood so far.

The *A. thaliana* genome contains at least 12 GLP loci corresponding to 39 expressed sequence tags (ESTs) (Carter *et al.*, 1998). *AtGLPs* are differentially expressed in plant tissues and regulate plant growth and development. In 1997, Membré *et al.* reported that the *AtGER3* promoter is expressed in plant leaves but not roots, and it is involved in circadian clock regulation (Membré *et al.*, 1997). Later, Schlesiel *et al.* demonstrated the existence of *AtGER3* protein in *A. thaliana* root extracts through proteome analysis (Schlesier *et al.*, 2004). Furthermore, ectopic expression of a sugar beet germin-like protein BvGLP1 into *A. thaliana* resulted in an enhanced plant disease resistance to *V. longisporum* and *Rhizoctonia solani* (Knecht *et al.*, 2010), suggesting a general role of GLPs in plant response to pathogens.

Flg22 is a synthetic 22-amino acid peptide corresponding to a conserved bacteria flagellin domain adequate to trigger plant defense response (Naito *et al.*, 2007). The gene induction by flg22 is rapid and transient, as the response diminished slowly and returned to the basal level by 24h (Denoux *et al.*, 2008). In *A. thaliana*, the perception of flg22 is based on the flagellin-sensitive 2 (FLS2) receptor, as site mutations in FLS2 caused abolished flg22 binding. Moreover, the plant receptor kinase BAK1/SERK3 (BRI1-associated kinase 1) was identified as a coreceptor of ligand-binding leucine-rich repeat receptor kinases, such as the immune receptor FLS2 and the brassinosteroid receptor BRI1 (Chinchilla *et al.*, 2009). *bak1* mutants show standard flagellin binding but are less sensitive to flg22 treatment (Chinchilla *et al.*, 2007).

In our previous work, we demonstrated that *AtGLP5* is predominantly expressed in the root but activated in leaves by diverse stress factors, including pathogen attacks. To address the transcriptional regulation of *AtGLP5*, we characterized the *AtGLP5* promoter in transgenic *Arabidopsis* expressing a series of  $P_{AtGLP5}::GUS$  constructs and challenged them with different stress factors, including the infection with *Verticillium longisporum* and treatment by flg22, respectively. We report that *AtGLP5* was repressed in the root tissues by infection of *V.*

*longisporum*. We observed that in leaves, the expression of *AtGLP5* could be activated explicitly by pathogen attacks, as demonstrated by flg22 leaf spraying. Furthermore, we report that the FLS2/flg22-mediated signaling pathway enhances the expression of *AtGLP5*. Finally, we describe several *cis*-elements in a 240 region of *AtGLP5* promoter, which may play an essential role in the regulation of *AtGLP5* expression.

## 4.3 Materials and Methods

### 4.3.1 Plant materials and cultivation

*A. thaliana* was grown at 22 °C in a climate chamber under short-day condition (8 h light/ 16 h dark). Transgenic plants expressing GUS-tagged full-length or truncated *AtGLP5* promoter were generated in our lab. Zheng Zhou kindly provided the *fls2* mutant and *bak1* mutant in the Col-0 background.

### 4.3.2 Plasmid construction and plant transformation

The constructs in our study were cloned through Gateway cloning (Gateway® technology, Invitrogen). The *AtGLP5*-related fragments were first amplified using Phusion® High-Fidelity Polymerase (Thermo Fisher Scientific) with corresponding primers (**Supplementary Table S1**) and cloned into the pDONR201 entry vector using BP clonase II enzyme mix. The positive clones were sequenced to ensure that no mutations were introduced during PCR amplification. Entry vectors with the right sequence were then combined into different destination vectors (pGWBs) by LR reaction for various research purposes (Nakagawa *et al.*, 2007). For example, pGWB433 was used for promoter::GUS analysis, pGWB451 for GFP-tagged subcellular localization analysis, and pGWB414 was used for overexpression of *AtGLP5*. All binary vector constructs were introduced into *Agrobacterium tumefaciens* GV3101 strain and transformed into *A. thaliana* Col-0 plants through the floral dip method (Clough and Bent, 1998). Additionally, full-length *P<sub>AtGLP5</sub>::GUS* construct was also transformed into the *fls2* mutant. The T<sub>0</sub> generation seeds were first selected on 1/2 MS plates with appropriate antibiotics and further confirmed by PCR.

### 4.3.3 *V. longisporum* infection on agar plates

*V. longisporum* isolate VL43 was provided by Dr. Elke Diederichsen (FU Berlin, Germany). The infection process was described by Behrens (Behrens *et al.*, 2019). Shortly, four days after plant germination, Arabidopsis seedling were transferred onto squared plates containing 1/2 MS medium and grown vertically in the growth chamber for more days. On the day of inoculation, 22% glycerol conidia stocks were thawed, centrifuged at 5,000 rpm for 10 min, and resuspended in sterile Czapek-Dox medium to a final concentration of  $2 \times 10^6$  conidia/ml. Then the conidia suspension was evenly spread on the roots with a sterilized brush. After 3, 6, and 9 days, the

whole seedlings were stained with GUS staining solution to observe GUS activity change during different infection stages. Non-infected plants were used as control.

#### **4.3.4 Flg 22 treatment**

One mM flg22 was prepared and sprayed evenly onto *A. thaliana* seedling (Zhou *et al.*, 2017). Briefly, *A. thaliana* seedlings were grown in soil for three weeks. The experimental groups were then evenly sprayed with 1 mM flg22, while the control groups were sprayed with water. Five hours after flg22 treatment, the plant leaves were then used for either GUS staining or qPCR analysis.

#### **4.3.5 *S. sclerotiorum* infection**

The *S. sclerotiorum* isolate used throughout this work was obtained from Professor W. Qian (Mei *et al.*, 2011). *S. sclerotiorum* was grown on a minimal medium at 22 °C. Three days later, agar-plugs were punched from the expanding margin of the mycelium using a 6 mm cork borer and placed on fully developed leaves of 4-week-old plants with the mycelium facing the leaf surface. Plants were covered with a transparent lid to keep high humidity. After 48 hours, the leaves were detached from the plants and used for GUS staining.

#### **4.3.6 Wounding and plant hormone treatment**

For the wounding treatment, 4-week-old plant leaves were cut with a pair of scissors. After 5 hours, the leaves were detached from the plants and used for GUS staining. For the SA treatment, 4-week-old plant leaves were sprayed with 1 mM SA or water as a control. After 24 hours, the leaves were detached from the plants and used for GUS staining. For the IAA and IBA treatment, 7-day-old seedlings were incubated in 10 µM IAA or 10µM IBA solutions, 0.1% ethanol was used as control. After 6 and 24 hours, the whole seedlings were used for GUS staining.

#### **4.3.7 Gus staining assays**

For histochemical GUS analysis, plant samples were incubated in fresh GUS staining buffer overnight at 37 °C. On the next day, the GUS staining buffer was carefully removed and replaced with 70% ethanol to bleach the green chlorophyll. Several changes of ethanol may be necessary until the tissues turned white. The stained samples were examined under a stereomicroscope (Zeiss).

### 4.3.8 Isolation of plant genomic DNA

For DNA isolation, plant samples were collected in 1.5 mL microcentrifuge tubes and immediately frozen in liquid nitrogen and then ground using a plastic pestle in the tube itself. DNA was isolated following the cetyltrimethylammonium bromide (CTAB) DNA Miniprep protocol as described (Clarke, 2009).

### 4.3.9 Isolation of plant RNA and qRT-PCR analysis

Total RNA was extracted from plant tissues with TRIzol<sup>®</sup> Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The purity and concentration of RNA were measured with GE Healthcare NanoVue Plus UV-Vis Spectrophotometer and evaluated by electrophoresis on an agarose gel. Subsequently, 1 µg of RNA was digested with DNase I (Thermo Scientific) for 30 min and reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The cDNA quality was checked by RT-PCR using the reference gene *Actin2*, and a 1:20 dilution of the cDNA was made as a PCR template. qRT-PCR was conducted in 10 µl reactions with Maxima SYBR Green qPCR Master Mix (Thermo Scientific) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following conditions: 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Relative expression of mRNA was calculated by the delta-delta CT method (Livak and Schmittgen, 2001). And all values for the mRNA species were normalized to *Actin2*. qRT-PCR primers can be found in **Supplementary Table S2**.

### 4.3.10 Statistical analysis

GraphPad Prism 8 computer software was used to perform all statistical analyses (GraphPad Prism, San Diego, CA, USA). The results were represented as means ± S.E., and different numbers of asterisks indicate significantly different values. The significant difference between the two groups was performed using the student's *t*-test. Multiple comparisons of means were analyzed by one-way ANOVA, followed by Dunnett's multiple comparisons test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).



## 4.4 Results

### 4.4.1 *In silico* analysis of the *AtGLP5* promoter sequence

To gain insight into the *cis*-regulation mechanism of *AtGLP5* expression, we undertook an *in silico* analysis of a ~1.5 kb sequence upstream to the start codon of *AtGLP5* using the PlantCARE online program (Lescot *et al.*, 2002). Several putative regulatory motifs related to different tolerance factors were identified within this region (**Fig. 1**). For example, a W-box (fungal elicitor responsive element) was located at -206 bp, and there were also many light-responsive elements (ACE, MRE, GT1-motif, Box 4, CCAAT-box) as well as wound-responsive elements (WUN-motif, WRE3). Moreover, several other types of *cis*-elements were also found in the promoter sequence, including a few MYB binding sites (CCAAT-box, MYB, MRE), a circadian element, an O<sub>2</sub>-site (zein metabolism regulation), an ARE motif (involved in anaerobic induction) and a CAT-box which is related to meristem expression. Multiple copies of core promoter elements like TATA-box and CAAT-box were also identified. In **Fig. 1**, some motifs were selected and visualized using a cisHighlight tool written by Sebastian Wolf (<https://msbi.ipb-halle.de/cisHighlight/>). The presence and frequency of these regulatory elements suggested that the promoter of *AtGLP5* might be involved in plant pathogen, wounding, and light-related responsivenesses.

### 4.4.2 *V. longisporum* interferes with the expression of *AtGLP5*

*V. longisporum* is a soilborne fungal plant pathogen which starts its infection through plant root, then gradually colonize the xylem vessels and finally spread into plant stems and leaves. In *P<sub>AtGLP5</sub>::GUS* transgenic *A. thaliana* seedlings, GUS expression was originally detected in the roots but not in the leaves, consistent with the transcript analysis (**Fig. 2B**). Interestingly, we noticed that at 6 days after infection of *V. longisporum*, when the fungi reached the upper part of the plant and leaves, GUS activity was repressed in the roots (**Fig. 2C**). However, a strong induction of GUS activity was concurrently given in the infected leaves (**Fig. 2B**). A similar suppression of GUS expression in the roots could also be observed in syncytia induced by *H. schachtii* (data not shown).

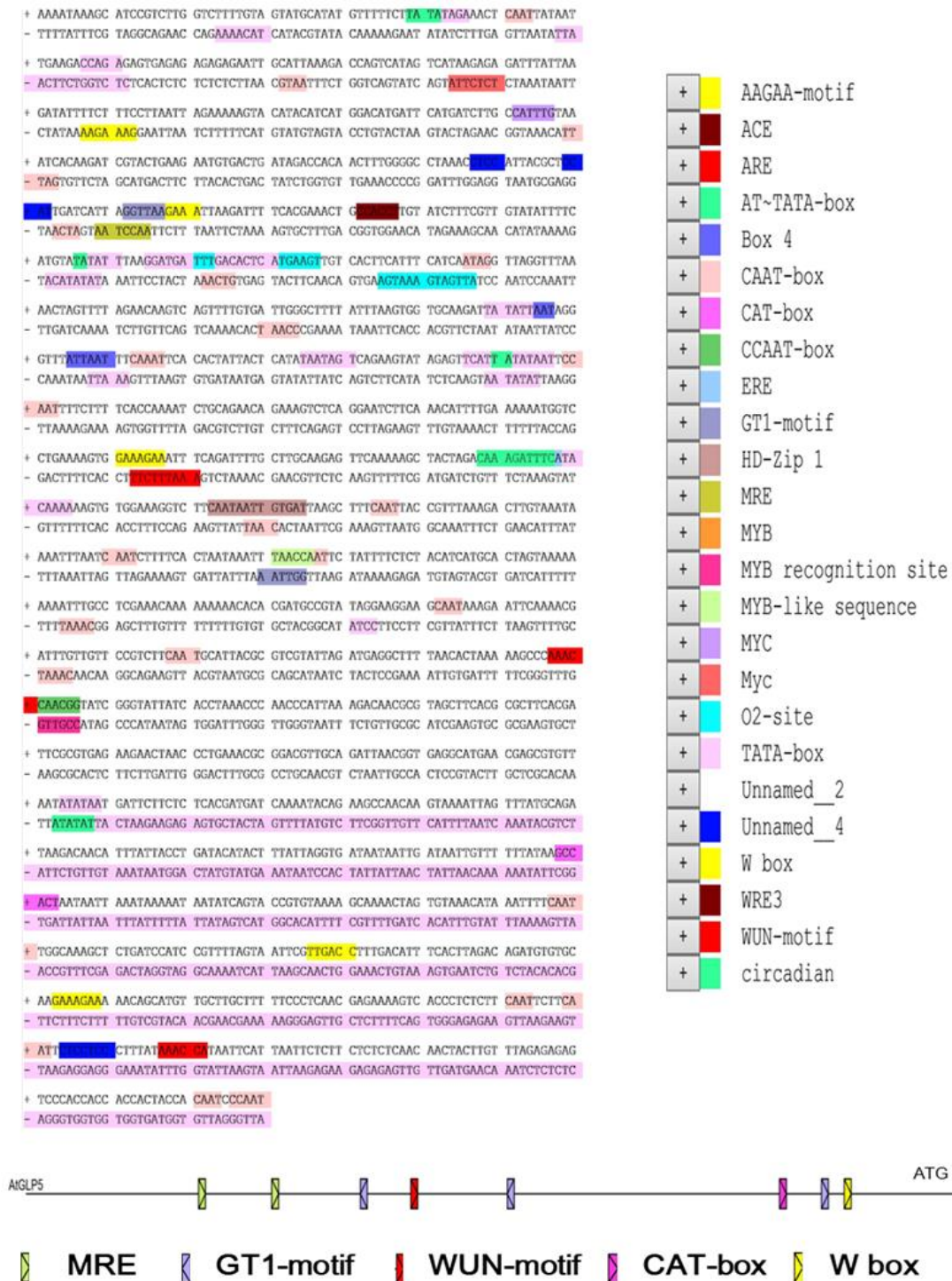


Fig. 1 Putative *cis*-acting elements within the *AtGLP5* promoter sequence were analyzed using the PlantCARE online program. Different colors represented different motifs. Some were then selected and visualized with a cisHighlight tool written by Sebastian Wolf at IPB Halle. The arrows in the boxes indicated sense or antisense direction.

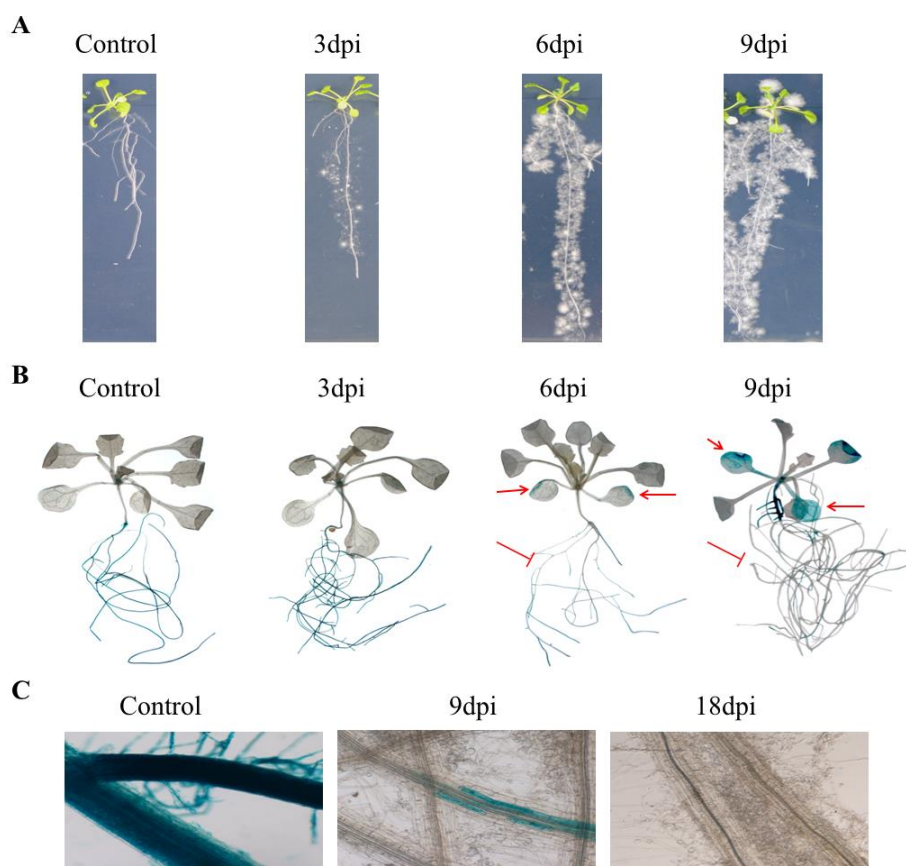


Fig. 2 GUS activity was induced in leaves but suppressed in roots upon *V. longisporum* infection. (A) The non-stained plants can indicate the infection progress of *V. longisporum*. (B) GUS activity can be observed after GUS staining in the root but not in the leaves in non-infected seedlings. At three days post-inoculation, there is no noticeable change in GUS activity. However, as the pathogen colonized the plant, GUS activity was inhibited in the root but induced in the infected leaves. (C) GUS activity was repressed in the roots.

#### 4.4.3 The induction of *AtGLP5* by flg22 is interrupted in two receptor mutants

In *A. thaliana*, the plant flg22 signaling is mediated by the receptor FLS2 and its coreceptor BAK1. Our previous transcript analysis revealed that flg22 could induce *AtGLP5* expression in Arabidopsis plants and activate GUS expression in the transgenic  $P_{AtGLP5}::GUS$  plants. Moreover, the abolishment of the flg22-induced *AtGLP5* expression in Arabidopsis *fls2* mutant supports that *AtGLP5* is involved in the FLS/flg22-dependent signaling pathway. To gain a deeper insight into the role of *AtGLP5* we further analyzed the expression of *AtGLP5* in both *fls2* and *bak1* mutants. As shown in **Fig. 3A**, knockout of FLS2 and BAK1 did not affect *AtGLP5* expression. Upon flg22 treatment, *AtGLP5* was upregulated in the wild-type plant. However, it was almost abolished in the *fls2* mutant. The expression level of *AtGLP5* in the *bak1* mutant was markedly lower compared with Col-0 (**Fig. 3**). This result suggests that the flg22 sensing to induce *AtGLP5* expression strictly relies on a functional FLS2 receptor.

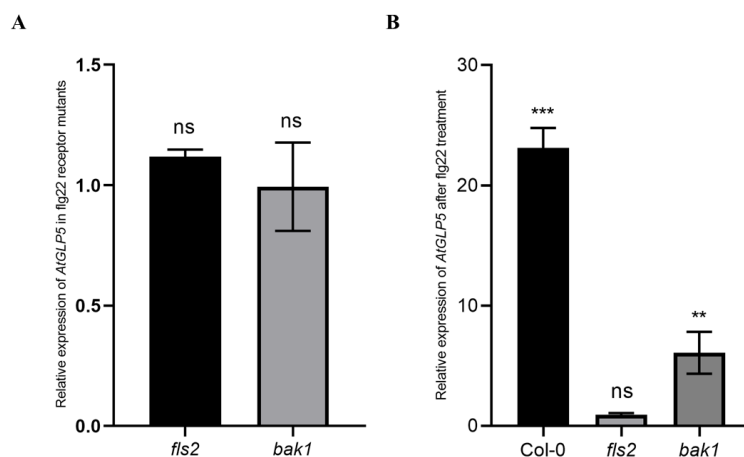


Fig.3 *AtGLP5* expression in *fls2* and *bak1* mutants. (A) The expression of *AtGLP5* was not altered in the *flg22* receptor mutants. (B) *AtGLP5* can be induced in Col-0 and *bak1* mutant by *flg22* but not in *fls2* mutant. Results are shown as means  $\pm$  SE and bars represent standard errors based on three independent experiments. Asterisks indicate the significant differences determined with unpaired Student's t-test (ns = not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

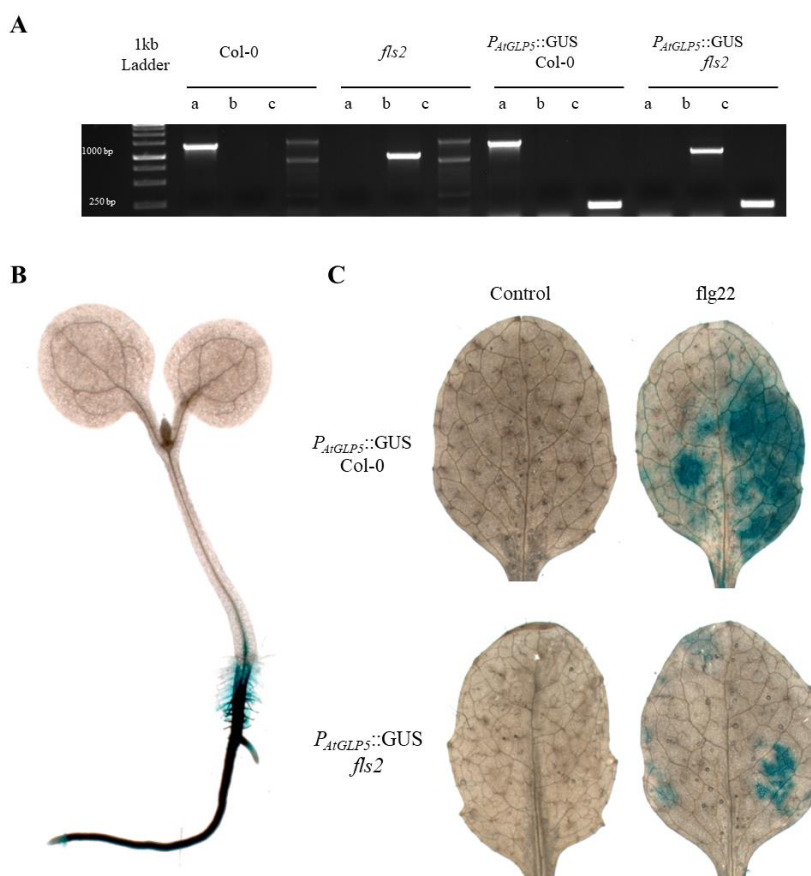


Fig. 4 GUS activity can be induced by *flg22* in the transgenic *fls2* mutant transformed with *AtGLP5* promoter. (A) Transgenic *fls2* mutant harboring  $P_{AtGLP5}::GUS$  construct was confirmed by PCR. Col-0 served as a negative control of both the *GUS* gene and T-DNA insertion. (B) GUS activity was mainly detected in the roots in the transgenic *fls2* mutant expressing  $P_{AtGLP5}::GUS$ . (C) GUS expression was activated by *flg22* in the  $P_{AtGLP5}::GUS$ -*fls2* transgenic plants, though not as pronounced as in transgenic Col-0 plants expressing  $P_{AtGLP5}::GUS$ .

Next, we transformed the  $P_{AtGLP5}::GUS$  construct into Arabidopsis *fls2* mutant plants (**Fig. 4A**). Similarly, GUS expression could be detected in the roots of *fls2* transgenic plants expressing  $P_{AtGLP5}::GUS$  (**Fig. 4B**). Interestingly, as shown in **Fig. 4C**, an unexpected induction of GUS activity by *flg22* was observed in the *fls2* mutant plants transformed with  $P_{AtGLP5}::GUS$ . However, the signal is less intense than transgenic Col-0 plants expressing  $P_{AtGLP5}::GUS$  (**Fig. 4C** and **Fig. S1**). This result may reflect the effect of an ectopic expression of *AtGLP5* promoter in Arabidopsis and the complexity of *flg22* sensing induced signaling pathways in plants.

#### 4.4.4 Deletion analysis of *AtGLP5* promoter reveals a 240bp region crucial for the regulation of *AtGLP5* expression

To identify the necessary region for the function of the *AtGLP5* promoter, we constructed three truncated promoters with 5' serial deletions tagged with the GUS reporter gene and transformed them into *A. thaliana* (**Fig. 5A**). All the three promoter constructs were able to drive a root-specific GUS expression as observed with the full-length promoter, except that the GUS expression intensity was dramatically reduced in the P3 promoter construct (**Fig. 5B**).

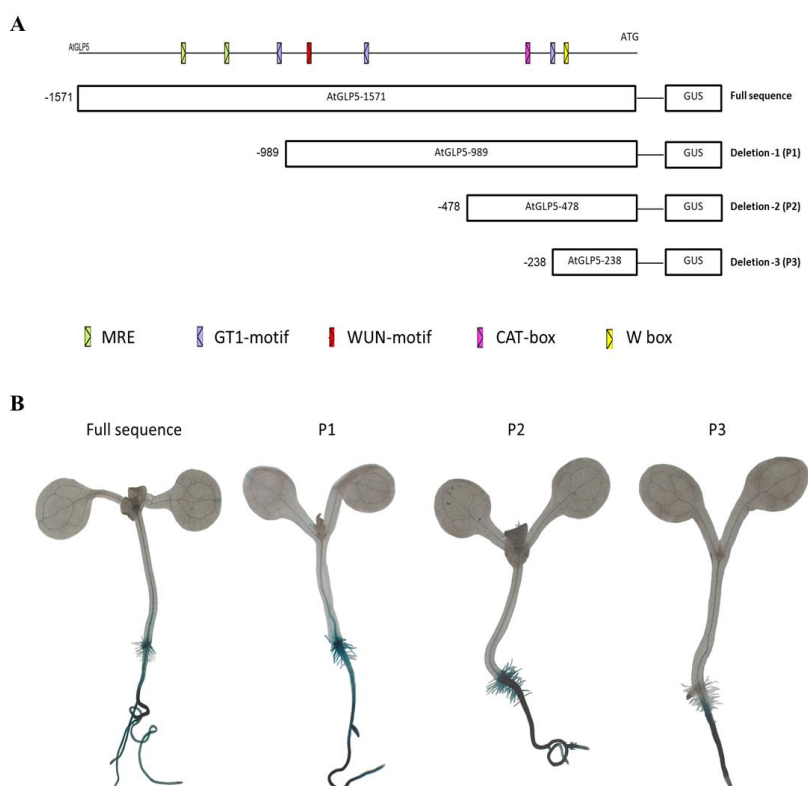


Fig.5 Progressive 5' serial deletions of the *AtGLP5* promoter. (A) Schematic representation of the three truncated fragments (P1, P2, and P3) fused to the GUS. Different colors represent different motifs predicted with PlantCARE online program. (B) GUS activity can be observed in the roots of all the three truncated promoters. However, the shortest P3 promoter showed dramatically reduced GUS intensity.

Next, we challenged the transgenic plants expressing different *AtGLP5* promoter deletion constructs with various biotic/abiotic stimuli. As shown in **Fig. 6**, the GUS expression was detected in none of the untreated control leaves. However, after treatments with *S. sclerotiorum*, SA, wounding, and flg22 leaf spraying, P1 and P2 promoters gave a similar GUS expression as observed with the full-length promoter. However, the P3 promoter, which is only 240 bp shorter than P2, could not trigger the GUS expression in transgenic plants. Thus, we conclude that the 240 bp region in the promoter sequence (P2) is pivotal for the regulation of *AtGLP5* expression.

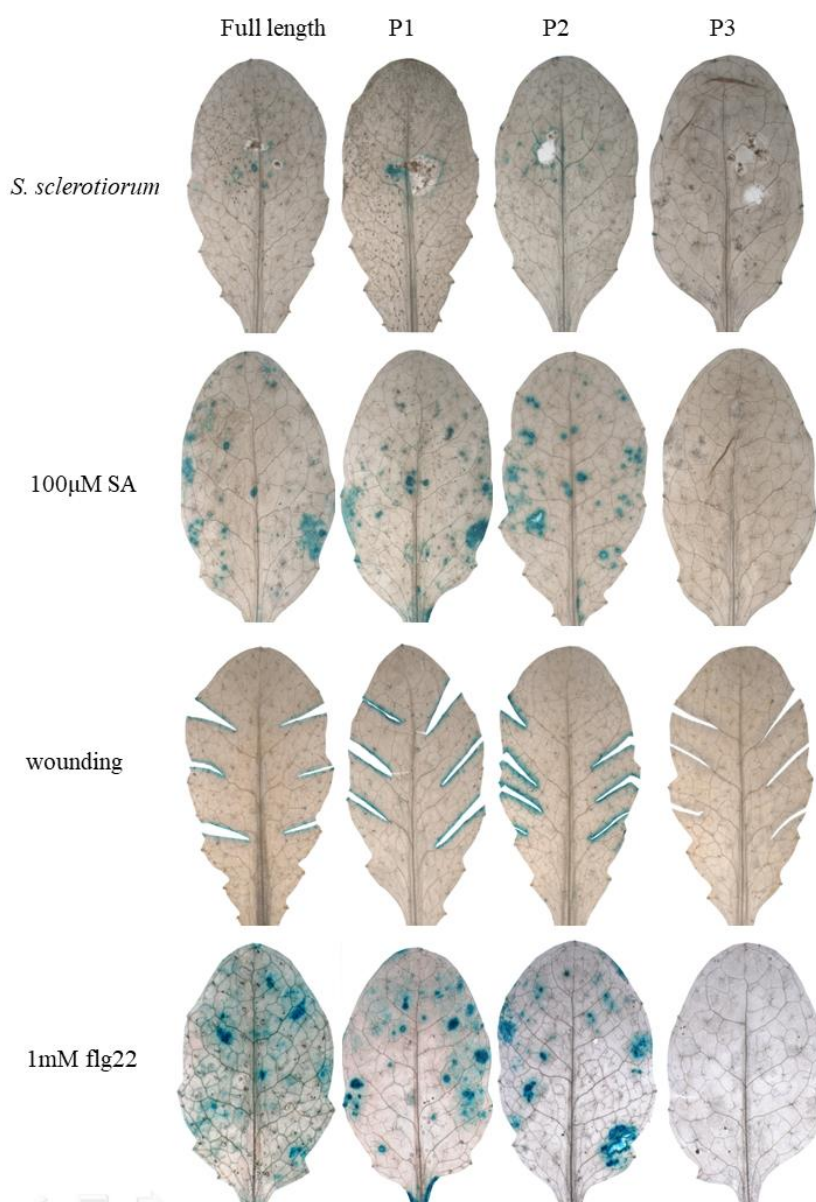


Fig. 6 Analysis of 5' deletions of *AtGLP5* promoter revealed a 240 bp region that was important for promoter activity. Upon *S. sclerotiorum*, SA, wounding, and flg22 treatments, P1 and P2 promoters showed similar stress-responsive patterns with the full-length promoter. In contrast, the shortest P3 promoter didn't respond to any of these stimuli.

## 4.5 Discussion

In our previous study, we found that *AtGLP5* is constitutively expressed in plant roots but not in leaves. The model of the function of *AtGLP5* in plant development and defense response remains unsolved. To address the transcriptional regulation of *AtGLP5*, we performed the *AtGLP5* promoter analysis in transgenic Arabidopsis expressing  $P_{AtGLP5}::GUS$  construct by challenging the plants with *Verticillium longisporum* and flg22 leaf-spraying, respectively.

The fungus *V. longisporum* enters the plants via the root and colonizes the plant vascular system. After reaching the plant's aerial parts, the fungus starts to invade leaf tissues and switches from biotrophic to a necrotrophic lifestyle (Reusche *et al.*, 2013). Recent studies suggest that different signaling pathways are involved in the host's response to *V. longisporum* (Häffner *et al.*, 2014). In *A. thaliana*, the ABA biosynthesis gene *NCED3* is upregulated in Arabidopsis roots and leaves upon *V. longisporum* infection. But in oilseed rape roots, this upregulation is only observed in the leaves (Behrens *et al.*, 2019). A constitutive expression of *AtGLP5* in *A. thaliana* roots may reflect its functional role in plant innate immunity protecting plant roots from pathogen attacks. In support of this scenario, the expression of *AtGLP5* in leaves is merely activated in response to the pathogen infection. A pathogen-induced expression of *AtGLP5* in leaves offers a unique and valuable system for a more in-depth characterization of the regulatory mechanism governing the *AtGLP5* expression.

The activation of the defense response by flg22 relies on the membrane resident receptor-like kinase FLS2 (Gómez-Gómez *et al.*, 2001; Gómez-Gómez and Boller, 2000). Shortly after flagellin perception by FLS2, BAK1 is recruited into the receptor complex functioning as a coreceptor in assisting the ligand-binding receptor in transmitting the signal from the outside of the cells to the inside (Chinchilla *et al.*, 2009; Heese *et al.*, 2007). It has been shown that Arabidopsis *bak1* mutants show normal binding but reduced sensitivity to flg22, indicating that BAK1 acts as a positive regulator in signaling (Chinchilla *et al.*, 2007). The residual sensitivity to flg22 observed in *bak1* mutants suggested that there might be a functional redundancy with other SERK proteins (Albert *et al.*, 2015). In accordance, we observed that the induction of *AtGLP5* in the *fls2* mutant is abolished and reduced in the *bak1* mutant. However, the observation of limited GUS expression occurring in the transgenic *fls2* mutants expressing the *AtGLP5* promoter by

flg22 supports for a complex and redundant mechanism of flg22 perception and signal transduction and activation of plant defense response in plants. This result can also explain why *Arabidopsis bak1* mutants did not exterminate *AtGLP5* induction upon flg22 treatment. It would be highly interesting to clarify the flg22-activated *AtGLP5* expression and its role in plant immunity.

An important finding of this study is the identification of a 240 bp region of the *AtGLP5* promoter sequence that was essential for the regulation of *AtGLP5*. A similar GUS expression pattern activated by different stimuli observed in the full-length, P1, and P2 promoter constructs, suggesting that the P2 promoter still contains the essential *cis*-elements responsible for the regulation of *AtGLP5* expression, while the P3 promoter construct was not sufficient to induce GUS expression under all given treatments. Therefore, we analyzed the promoter region from -478 to -238 in more detail and propose it is crucial for the regulation of *AtGLP5*. Transcription of genes was regulated by transcription factors that specifically bind to *cis*-regulatory elements. The W box [(T)TGAC(C/T)] is the binding site for WRKY transcription factors and represents a major class of *cis*-acting elements responsible for the pathogen- or SA-induced gene expression in plants (Li *et al.*, 2006; Rushton *et al.*, 1996). It was shown that wound- and pathogen-induced signaling consists of networks with some shared components (Romeis *et al.*, 1999). Our *in silico* analysis of the *AtGLP5* promoter sequence identified a subset of *cis*-elements related to defense response. Among them, there exists a W box at the position of -206 bp. The motif GT-1 is known to mediate pathogen and light-induced gene expression (Lam and Chua, 1990; Park *et al.*, 2004). A meristem-specific element CAT-box is present in the 240bp region (Hong *et al.*, 2011). Thus, it is reasonable to believe that the response of *AtGLP5* to various stimuli might be controlled by the *cis*-elements like the GT-1 motif or CAT-box located within the 240bp region. Further analysis is needed to understand the regulation of *AtGLP5* expression regarding its functional relevance.

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## Supplementary data

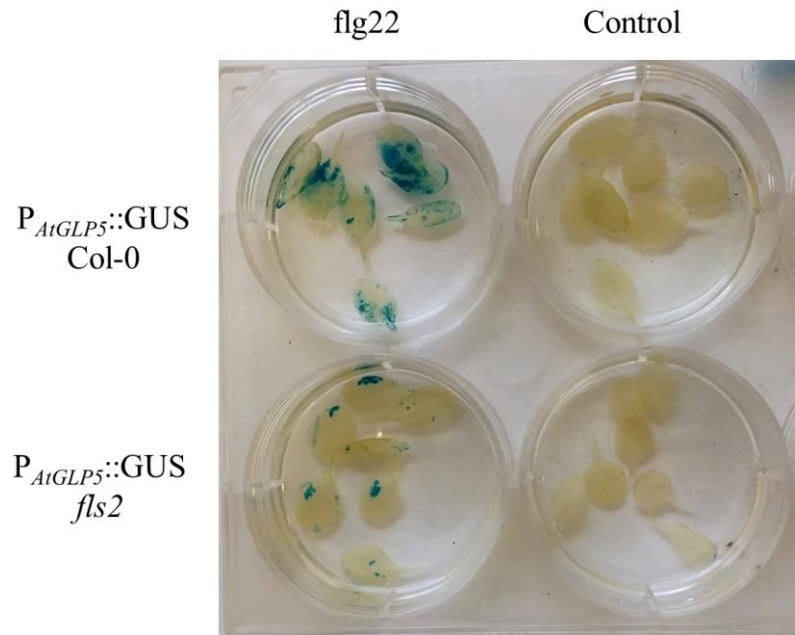


Fig. S1 Transformation of  $P_{AtGLP5}::GUS$  construct into the *fls2* mutant. In transgenic Col-0 plants expressing *AtGLP5* promoter, GUS activity was induced by flg22. Unexpectedly, in the transgenic *fls2* mutant transformed with  $P_{AtGLP5}::GUS$  construct, GUS activity was also induced after flg22 treatment. Plants sprayed with water served as control.

Table S1 Primers used in this study.

Primer	Sequence	Purpose
$P_{AtGLP5}$ -F1	GGGGACAAGTTTGTACAAAAAAGCAGGC TGCAGAACAGAAAGTCTCAGG	Cloning F1 <i>AtGLP5</i> promoter
$P_{AtGLP5}$ -F2	GGGGACAAGTTTGTACAAAAAAGCAGGC TTAACGGTGAGGCATGAACG	Cloning F2 <i>AtGLP5</i> promoter
$P_{AtGLP5}$ -F3	GGGGACAAGTTTGTACAAAAAAGCAGGC TCAAAGCTCTGATCCATCCG	Cloning F3 <i>AtGLP5</i> promoter
$P_{AtGLP5}$ -R	GGGGACCACTTTGTACAAGAAAGCTGGG TCTGGGATTGTGGTAGTGGTG	Cloning of <i>AtGLP5</i> promoters
<i>ACT2</i> -F	ACCTTGCTGGACGTGACCTTACTGAT	qRT-PCR
<i>ACT2</i> -R	GTTGTCTCGTGGATTCCAGCAGCTT	qRT-PCR
<i>AtGLP5</i> -F	CAAAATCAATGGCTTCCCTTGC	qRT-PCR
<i>AtGLP5</i> -R	CGAGGACGAAAACGACCTCAGT	qRT-PCR
<i>Gus</i> -F	GTTGACTGGCAGGTGGTGG	qRT-PCR
<i>Gus</i> -R	GGTAGATATCACACTCTGTCTGG	qRT-PCR
<i>fls2</i> _LP1	TGTCCGGTGATGTTCCCTGAG	Genotyping the <i>fls2</i> mutant
<i>fls2</i> _RP1	CAGCTCTCCAGGGATGGTTC	Genotyping the <i>fls2</i> mutant
LBb1.3	ATTTTGCCGATTTTCGGAAC	Genotyping the <i>fls2</i> mutant

## Chapter 5

### General Discussion

This study utilized the *Arabidopsis thaliana-Sclerotinia sclerotiorum* pathosystem to discover valuable rapeseed stem rot disease resistance engineering traits with activation-tagging, transgenic overexpressing, and T-DNA insertion knock-out mutants. By comparing their different disease resistance phenotypes and analyzing the expression of several pathway associated genes, we have got a relatively detailed knowledge about the molecular mechanism for the anti-pathogen function of the two membrane-localized proteins, AtGLP5 and AtSUC7. AtGLP5 is also further demonstrated to possess SOD activity and has a pivotal role in FLS2-associated flg22 signaling particularly. *AtSUC7*, previously regarded as a pseudogene encoding non-functional sucrose transporter, is proved to be involved in disease resistance and flowering time control.

#### **5.1 Plasma membrane proteins play a vital role in plant defense response and are promising candidates for genetic engineering increased disease resistance in plants**

Plasma membrane (PM), or plasmalemma, is a thin (typically between 4 and 10 nm) but highly elaborated structure that forms a living cell's protective outer boundary. It can serve as an obstacle keeping the cells' ingredients in and unnecessary elements out, or an entrance transporting fundamental nutrients into the cell and delivering the wastes out of the cell (Murphy *et al.*, 2010). According to the Singer-Nicolson fluid-mosaic membrane model, the PM is a mosaic of components, including phospholipids, cholesterol, and proteins that move freely and fluidly in the plane of the membrane. The lipid bilayer builds up a fundamental two-dimensional matrix consisting of amphipathic lipid molecules with a polar or hydrophilic head group and a non-polar or hydrophobic tail (Nicolson, 2014; Singer and Nicolson, 1972). Embedded in the lipid bilayer are large proteins associated with the cell membrane (Robertson, 2018). These proteins can function either as paths or transporters to facilitate the movement of compounds across the plasma membrane, either as receptors or structural proteins (Luschnig and Vert, 2014; Ren *et al.*, 2019; Sussman, 1994).

To determine the subcellular localization of AtGLP5 and AtSUC7, genes encoding both proteins were placed in a plant expression vector pGWB405 with a 3' fluorescent protein (GFP) tag and then introduced into *A. thaliana*. Via fluorescent microscopy, we could observe a PM localization of both proteins. Since the PM outlines the boundary between the cell and its surroundings, it serves as the fundamental site for signal perception and transduction to downstream signaling cascades (Furt *et al.*, 2011). The sensing of danger signals is the initial step in the activation of plant immune responses. After *S. sclerotiorum* inoculation, both *AtGLP5* and *AtSUC7* were induced within 3 hours and then went down after 12 or 24 hours, indicating that they are active at the early stages of the infection process. Therefore, it is reasonable to assume that the early induction of *AtGLP5* and *AtSUC7* leads to the timely activation of defense to restrict fungal growth and colonization. In support of this, transgenic plants overexpressing both *AtGLP5* and *AtSUC7* show enhanced resistance against *S. sclerotiorum* infection in *A. thaliana*. Furthermore, transcription analysis of several defense-related genes revealed that both *PDF1.2* and *WRKY 53* were constitutively upregulated in *AtGLP5-OE* and *AtSUC7-OE* transgenic plants, respectively. *PDF1.2* is a marker gene of the JA/ET signaling pathway (Kravchuk *et al.*, 2011). This result suggests that both *AtGLP5* and *AtSUC7* are involved in activating the JA signaling pathway known for plant resistance against necrotrophs (El Oirdi *et al.*, 2011). *WRKY 53* is a transcription factor involved in leaf senescence (Miao *et al.*, 2004) and a positive regulator of plant basal resistance (Hu *et al.*, 2012; Murray *et al.*, 2007). We found that, in *AtGLP5-OE* transgenic plants, the expression level of *ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1)* was elevated at about 2 times as compared with wild-type, while a strong downregulation of *EDS1* was given in *AtSUC7-OE* transgenic plants. *EDS1* and its interacting partner, *PHYTOALEXIN DEFICIENT 4 (PAD4)*, encode lipase-like proteins, are believed to control signal antagonism between SA and JA defenses (Xing and Chen, 2006). The *MAP kinase 4 (MPK4)* gene negatively regulates the expression of *EDS1* and SA-dependent defense responses but stimulates the JA signaling pathway conferring resistance towards the necrotrophic fungus *Alternaria brassicicola* (Brodersen *et al.*, 2006; Petersen *et al.*, 2000). In similar, we observed that *AtGLP5* and *AtSUC7*, seem to interact with *EDS1* and enhance the expression of *PDF1.2*, a marker gene of JA signaling pathway (Zhang *et al.*, 2020), strongly indicating a functional role of both *AtGLP5* and *AtSUC7* in activating plant resistance response to *S. sclerotiorum* via enhancing JA signaling pathway. Nevertheless, the

transcription level of *PRI*, a signaling pathway marker gene (Zhang *et al.*, 2020), was about 2 times higher in *AtSUC7-OE* transgenic plants than the wild-type plants. Thus, it is likely a reflection of distinct function models of both genes in regulating signaling pathways.

## 5.2 *AtGLP5* exhibited SOD activity and elevated $H_2O_2$ production in transgenic

### *AtGLP5*-overexpressing plants upon pathogen attack

Initially, “true germins” present only in true cereals such as wheat and barley were proved to possess oxalate oxidase (OxO) activity, which can oxidize oxalic acid (OA) into hydrogen peroxide ( $H_2O_2$ ), making them potential resistance candidate genes against OA-secreting pathogens such as *S. sclerotiorum* (Davidson *et al.*, 2009). Apart from the ability to clear the toxin OA secreted by the fungus, the production of  $H_2O_2$ , which is an important second messenger, also contribute to plant defense response by a direct toxic action towards pathogens, activating cell wall cross-linking of polysaccharides and proteins that strengthen the cell wall barrier, and triggering stomatal closure and appropriate cellular-level responses to restrict pathogen colonization (Marcec *et al.*, 2019). Although sharing a high sequence similarity, germin-like proteins (GLPs), which are quite ubiquitous in many land plant species, including monocots, dicots, and moss, have not been shown to display OxO activity. Instead, several studies demonstrated that some GLPs possess superoxide dismutase (SOD) enzymatic property, which can produce hydrogen peroxide by converting superoxide anions into  $H_2O_2$  (Barman and Banerjee, 2015). BnGLP3, a germin-like protein exhibiting SOD activity in *B. napus*, was upregulated at 6h after *S. sclerotiorum* infection in both susceptible ‘Falcon’ and tolerant ‘Zhongshuang 9’ *B. napus* varieties, suggesting that BnGLP3 might be responsible for plant basal defense against *S. sclerotiorum* invasion at early stages (Rietz *et al.*, 2012). In our current research, the SOD enzymatic activity of *AtGLP5* and the higher accumulation level of  $H_2O_2$  observed in *A. thaliana* leaves of *AtGLP5-OE* plants compared with Col-0 in response to *S. sclerotiorum* might also contribute to the antifungal property of *AtGLP5*. As shown in **Fig. 1**, upon pathogen infection, *AtGLP5*, which exhibits SOD enzymatic activity, rapidly converts superoxide anions into  $H_2O_2$ , apoplastic  $H_2O_2$  is then transported into the cytoplasm probably by aquaporin and act as a transducing signal in cytosolic signaling cascades to trigger downstream defense responses (Qi *et al.*, 2018).

### 5.3 *AtSUC7* plays a role in *S. sclerotiorum* resistance

The *A. thaliana* genome expresses a total of nine sucrose transporters, *AtSUC1-9*. *AtSUC1-5* shows low affinity, while *AtSUC8* and *AtSUC9* show a higher affinity to sucrose and maltose (Sauer, 2007). Despite transporter activity in phloem loading, the well-characterized *AtSUC2* can also be induced by several abiotic stresses such as salt, osmotic, low temperature, and exogenous phytohormone ABA treatment, and the promoter of *AtSUC2* was further established as a powerful tool to study phloem physiology and development (Stadler and Sauer, 2019). At first, *AtSUC6* and *AtSUC7* have been described as pseudogenes encoding aberrant proteins without functional sucrose transporter activity (Sauer *et al.*, 2004). Through heterologous expression in yeast, *AtSUC6* was then demonstrated to take up sucrose and maltose, although at a much lower pH value (Rottmann *et al.*, 2018). However, until now, *AtSUC7* does not show any transporter activity. Here, we presented that *AtSUC7* encoded a plasma membrane-localized protein involved in plant defense against the necrotrophic fungi *S. sclerotiorum* and plant flowering time control (**Fig. 1**). Upon *S. sclerotiorum* infection, *AtSUC7* transcription was immediately activated in 3h after inoculation. Overexpression of *AtSUC7* in transgenic plants constitutively activated the expression of *PDF1.2*, which is a JA/ET signaling pathway associated marker gene, thus enhanced plant disease resistance against *S. sclerotiorum*.

Moreover, we compared the transcriptional level of all the other *AtSUCs* between *AtSUC7-OE* transgenic and wild-type plants. We observed significantly upregulated expression of other sucrose symporter genes, such as *AtSUC6*, *AtSUC1*, *AtSUC2*, *AtSUC3*, and *AtSUC5*. The transcript level of other genes, *AtSUC4*, *AtSUC8*, and *AtSUC9*, remained unchanged. These results indicated somehow the interaction between *AtSUC7* and the other sucrose symporter members, especially *AtSUC6*.

### 5.4 *AtGLP5* and *AtSUC7* are involved in plant development and defense trade-offs

Apart from the disease resistance phenotypes, we were also able to observe a stunted root growth of *AtGLP5-OE* transgenic plants and early flowering in *AtSUC7-OE* transgenic and the IRS30 activation-tagging mutant.

Both *AtGLP5-OE* and *AtGLP5-GFP* transgenic plants displayed abnormal development with



disrupted root growth. Histochemical analysis of the *AtGLP5* promoter demonstrated that *AtGLP5* was mainly expressed in the root but not at the root apex. Plant roots are necessary for the general development and growth of the plants by providing anchorage in the soil and the assimilation of nutrients and water. Root growth is the product of iterative cell division, elongation, and differentiation (Sebastian and Lee, 2013). The absent expression of *AtGLP5* at the root apical meristem implies that *AtGLP5* is probably not involved in the root development.

An interesting observation is that both the *AtSUC7* activation-tagging mutant (IRS30) and *AtSUC7-OE* transgenic plants showed early flowering, differing from the report made by Sivitz et al. (Sivitz *et al.*, 2007) that knock out of the sucrose transporter gene *AtSUC9* in *A. thaliana*, which encodes an ultrahigh-affinity sucrose transporter resulted in early flowering under short-day but not long-day conditions. It is discussed that *AtSUC9* might prevent early flowering by retaining a low level of extracellular sucrose. Sugars play essential roles as nutrients and signal molecules (Morkunas and Ratajczak, 2014; Smeekens *et al.*, 2010). Until now, *AtSUC7* did not show any kind of sucrose substrate transporter activity (Rottmann *et al.*, 2018). Thus, the opposite effects of *AtSUC7* and *AtSUC9* in this regard might illustrate a functional antagonism between two sucrose transporter family members. It is likely that *AtSUC7* accelerates the plant flowering process by maintaining a higher level of extracellular sucrose at a cellular level. Overexpression of a SWEET gene family member *AtSWEET10* accelerates flowering through the activation of the photoperiodic pathway involving regulation of *FT* and *SOC1* (Andrés *et al.*, 2020). We examined the expression of several floral genes, including *FT*, *FLC*, *SOC1*, and *LFY*. We found that the flowering repressor gene *FLC* was significantly repressed, while the positive regulator gene *SOC1* was upregulated in the both IRS30 mutant and *AtSUC7-OE* transgenic plants. Thus, we conclude that *AtSUC7* is functionally involved in the plant flowering control by, amongst others, regulating *FLC* and *SOC1*.

Plants have developed a fine-tuned immune system to fight against stress factors, which often compromises growth and development. Therefore, a tradeoff between growth /development and defense response is often modulated by crosstalk between diverse signaling pathways (Luo *et al.*, 2019). Recent researches demonstrate that plant hormones like SA can stimulate plant immunity to various pathogens but suppress growth and development (van Butselaar and Van den

Ackerveken, 2020). The tradeoff also works the other way round, where active growth and developmental processes can suppress SA production and plant immunity (van Butselaar and Van den Ackerveken, 2020). Upon *Pst* DC3000 infection, *EDS1* quickly enhances SA biosynthesis and activates expression of defense-related genes, whereas pathogen infection can stabilize DELLA proteins to restrict plant growth. However, the stabilized DELLA proteins can interact with EDS1 to suppress the excessive production of SA, thus forming a feedback regulatory loop by which plants maintain the subtle balance between defense and growth in response to constant pathogen attack (Li *et al.*, 2019). Recently, researchers from different groups demonstrated that NAA50, an N-terminal acetyltransferase, can suppress Arabidopsis development and induce stress defense (Feng *et al.*, 2020; Hartman, 2020). NAA50 can directly interact with ENHANCED DISEASE RESISTANCE 1 (EDR1), a negative regulator of SA and ET pathways, and negatively regulates defense response (Neubauer and Innes, 2020). Loss of function of *naa50* represses plant growth and caused accumulation of proteins involved in stress signaling, and this phenotype could be rescued by the expression of active HsNAA50 from human and AtNAA50 from plants but not ScNAA50 from yeast (Armbruster *et al.*, 2020). Here, we presented that overexpression of *AtGLP5* and *AtSUC7* enhances disease resistance but caused either aberrant root growth or accelerated bolting. Constant activation of the JA signaling pathway and upregulation of WRKY transcription factors in the transgenic plants might explain these transgenic plants' abnormal growth and development.

### **5.5 *AtGLP5* is a novel player in FLS2-flg22 interaction**

A critical step that distinguishes between resistant and susceptible plants is the timely recognition of pathogen attack and the rapid activation of downstream defense signaling pathways. The slower and weaker response to pathogen invasion leads to the failure to restrict pathogen growth and spread, and even be killed by pathogen infection (Yang *et al.*, 1997). The activation of defense responses in plants is launched by the perception of elicitors (Bent and Mackey, 2007; Jones and Takemoto, 2004). Many elicitors from *S. sclerotiorum* have been reported in the past decade, as summarized in chapter 1. Still, less has been known on plants, such as plant resistance genes, resistance response, and molecular plant-fungus interactions.

A well-known plant pattern recognition receptor (PRR) is the Arabidopsis receptor-like kinase

FLS2, which recognizes a conserved 22 amino acid N-terminal sequence of flagellin (Gómez-Gómez *et al.*, 2001; Gómez-Gómez and Boller, 2000). Both the extracellular leucine-rich repeat domain and the kinase activity of FLS2 are required for specific flagellin binding and defense response activation (Gómez-Gómez *et al.*, 2001). The plant receptor kinase BAK1 (BRASSINOSTEROID INSENSITIVE 1-associated kinase 1) can bind directly to FLS2 and act as a co-receptor by recognizing the C terminus of the FLS2-bound flg22 to induce plant immunity (Sun *et al.*, 2013). Plants carrying *bak1* mutations show normal flagellin binding but are less sensitive to flg22 treatment, indicating that BAK1 is a positive regulator in this signaling (Chinchilla *et al.*, 2007). In our study, the expression of *AtGLP5* is induced in the wild-type Col-0 but abolished in *fls2* mutant and reduced in *bak1* mutant upon flg22 treatment. These results suggest that *AtGLP5* expression is regulated by the FLS2/flg22 PTI signaling pathway. Also, promoter::GUS assays reveal a slightly induced GUS activity by flg22 in the *fls2* mutant transformed with  $P_{AtGLP5}$ ::GUS construct compared with transgenic  $P_{AtGLP5}$ ::GUS plants in the Col-0 background. Thus the deficiency of *fls2* mutant to bind flg22 seems to some extent rescued by the overexpression of *AtGLP5*. This partially functional complementation of FLS2 by *AtGLP5* in *fls2* mutant may highlight a more valuable role and function of *AtGLP5* in participating in plant immune system in general. However, further experiments are needed to elucidate underlying mechanisms.

## 5.6 Perspectives

Taken together, an enhanced disease resistance against *S. sclerotiorum* accompanied by growth and development trade-offs was demonstrated by enhancing the expression of *AtGLP5* and *AtSUC7* in *A. thaliana*. Based on our present results, we propose a model to illustrate the role of *AtGLP5* and *AtSUC7* in plant early immune responses when challenged with *S. sclerotiorum* (**Fig. 1**). Attacks on *A. thaliana* plants by *S. sclerotiorum* can induce the expression of both *AtGLP5* and *AtSUC7*. Overexpression of *AtGLP5* stimulated H<sub>2</sub>O<sub>2</sub> production, which can further inhibit pathogen growth, stimulate cell wall cross-linking, and mediate signaling for transcriptional changes of PR genes. Moreover, there was a positive association between *AtGLP5* and FLS2, an flg22 receptor, since we demonstrated that *AtGLP5* could partially rescue the function of FLS2 in the *fls2* mutant. *AtGLP5* overexpression constitutively upregulated the expression of JA pathway

marker gene *PDF1.2* and ABA pathway marker gene *NCED3*, as well as many of the *WRKY* transcription factors such as *WRKY18*, *WRKY22*, *WRKY33*, *WRKY40*, and *WRKY53*, which is related to plant senescence. Overexpression of *AtSUC7* conferred disease resistance mainly by activating JA signaling pathway marker gene *PDF1.2*. *AtSUC7* also upregulated the expression of *WRKY53*. Moreover, *AtGLP5* and *AtSUC7* are involved in the trade-offs between plant defense and development, since overexpression of *AtGLP5* represses root growth, and overexpression of *AtSUC7* accelerates plant flowering development. A reduced *FLC* expression might explain the molecular mechanism of the early flowering phenotype of *AtSUC7*-overexpressing plants.

This finding will increase our knowledge about the plant-fungus interaction and offer novel strategies facilitating breeding resistance against *S. sclerotiorum*, e.g., rapeseed. Nevertheless, Both *AtGLP5* and *AtSUC7* belong to large gene families. Thus, it is imperative to distinguish each member's role and mode of function in plant-pathogen and plant growth and development and their expression patterns in response to various stress factors. For this, further molecular characterization and genetic modifications with the help of transgenic approaches (overexpression and knock-out) as well as plant genome editing by CRISPR-cas9 may shed more light on underlying mechanisms and potential for the plant breeding practice (Ahmad *et al.*, 2020; Pröbsting *et al.*, 2020; Shehryar *et al.*, 2020), which may significantly contribute to integrated disease management that combines genetic engineering and effective fungicide applications to protect crops from the threat made by *S. sclerotiorum* infection and sustainable agriculture.

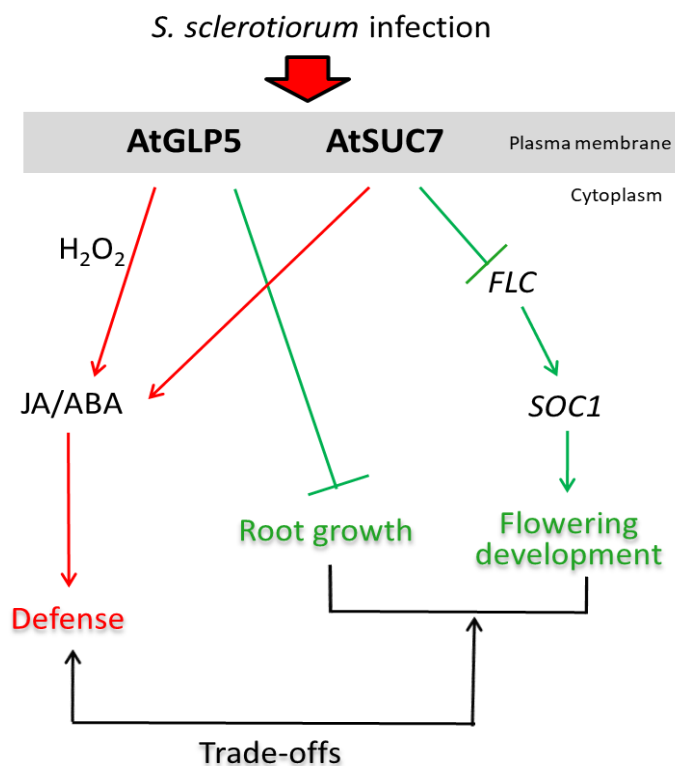


Fig. 1 A proposed working model for AtGLP5 and AtSUC7 in regulating plant defense and development. Upon *S. sclerotiorum* infection, both of AtGLP5 and AtSUC7 were upregulated at early stages, suggesting a potential role in regulating plant early defense response. AtGLP5 encodes a SOD enzyme, which can catalyze the production of H<sub>2</sub>O<sub>2</sub>, an important signaling molecule triggering downstream defense signals. Overexpression of AtGLP5 can activate *PDF1.2* and *NCED3*, involved in JA- and ABA-signaling pathways. While overexpression of AtSUC7 can positively regulate JA-signaling pathway marker gene *PDF1.2*, leading to defense response. Moreover, AtGLP5 and AtSUC7 are involved in the trade-offs between plant defense and development, since overexpression of AtGLP5 represses root growth, and overexpression of AtSUC7 accelerates plant flowering development.

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## Summary

Due to their sessile nature, plants cannot escape from adverse environmental conditions and are frequently confronted with diverse abiotic and biotic stress factors. The production of oilseed rape (*Brassica napus*), a major vegetable oil source, is continuously threatened by various diseases. Sclerotinia stem rot, which is caused by *Sclerotinia sclerotiorum*, is one of the major diseases of *B. napus*. It can lead to yield losses of 10-20 % and up to 80 % in severely infected fields. *S. sclerotiorum* represents one of the most devastating fungal pathogens and can colonize more than 400 plant species, including many economically important crops in almost every continent of the world. So far, no efficient and effective resistance against *S. sclerotiorum* is available. It remains a significant challenge to protect crops from this fungal disease. The establishment of *Arabidopsis thaliana* as a model plant to study the plant-*S. sclerotiorum* interaction has accelerated our molecular understanding of plant disease and defense response, as well as the identification of candidate genes. A deeper understanding of the mechanism underlying the plant-*S. sclerotiorum* interaction may help developing novel strategies and approaches to control this disease. In this thesis, in addition to the general introduction (Chapter 1) and general discussion (Chapter 5), three research chapters are presented, including the main results regarding the molecular identification and characterization of candidate genes involved in plant resistance response to *S. sclerotiorum* infection in *A. thaliana*.

**Chapter 2** is titled “AtGLP5, a germin-like protein of *Arabidopsis thaliana*, is a superoxide dismutase and plays an important role in plant development and innate immune response”. We report that AtGLP5, an ortholog of BnGLP3 of *B. napus*, is transcriptionally upregulated in both susceptible and tolerant *B. napus* varieties upon *S. sclerotiorum* infection. BnGLP3 exhibits a superoxide dismutase (SOD) activity that can activate plant defense by producing H<sub>2</sub>O<sub>2</sub>. Transcript profiling and promoter::GUS analysis revealed that *AtGLP5* was mainly expressed in the roots. Additionally, GUS activity was also detected in the shoot apical stem, hydathode of the cotyledons, and floral organs such as nectary and gynoecium. The expression of *AtGLP5* was activated by diverse biotic and abiotic stress factors, such as plant pathogens *S. sclerotiorum* and *Pst* DC3000, salicylic acid, wounding, and flg22 leaf spray. Moreover, we demonstrate for the

first time that AtGLP5 possesses SOD activity and is localized at the plasma membrane. AtGLP5 also represents a novel player in the FLS-mediated plant immunity. Overexpression of AtGLP5 disturbed plant root growth. Furthermore, the overexpression of *AtGLP5* in *A. thaliana* enhanced the expression of several defense-related genes. It increased plant disease resistance in association with a higher level of H<sub>2</sub>O<sub>2</sub> in plants, while its knockout mutants showed enhanced susceptibility. These data strongly suggest a crucial role of *AtGLP5* in plant defense response.

**Chapter 3** describes the finding that “Enhancing expression of *AtSUC7* encoding a sucrose symporter confers plant defense against *Sclerotinia sclerotiorum* and promotes early flowering in *Arabidopsis thaliana*”. The IRS30 mutant was obtained by screening a population of *A. thaliana* activation-tagging mutants. The IRS30 mutant plants showed increased disease resistance accompanied by an early flowering phenotype. The IRS30 mutant contains a T-DNA insertion at the last intron of *AtSAG24*, resulting in an upregulated expression of a sucrose symporter gene, *AtSUC7*. The functional characterization of *AtSUC7* revealed that it encodes a plant cell membrane-localized protein and is activated in response to pathogen infection. With both the IRS30 mutant and transgenic *AtSUC7*-overexpressing plants, we demonstrated a dual role of *AtSUC7* in enhancing plant disease resistance and accelerating flowering time. We showed that an enhanced expression of *AtSUC7* led to the upregulation of plant defense-related genes like *PDF1.2*, and *AtWRKY53*, a vital transcription factor involved in plant defense and senescence control. In contrast, a SA signaling pathway-associated gene *EDS1* was drastically repressed. We proved that an enhanced *AtSUC7* expression affects flowering time through fine-tuning of the autonomous pathway leading to the repression of the negative regulator *FLC* but the activation of the positive regulator *SOCl*. Our results highlight the crucial role of plant sucrose symporters in balancing plant defense and growth tradeoffs.

**Chapter 4** reports that “Promoter analysis reveals a comprehensive regulatory mechanism of *AtGLP5* in plant defense response”. Our previous study demonstrated that *AtGLP5* was predominantly expressed in the root but activated in leaves by diverse stress factors, including pathogen attacks. The regulation mechanism underlying remains unsolved. To address the transcriptional regulation of *AtGLP5*, we performed the *AtGLP5* promoter analysis in transgenic *Arabidopsis* expressing *P<sub>AtGLP5</sub>::GUS* construct by challenging the plants with *Verticillium longisporum* and by flg22 leaf-spraying. Much to our surprise, we found that GUS expression was

depressed in roots, along with the infection progression of *V. longisporum*. However, the GUS activity in leaves was first detected at the late stage of *V. longisporum* infection and by direct spraying of flg22 on leaves as well, thus supporting its pathogen-responsive expression. Besides, the flg22-induced GUS expression was significantly mitigated in Arabidopsis leaves in the *fls2* background, thus evidencing for direct involvement of *AtGLP5* in the FLS2/flg22-mediated signaling pathway and plant immunity. Further on, the promoter deletion analysis unveiled several *cis*-element candidates within a 240bp region of the *AtGLP5* promoter, which is probably responsible for the complex regulation of the *AtGLP5* expression.

Taken together, this work demonstrates a vital role of *AtGLP5* and *AtSUC7* in plant defense response to pathogen attacks. It highlights a valuable strategy for employing candidate genes from *A. thaliana* to improve plant disease resistance. Molecular and functional characterization on *AtGLP5* and *AtSUC7* also provides an in-depth insight into the complex plant-pathogen interaction, emphasizing their roles in plant defense response and balancing the tradeoff between growth /development and defense response of plants.

## Zusammenfassung

Aufgrund ihrer sessilen Lebensweise können Pflanzen ungünstigen Umweltbedingungen nicht entkommen und sind häufig mit diversen abiotischen und biotischen Stressfaktoren konfrontiert. Die Produktion von Ölrapen (*Brassica napus*), einer wichtigen Pflanzenölquelle, ist ständig von verschiedenen Krankheiten bedroht. Sklerotinia Stängelfäule, die durch den Pilz *Sclerotinia sclerotiorum* verursacht wird, ist eine der Hauptkrankheiten von *B. napus*. Sie kann zu Ertragsverlusten von 10-20 % und auf stark infizierten Feldern bis zu 80 % führen. *S. sclerotiorum* ist einer der verheerendsten Pilzpathogene und kann mehr als 400 Pflanzenarten besiedeln, darunter viele wirtschaftlich wichtige Nutzpflanzen auf fast allen Kontinenten der Welt. Bislang ist keine effiziente und effektive Resistenz gegen *S. sclerotiorum* verfügbar. Es bleibt daher eine große Herausforderung, Nutzpflanzen vor dieser Pilzkrankheit zu schützen. Die Etablierung von *Arabidopsis thaliana* als Modellpflanze zur Untersuchung der Wechselwirkung Pflanze-*S. sclerotiorum* hat unser molekulares Verständnis von Pflanzenkrankheiten und Abwehrreaktionen sowie die Identifizierung von Kandidatengenen beschleunigt. Ein tieferes Verständnis des Mechanismus, der der Interaktion zwischen Pflanze und *S. sclerotiorum* zugrunde liegt, kann dazu beitragen, neue Strategien und Ansätze zur Bekämpfung dieser Krankheit zu entwickeln. In der vorliegenden Dissertation werden neben der allgemeinen Einführung (Kapitel 1) und der Schlussdiskussion (Kapitel 5) drei Forschungskapitel vorgestellt. Diese behandeln die wichtigsten Ergebnisse der molekularen Identifizierung und Charakterisierung von Kandidatengenen, die an der pflanzlichen Resistenzantwort auf eine Infektion mit *S. sclerotiorum* bei *A. thaliana* beteiligt sind.

**Kapitel 2** trägt den Titel "AtGLP5, a germin-like protein of *Arabidopsis thaliana*, is a superoxide dismutase and plays an important role in plant development and innate immune response". Wir konnten zeigen, dass AtGLP5, ein Ortholog von BnGLP3 in *B. napus*, nach einer Infektion mit *S. sclerotiorum* sowohl bei anfälligen als auch bei toleranten *B. napus*-Sorten transkriptionell hochreguliert wird. BnGLP3 weist eine Superoxiddismutase-Aktivität (SOD) auf, die die Pflanzenabwehr durch die Produktion von H<sub>2</sub>O<sub>2</sub> aktivieren kann. Transkript-Profiling und eine Promotor::GUS-Analyse ergab, dass *AtGLP5* hauptsächlich in den Wurzeln exprimiert wird.

Darüber hinaus wurde GUS-Aktivität auch am apikalen Sprossstiel, an der Hydathode der Keimblätter und an floralen Organen wie Nektarium und Gynoeceum nachgewiesen. Die Expression von *AtGLP5* wurde durch verschiedene biotische und abiotische Stressfaktoren aktiviert, wie z.B. durch die Pflanzenpathogene *S. sclerotiorum* und *Pst* DC3000, Salicylsäure, mechanische Verwundung und das Besprühen mit flg22. Darüber hinaus zeigen wir zum ersten Mal, dass *AtGLP5* SOD-Aktivität besitzt und an der Plasmamembran lokalisiert ist. *AtGLP5* stellt auch einen neuartigen Akteur in der FLS-vermittelten Pflanzenimmunität dar. Pflanzenimmunität dar. Die Überexpression von *AtGLP5* störte das Wurzelwachstum der Pflanzen. Darüber hinaus erhöhte die Überexpression von *AtGLP5* in *A. thaliana* die Expression mehrerer abwehrrelevanter Gene. Sie erhöhte die Resistenz gegen Pflanzenkrankheiten in Verbindung mit einem höheren H<sub>2</sub>O<sub>2</sub>-Gehalt in Pflanzen, während ihre Knockout-Mutanten eine erhöhte Anfälligkeit zeigten. Diese Daten deuten stark auf eine entscheidende Rolle von *AtGLP5* bei der Abwehrreaktion in Pflanzen hin.

**Kapitel 3** beschreibt die Feststellung, dass “Enhancing expression of *AtSUC7* encoding a sucrose symporter confers plant defense against *Sclerotinia sclerotiorum* and promotes early flowering in *Arabidopsis thaliana*”. Die IRS30-Mutante wurde durch Screening einer Population von *A. thaliana*-Aktivierungsmutanten gewonnen. Die Pflanzen mit IRS30-Mutation zeigten eine erhöhte Krankheitsresistenz, begleitet von einem frühblühenden Phänotyp. Die IRS30-Mutante enthält eine T-DNA-Insertion im letzten Intron von *AtSAG24*, was zu einer hochregulierten Expression eines Saccharose-Symportergens, *AtSUC7*, führt. Die funktionelle Charakterisierung von *AtSUC7* ergab, dass es für ein in der Pflanzenzelle membranlokalisiertes Protein kodiert und als Reaktion auf eine Pathogeninfektion aktiviert wird. Sowohl mit der IRS30-Mutante als auch mit den transgenen *AtSUC7*-überexprimierenden Pflanzen konnten wir eine Doppelrolle von *AtSUC7* bei der Verbesserung der Resistenz gegen Pflanzenkrankheiten und der Beschleunigung der Blütezeit nachweisen. Wir zeigten, dass eine erhöhte Expression von *AtSUC7* zur Hochregulierung von Genen der Pflanzenabwehr wie *PDF1.2* und *AtWRKY53* führte, einem lebenswichtigen Transkriptionsfaktor, der an der Pflanzenabwehr und der Seneszenzkontrolle beteiligt ist. Im Gegensatz dazu wurde ein SA-Signalweg-assoziiertes Gen *EDS1* stark unterdrückt. Wir konnten nachweisen, dass eine verstärkte *AtSUC7*-Expression die Blütezeit durch eine Feinabstimmung des autonomen Pfades beeinflusst, was zur Unterdrückung des negativen Regulators *FLC*, aber

zur Aktivierung des positiven Regulators *SOCI* führt. Unsere Ergebnisse unterstreichen die entscheidende Rolle der Pflanzen-Saccharose-Sympathisanten beim Ausgleich zwischen Pflanzenschutz und Wachstumskompromissen.

In **Kapitel 4** wird berichtet, dass “Promoter analysis reveals a comprehensive regulatory mechanism of *AtGLP5* in plant defense response”. Unsere frühere Studie zeigte, dass *AtGLP5* vorwiegend in der Wurzel exprimiert wird, aber in den Blättern durch verschiedene Stressfaktoren, darunter auch durch Pathogenangriffe, aktiviert wurde. Der zugrundeliegende Regulierungsmechanismus bleibt ungelöst. Um die Transkriptionsregulation von *AtGLP5* zu untersuchen, führten wir eine Analyse des *AtGLP5*-Promotors in transgenen Arabidopsis-Pflanzen durch, die ein  $P_{AtGLP5}::GUS$ -Konstrukt exprimieren. Diese Pflanzen wurden mit *Verticillium longisporum* infiziert, sowie besprüht mit flg22. Zu unserer großen Überraschung stellten wir fest, dass die Expression des GUS in den Wurzeln zusammen mit dem Infektionsverlauf von *V. longisporum* depressiv war. Die GUS-Aktivität in den Blättern wurde jedoch erst im Spätstadium der *V. longisporum*-Infektion und durch direktes Besprühen mit flg22 auch auf den Blättern nachgewiesen, wodurch die auf den Erreger ansprechende Expression unterstützt wurde. Außerdem war die flg22-induzierte GUS-Expression in Arabidopsis-Blättern mit *fls2*-Hintergrund signifikant abgeschwächt, was auf eine direkte Beteiligung von *AtGLP5* am FLS2/flg22-vermittelten Signalweg und an der pflanzlichen Immunität hindeutet. Im weiteren Verlauf enthüllte die Analyse mehrere *cis*-Element-Kandidaten innerhalb einer 240bp-Region des *AtGLP5*-Promotors, die wahrscheinlich für die komplexe Regulierung verantwortlich sind.

Zusammengenommen demonstriert diese Arbeit eine entscheidende Rolle von *AtGLP5* und *AtSUC7* bei der Pflanzenabwehr als Reaktion auf Pathogenangriffe. Sie gibt weiterhin wertvolle Anreize für die Untersuchung von Kandidatengen von *A. thaliana* zur Verbesserung der Resistenz gegen Pflanzenkrankheiten. Die molekulare und funktionelle Charakterisierung von *AtGLP5* und *AtSUC7* bietet auch einen vertieften Einblick in die komplexe Pflanze-Pathogen-Interaktion, wobei ihre Rolle bei der Abwehrreaktion von Pflanzen hervorgehoben wird und der Kompromiss zwischen Wachstum/Entwicklung und Abwehrreaktion von Pflanzen ausgeglichen wird.



## Materials and Methods

This chapter includes several tables listing all the materials, such as machines, chemicals, organisms, software, databases, and some comprehensive protocols for the chief procedures used in this study. A brief description of all the methods used in each chapter can be found in the “Materials and methods” section of the corresponding chapter.

### Materials

Table 1 Machines and devices used in this study.

Machine	Model	Company
Aerospace Digimatic Vernier Caliper	0-150 mm	Aerospace
Autoclave	VX-75	Systec
Blotting cell	Trans-Blot <sup>®</sup> SD Semi-Dry Electrophoretic	Bio-rad
Camera	D3000	Nikon
Centrifuge	mini G	IKA <sup>®</sup>
Centrifuge	5417 R	Eppendorf
Centrifuge	Heraeus Multifuge X3R	Thermo Fisher Scientific
Climate chamber	VB0714	V ösch Industrietechnik
CoolNat chip ice machine	ZBE 30-10	ZIEGRA
Drying oven	Venti-line VL115	VWR
Gel documentation	Gel Doc <sup>™</sup> XR+	Bio-Rad
Gel electrophoresis chamber	Mini-Sub <sup>®</sup> Cell GT	Bio-Rad
Gel electrophoresis power supply	PowerPac <sup>™</sup> Basic	Bio-Rad
Homogenizer	D-8	MICCRA
Homogenizer	Precellys <sup>®</sup> Evolution	Bertin Technologies
Incubator	Excellent UFE 400-800	Memmert
Incubator, shaking	CERTOMAT <sup>®</sup> IS	Sartorius Stedim Biotech
Light Thermostats germination chamber	1301	Rubarth Apparate
Magnetic stirrer	Combimag REO	IKA <sup>®</sup>
Microscope	Stereo Discovery.V20	Carl Zeiss
Microwave	hNN-E235M	Panasonic
PCR thermocycler	C1000 Touch <sup>™</sup>	Bio-Rad
PCR thermocycler	Biometra TOne 96	Analytik Jena
pH meter	inoLab pH 720	WTW
Pipettes	2.5 µl, 20 µl, 200 µl, 1000 µl, 5ml	Eppendorf
Realtime PCR System	CFX96 Touch Real-Time PCR Detection System	Bio-Rad
Scale	ABJ80-4M	KERN & SOHN
Scale	KB1200-2	KERN & SOHN
Shaker	Vibramax 100	Heidolph Instruments

Table 1 continued

<b>Machine</b>	<b>Model</b>	<b>Company</b>
Spectrophotometer	NanoVue Plus	GE Healthcare Life Science
Thermomixer	Biometra TSC ThermoShaker	Analytik Jena
Upright Microscope	Eclipse Ni	Nikon
UV-VIS Spectrophotometer	UVmini-1240	Shimadzu
Vortex	MS 2	IKA®
Water bath	Immersion Circulators Model 1112A	VWR
Water bath, shaking	1083	GFL Gesellschaft für Labortechnik mbH
Workbench, sterile	HERAsafe™ KS 12	Thermo Fisher Scientific

Table 2 Chemicals used in this study.

<b>Chemical</b>	<b>Article number</b>	<b>Company</b>
30% Acrylamide	1610158	Bio-Rad
Acetic acid	3738.2	Roth
Acetosyringone	38766	Fluka
Ammonium acetate	7869.2	Roth
Ampicillin	K0291.1	Roth
APS (Ammonium peroxodisulphate)	9592.2	Roth
Bacto-Agar	214010	OTTO NORDWALD
Biozym LE-Agarose	840004	Biozym
Bromophenol blue	A512.1	Roth
CaCl <sub>2</sub> (Calcium chloride)	CN93.2	Roth
CaCl <sub>2</sub> O <sub>2</sub> (Calcium hypochlorite)	5164.1	Roth
Chloroform	3313.2	Roth
CTAB (cetyltrimethylammonium bromide)	9161.2	Roth
Czapek Dox Broth	C1714.1000	Duchefa Biochemie
D(+)-Saccharose	4621.2	Roth
D(+)-Sucrose	4321.2	Roth
DAB (3 3'-diaminobenzidine tetrahydrochloride)	CN75.3	Roth
DEPC (diethyl pyrocarbonate)	K028.2	Roth
DL malic acid	1003820250	Merk
DMSO (dimethyl sulfoxide)	4720.2	Roth
dNTP Mix	R0181	Thermo Fisher Scientific
EDTA (ethylenediaminetetraacetic acid)	8043.2	Roth
Ethanol	9065.5	Roth
Ethidium bromide	2218.2	Roth
GeneRuler DNA Ladder 1kb	SM0311	Thermo Fisher Scientific
Gentamycin	G0124.0025	Duchefa Biochemie
Gibberellin	G0907-0001	Duchefa Biochemie
Glycerol	4043.1	Roth
H <sub>3</sub> BO <sub>3</sub> (Boric acid)	6943.1	Roth

Table 2 continued

<b>Chemical</b>	<b>Article number</b>	<b>Company</b>
HPLC water	A511.3	Roth
IAA	I0901.0005	Duchefa Biochemie
IBA	I0902.0005	Duchefa Biochemie
Isoamyl alcohol	8930.1	Roth
Isopropanol	20842.330DB	VWR
Jasmonic acid	J0936.0250	Duchefa Biochemie
Kanamycin	T832.3	Roth
KH <sub>2</sub> PO <sub>4</sub> (Potassium dihydrogen phosphate)	3904.1	Roth
KNO <sub>3</sub> (Potassium nitrate)	8001.3	Roth
KOH (potassium hydroxide)	6751.3	Roth
MES	4256.2	Roth
Methanol	4627.5	Roth
MgCl <sub>2</sub> (magnesium chloride)	kk36.2	Roth
MgSO <sub>4</sub> (magnesium sulfate)	P027.3	Roth
MOPS (3-(N- morpholino)-propane-sulfonic acid)	6979.3	Roth
MS basal salts including Vitamines and MES	M0255.0050	Duchefa Biochemie
Na <sub>2</sub> HPO <sub>4</sub> (disodium phosphate)	X987.2	Roth
NaCl (sodium chloride)	3957.2	Roth
NaH <sub>2</sub> PO <sub>4</sub> (monosodium phosphate)	K300.2	Roth
NaOH (sodium hydroxide)	6771.2	Roth
NH <sub>4</sub> NO <sub>3</sub> (Ammonium nitrate)	A9642-500g	Sigma-Aldrich
PDB (Potato-dextrose broth)	CP74.2	Roth
Phenol	0038.3	Roth
Phyto Agar	P1003	Duchefa Biochemie
Potassium acetate	T874.1	Roth
Rifampicin	R0146.0005	Duchefa Biochemie
Salicylic acid	S1367.0506	Duchefa Biochemie
SDS (sodium dodecyl sulfate)	4360.2	Roth
Silwet Gold	AM6A12W004	Spiess Urania
Spectinomycin	S4014-5g	Sigma-Aldrich
TEMED (tetramethylethylenediamine)	2367.3	Roth
TRIS (tris(hydroxymethyl)aminomethane)	5429.3	Roth
TRIS-HCl (Tris-hydrochloride)	9090.3	Roth
Triton X 100	3051.3	Roth
TRIzol <sup>®</sup>	15596018	Life Technologies
Tryptone/Peptone	8952.3	Roth
Tween 20	9127.1	Roth
Water	3175.2	Roth
X-glcA	X1405.1000	Duchefa Biochemie
Yeast Extract	2904.3	Roth
β-mercaptoethanol	4227.1	Roth

## Materials and Methods

Table 3 Enzymes and kits used in this study.

Enzyme / Kit	Article number	Company
DNase I	EN0521	Thermo Fisher Scientific
Dream Taq DNA Polymerase	EP0704	Thermo Fisher Scientific
Gateway™ BP Clonase™ II Enzyme mix	11789020	Invitrogen
Gateway™ LR Clonase™ II Enzyme mix	11791020	Invitrogen
Lumi-LightPLUS Western Blotting Kit	12015218001	Roche
Maxima SYBR Green/ROX qPCR Master Mix (2X)	K0222	Thermo Fisher Scientific
NucleoSpin Gel and PCR Clean-Up	740609250	Macherey-Nagel
NucleoSpin Plasmid Kit	740588.250	Macherey-Nagel
Phusion™ High-Fidelity DNA Polymerase	F530S	Thermo Fisher Scientific
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Fisher Scientific

Table 4 Softwares and online databases or tools used in this study.

Software/ Database	Company/ Source
Arabidopsis eFP Browser	<a href="http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi">http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi</a>
AxioVision rel. 4.8	Carl Zeiss
BLAST	<a href="https://blast.ncbi.nlm.nih.gov/Blast.cgi">https://blast.ncbi.nlm.nih.gov/Blast.cgi</a>
CFX Maestro™ Software for CFX Real-Time PCR Instruments	Bio-Rad
cisHighlight	<a href="https://msbi.ipb-halle.de/cisHighlight/">https://msbi.ipb-halle.de/cisHighlight/</a>
DNAMAN	Lynnon Biosoft
Image Lab™	Bio-Rad
MegaX	<a href="https://www.megasoftware.net/">https://www.megasoftware.net/</a>
Microsoft® Office	Microsoft Corporation
NCBI	<a href="https://www.ncbi.nlm.nih.gov/">https://www.ncbi.nlm.nih.gov/</a>
PlantCARE	<a href="http://bioinformatics.psb.ugent.be/webtools/plantcare/html/">http://bioinformatics.psb.ugent.be/webtools/plantcare/html/</a>
Primer Premier 5	PREMIER Biosoft
Prism 8	GraphPad Software
SIGNAL T-DNA Primer Design	<a href="http://signal.salk.edu/tdnaprimers.2.html">http://signal.salk.edu/tdnaprimers.2.html</a>
The Arabidopsis Information Resource	<a href="https://www.arabidopsis.org/">https://www.arabidopsis.org/</a>

Table 5 Organisms used in this study.

Organisms	Isolate/ Genotype	Source
<i>Agrobacterium tumefaciens</i>	GV3101	lab stock
<i>Escherichia coli</i>	DH5a	lab stock
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	DC3000	provided by Prof. Dr. M. Sauter (CAU Kiel, Germany)
<i>Sclerotinia sclerotiorum</i>	1980	provided by Prof. Dr. W. Qian (SWU Chongqing, China)
<i>Verticillium longisporum</i>	VL43	provided by Dr. E. Diederichsen (FU Berlin, Germany)
<i>Arabidopsis thaliana</i>	Col-0	lab stock
	Col-2	lab stock
	<i>Atglp5</i> knock-out mutants (N738879; N863003)	NASC
	<i>AtGLP5-OE</i> (in Col-0)	self-provided
	<i>AtGLP5-GFP</i> (in Col-0)	provided by Dr. Steffen Rietz
	<i>P<sub>AtGLP5</sub>::GUS</i> -full sequence (in Col-0)	provided by Dr. Steffen Rietz
	<i>P<sub>AtGLP5</sub>::GUS-P1</i> (in Col-0)	self-provided
	<i>P<sub>AtGLP5</sub>::GUS-P2</i> (in Col-0)	self-provided
	<i>P<sub>AtGLP5</sub>::GUS-P3</i> (in Col-0)	self-provided
	Activation-tagging mutants (in Col-2)	NASC
	<i>AtSUC7-GFP</i> (in Col-0)	self-provided
	<i>AtSUC7-OE</i> (in Col-0)	self-provided
	<i>fls2</i> knock-out mutant (SALK_062054)	NASC
	<i>Nicotiana benthamiana</i>	

Table 6 Buffers and media used in this study.

Buffer/ Medium	Composition
2x CTAB	200 mM Tris/HCl (pH 7.5)
	20 mM EDTA (pH 8.0)
	1.4 M NaCl
	2% CTAB (w/v)
TE Buffer	10 mM Tris/HCl (pH 8.0)
	1 mM EDTA (pH 8.0)
GUS staining buffer	30 mg X-glcA in 300 µl DMSO for 100 ml
	50 mM Na <sub>2</sub> HPO <sub>4</sub> (pH 7)
	50 mM NaH <sub>2</sub> PO <sub>4</sub> (pH 7)
	10% Triton X 100 (500 µl/100 ml)

## Materials and Methods

Table 6 continued

<b>Buffer/ Medium</b>	<b>Composition</b>
10x Loading buffer	5 ml TA buffer 5 ml Glycerol 10 mg Bromophenol blue
Protein extraction buffer	50 mM Tris/HCl pH 7.5 2% SDS 50 mM DTT
Stacking gel (5%, 5 ml; for SDS-PAGE)	1.9 ml H <sub>2</sub> O 1.7 ml 30% Acrylamide 1.3 ml 1.5 M Tris, pH 8.8 0.05 ml 10% SDS 0.05 ml 10% APS 0.002 ml TEMED
Separating gel (10%, 1 ml; for SDS-PAGE)	0.68 ml H <sub>2</sub> O 0.17 ml 30% Acrylamide 0.13 ml 1 M Tris, pH 8.8 0.01 ml 10% SDS 0.01 ml 10% APS 0.001 ml TEMED
5x Gel loading buffer (without DTT; for SDS-PAGE)	0.25M Tris/HCl, pH 6.8 10% SDS 30% Glycerine 0.02 % Bromophenole blue
10x Running buffer (for SDS-PAGE)	15.15 g/l Tris 72 g/l Glycine 5 g/l SDS
10x Transfer buffer (400ml)	29.9 g Tris 14.65 g Glycine 25 ml 10% SDS
10x TBST	12.11 g/l Tris 87.6 g/l NaCl, pH to 7.5 1% Tween 20
Reaction solution (for detection of SOD activity)	100 mM Potassium phosphate buffer, pH 7.8 5 mM Riboflavin 100 µl/30 ml TEMED
Infiltration medium (for transient expression in tobacco)	10 mM MES 10 mM MgCl <sub>2</sub> 0.15 mM Acetosyringone
YEB medium	5 g/l Beef extract 1 g/l Yeast extract 5 g/l Sucrose 0.49 g/l MgSO <sub>4</sub> x 7 H <sub>2</sub> O

Table 6 continued

<b>Buffer/ Medium</b>	<b>Composition</b>
NYGA medium (for <i>Pst</i> DC3000)	5 g/l Tryptone/Peptone 3 g/l Yeast extract 20 ml/l Glycerol 15 g/l Bacto Agar
½ MS medium (for plants)	0.5x MS salts incl. Vitamines and MES 5 g/l Sucrose pH 5.8 (adjust with KOH) 8 g/l Phyto agar
PDA medium (for <i>S. sclerotiorum</i> )	1x PDB (pH 5.6) 15 g/l Bacto agar
Czapek Dox medium (for <i>V. longisporum</i> )	1x CDB (pH 6.8)
Malic acid (MA) medium (for <i>S. sclerotiorum</i> )	1 g/l NaOH 2 g/l DL malic acid 1 g/l KH <sub>2</sub> PO <sub>4</sub> 2 g/l NH <sub>4</sub> NO <sub>3</sub> 0.1 g/l MgSO <sub>4</sub> x 7 H <sub>2</sub> O 0.5 g/l Yeast Extract pH 5.5 1.5 % Bacto agar
LB medium (for bacteria)	10 g/l Tryptone/Peptone 5 g/l Yeast extract 10 g/l NaCl (pH 7) 15 g/l Bacto agar (for solid medium)

## Methods

### Isolation of plant genomic DNA with CTAB

Total gDNA was isolated following the cetyltrimethylammonium bromide (CTAB) DNA Miniprep protocol as described with slight modifications (Clarke, 2009).

1. Collect plant tissue and directly grind the tissue to a fine powder in 1.5 ml tubes either with a plastic pestle using liquid nitrogen or with a homogenizer (Precellys® Evolution).
2. Add 1 ml of prewarmed 2x CTAB buffer to each tissue sample and mix thoroughly with a vortex. Then transfer the tubes into a water bath at 65 °C for 30 min with intermittent shaking.
3. Add 200 µl of chloroform and mix thoroughly with a vortex. Incubate at RT for 5 min.
4. Centrifuge at 12,000 rpm for 10 min at RT.
5. Carefully transfer the aqueous layer into a clean 1.5 ml tube and repeat the extraction (Steps 3-5) using an equal amount of chloroform.
6. Add an equal amount of cold isopropanol, mix thoroughly with a vortex. Incubate the samples at -20 °C for 10 min.
7. Centrifuge at 12,000 rpm for 10 min at RT.

8. Discard the supernatant and wash the pellet with 1 ml of cold 70% ethanol. Centrifuge briefly to secure the pellet. Repeat the wash for one more time.
9. Remove all the ethanol and dry the pellet in the air for several minutes.
10. Dissolve the DNA in 20-50  $\mu$ l of HPLC water.

### **Isolation of plant total RNA with TRIzol**

Total RNA was isolated following the user guide for TRIzol<sup>®</sup> Reagent except that all the steps were performed on ice and centrifuged at 4  $^{\circ}$ C (Invitrogen).

1. Add 1 ml of TRIzol to the grounded tissue samples in 1.5 ml tubes and mix thoroughly with a vortex. Incubate on ice for 5 min to permit complete dissociation of the nucleoproteins complex.
2. Add 0.2 ml of chloroform to each tube, securely cap the tube, and then mix thoroughly with a vortex. Incubate on ice for 5 min.
3. Centrifuge at 12,000 rpm for 10 min at 4  $^{\circ}$ C.
4. Carefully transfer the aqueous phase containing the RNA to a new tube. Notice that it is very important to avoid transferring any of the interphase or organic layer into the pipette when removing the aqueous phase.
5. Add an equal amount of cold isopropanol to the aqueous phase and mix thoroughly with a vortex. Incubate at -20  $^{\circ}$ C for at least 30 min.
6. Centrifuge at 12,000 rpm for 10 min at 4  $^{\circ}$ C.
7. Discard the supernatant and wash the pellet with 1 ml of cold 70% ethanol (prepared with 0.1% DEPC-treated water to inactivate RNase).
8. Centrifuge briefly to secure the pellet. Repeat the wash for one more time.
9. Remove all the ethanol and dry the pellet in the air for several minutes.
10. Dissolve the RNA in 20-50  $\mu$ l of RNase-free water.

### **cDNA synthesis and qRT-PCR**

Synthesis of the first-strand cDNA from total RNA templates was performed following the user guide for The Thermo Scientific<sup>™</sup> RevertAid<sup>™</sup> First Strand cDNA Synthesis Kit (Thermo Scientific).

1. Assess RNA integrity before cDNA synthesis by electrophoresis of 0.5  $\mu$ g of total RNA on 1.5% agarose gel at 120V for 15 min. Both 18S and 28S rRNA should appear as sharp bands, and the 28S rRNA band should be approximately twice as intense as the 18S rRNA. A lower molecular weight of the 5S rRNA band can also be observed on the gel. New total RNA should be prepared if any smearing of RNA bands occurs.
2. The concentration of RNA was measured with GE Healthcare NanoVue Plus UV-Vis Spectrophotometer.
3. Remove gDNA from the RNA samples with DNase I by adding to an RNase-free tube:

RNA	1 $\mu$ g
10X reaction buffer with MgCl <sub>2</sub>	1 $\mu$ l
DNase I	1 $\mu$ l
RNase-free water	to 10 $\mu$ l



Incubate at 37 °C for 30 min. Add 1 µl of 50 mM EDTA and incubate at 65 °C for 10 min to terminate the reaction.

4. Add the following reagents into a nuclease-free tube on ice:

Template RNA	1 µg
Oligo (dT) <sub>18</sub> primer	1 µl
5X Reaction buffer	4 µl
RiboLock RNase Inhibitor	1 µl
10 mM dNTP Mix	2 µl
RevertAid M-MuLV RT	1 µl
RNase-free water	to 20 µl

Mix gently and centrifuge briefly. First, incubate at 42 °C for 60 min and then terminate the reaction by heating at 70 °C for 5 min. The cDNA quality was finally checked by RT-PCR using the reference gene *Actin2*, and a 1:20 dilution of the cDNA was made as a PCR template. cDNA was stored at -20 °C or -80 °C for longer storage.

qRT-PCR was conducted in 10 µl reactions with Maxima SYBR Green qPCR Master Mix (Thermo Scientific) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following conditions: 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Relative expression of mRNA was calculated by the delta-delta CT method (Livak and Schmittgen, 2001). And all values for the mRNA species were normalized to *Actin2*. qRT-PCR primers can be found in supplementary tables attached to each chapter.

The reaction tubes were prepared by adding the following components on ice:

Maxima SYBR Green qPCR Master Mix (2X)	5 µl
Forward Primer	1 µl
Reverse Primer	1 µl
Template cDNA	1 µl
Nuclease-free water	2 µl

#### **Plasmid vector preparation via Gateway cloning**

The overexpression (OE), and HA-, GFP- or GUS-tagged constructs of *AtGLP5* and *AtSUC7* were prepared via Gateway cloning using Invitrogen BP and LR cloning kits.

The sequences of interest were amplified from either gDNA (for promoter sequence) or cDNA of Arabidopsis plants with the correspondent primer pairs containing att-sites with Phusion™ High-Fidelity DNA Polymerase for the cloning into pDONR201 vector. From the pDONR201 entry clone, the sequences were then transferred into Gateway binary vectors such as pGWB414 producing a 35S promoter-driven gene construct with a 3xHA-tag coding region at the 3'-end for *AtGLP5*; pGWB402 producing a 35S promoter-driven gene construct for overexpression of *AtSUC7*; pGWB405 and pGWB451 for GFP-tagged subcellular localization analysis of *AtSUC7* and *AtGLP5*, respectively; and pGWB433 fused with a β-glucuronidase (GUS) coding region for

determination of tissue-specific expression patterns of *AtGLP5*. Table 7 describes the information of these pGWB vectors briefly.

Table 7 Commercial pGWB vectors used in this study.

Vector name	Gateway cassette	Bacterial selection	Markers for plant	Type
pGWB402	P <sub>35S</sub> - <i>attR1</i> -Cm <sup>r</sup> - <i>ccdB</i> - <i>attR2</i> -T <sub>NOS</sub>	Sp <sup>c</sup> <sup>r</sup>	Km <sup>r</sup>	35S pro, no tag
pGWB405	P <sub>35S</sub> - <i>attR1</i> -Cm <sup>r</sup> - <i>ccdB</i> - <i>attR2</i> -sGFP-T <sub>NOS</sub>	Sp <sup>c</sup> <sup>r</sup>	Km <sup>r</sup>	35S pro, C-sGFP
pGWB414	P <sub>35S</sub> - <i>attR1</i> -Cm <sup>r</sup> - <i>ccdB</i> - <i>attR2</i> -3xHA-T <sub>NOS</sub>	Sp <sup>c</sup> <sup>r</sup>	Km <sup>r</sup>	35S pro, C-3xHA
pGWB433	<i>attR1</i> -Cm <sup>r</sup> - <i>ccdB</i> - <i>attR2</i> -GUS-T <sub>NOS</sub>	Sp <sup>c</sup> <sup>r</sup>	Km <sup>r</sup>	no pro, C-GUS
pGWB451	P <sub>35S</sub> - <i>attR1</i> -Cm <sup>r</sup> - <i>ccdB</i> - <i>attR2</i> -G3GFP-T <sub>NOS</sub>	Sp <sup>c</sup> <sup>r</sup>	Km <sup>r</sup>	35S pro, C- G3GFP

1) Procedure for BP Recombination reaction:

1. Mix:

attB PCR product (20-50 fmoles)	0.5-3.5 µl
pDONR201 vector (150 ng/µl)	0.5 µl
1x TE Buffer, pH 8.0	4 µl

2. Vortex BP Clonase II briefly and add 1 µl to the mixture.

3. Incubate at 25 °C overnight.

4. Add 1 µl of 2 µg/µl Proteinase K and incubate at 37 °C for 10 min to improve efficiency.

5. Transform the reaction product into competent *E.coli* cells.

6. After transformation, the plasmid DNA of the positive clones were isolated with the NucleoSpin Plasmid Kit from Macherey-Nagel, further confirmed with vector- and gene-specific primers, and then sequenced.

2) Procedure for LR Recombination reaction:

1. Mix:

Entry clones (10 fmoles)	0.5-3.5 µl
pDONR201 vector (150 ng/µl)	0.5 µl
1x TE Buffer, pH 8.0	4 µl

2. Vortex LR Clonase briefly and add 1 µl to the mixture.

3. Incubate at 25 °C overnight.

4. Add 1 µl of 2 µg/µl Proteinase K and incubate at 37 °C for 10min to improve efficiency.

5. Transform the reaction product into competent *E.coli* cells.

6. After transformation, plasmid DNA of the positive clones were isolated, confirmed with vector- and gene-specific primers, and then transformed into competent *Agrobacterium tumefaciens* strain GV3101 cells.

7. After transformation into GV3101, positive clones were further checked with vector- and

gene-specific primers and finally used for plant transformation.

### ***Agrobacterium*-mediated transformation of *Arabidopsis thaliana* through the floral dip method**

All binary vector constructs were introduced into *Agrobacterium tumefaciens* GV3101 strain and transformed into *A. thaliana* plants through the floral dip method (Clough and Bent, 1998).

1. Grow healthy, vigorous (a major factor affecting transformation rates) *Arabidopsis* plants first under short-day conditions to produce strong plants and then move them into greenhouse under long-day conditions to accelerate flowering.
2. Clip first bolts to encourage the proliferation of many secondary bolts.
3. Grow the prepared bacterial in LB medium with antibiotics at 28 °C.
4. Spin down *Agrobacterium* at 1,000 rpm for 10 min. Resuspend to OD<sub>600</sub> = 0.8 in 5% sucrose solution.
5. Add Silwet Gold to a concentration of 0.05% and mix well before dipping.
6. Dip the inflorescence of the plants into the *Agrobacterium* solution for 10 s. Keep the plants at a high relative humidity in a black plastic bag for one day.
7. The next day, remove the plastic bag and return plants to their previous growing conditions. After one week, the plants might be dipped for one or two more times to achieve higher rates of transformation if they are still in very good condition.
8. Water and grow plants normally, harvest dry seeds.
9. After vernalization, seeds can be sterilized and grown on 1/2 MS plates with appropriate antibiotics to select positive transformants.

### **Cultivation and inoculation processes for *S. sclerotiorum*, *Pst* DC3000, and *V. longisporum***

Infection of *A. thaliana* leaves with *S. sclerotiorum* was either performed by agar plug infection or by spray infection with mycelia suspension. In the first approach, *S. sclerotiorum* was grown on PDA (for transcript analysis) or MA (for resistance assay) medium at 22 °C. After two or three days, agar-plugs were punched from the expanding margin of the mycelium using a 6 mm cork borer and placed on fully developed leaves of 5-week-old plants with the mycelium facing the leaf surface. Leaves treated with just plain agar-plugs served as control. After two days, a digital caliper was used to measure the major and minor diameters of the ellipse-shaped lesions. The area of lesions was estimated by multiplying half the length of the major axis by half the length of the minor axis, then multiplying by  $\pi$ . For the spray infection method, 15 pieces of agar-plugs containing mycelia were transferred into 70 ml 40% Czapek Dox medium and shaken for three days at 22 °C. Subsequently, the mycelium was homogenized with a homogenizer (D-8) for 35 s and centrifuged at 6,000 rpm for 10 min. The sedimented mycelia were weighed and resuspended

in 10 mM MgSO<sub>4</sub> at a concentration of 1 g/25 mL. Leaves of 5-week-old plants were sprayed with the mycelium suspension. Plants were covered with a transparent lid to keep high humidity. After two or three days, photos were taken to compare the severity of symptoms on each plant.

*Pst* DC3000 was cultivated on a Petri dish with NYGA medium plus appropriate antibiotics for 2 days at 28 °C. Bacteria were then collected by washing with 5 mL 10 mM MgCl<sub>2</sub> and adjusted to OD<sub>600</sub> = 0.2. After adding 0.04% (v/v) Silwet Gold, the prepared bacteria suspension was sprayed onto 5-week-old *A. thaliana* plants until saturation when the leaves appear evenly wet and then covered with a transparent lid. For control treatments, plants were sprayed with a solution containing 10 mM MgCl<sub>2</sub>, 0.04% Silwet Gold. To calculate the bacteria number, leaf discs from the infected leaves were excised at 3 dpi using a cork borer and shaken for 1 h in 500 µl of 10 mM MgCl<sub>2</sub> with 0.01% Silwet Gold. The bacteria number was calculated from serial dilutions grown on NYGA plates for two days.

The infection process of *V. longisporum* was described by Behrens (Behrens *et al.*, 2019). Shortly, four days after plant germination, Arabidopsis seedling were transferred onto squared plates containing 1/2 MS medium and grown vertically in the growth chamber for more days. On the day of inoculation, 22% glycerol conidia stocks were thawed, centrifuged at 5,000 rpm for 10 min, and resuspended in sterile Czapek-Dox medium to a final concentration of 2x10<sup>6</sup> conidia / mL. Then the conidia suspension was spread on the roots with a sterilized brush. After 3, 6, and 9 days, the whole seedlings were stained with GUS staining solution to allow us to observe the change of GUS activity during different infection stages. Non-infected plants were used as control.

#### **Plant treatment with flg22 and phytohormones**

For the different stresses, leaves of 4-week-old transgenic *A. thaliana* carrying *P<sub>AtGLP5</sub>::GUS* were collected at 24 hours after treatment of plant hormones (1 mM SA, 100 µM and 500 µM JA, 10 µM IAA or 10 µM IBA). As for the wounding and flg22 treatment, leaves were collected at an earlier stage, at 5 hours after cut with a pair of scissors or sprayed with 1 mM flg22, since plants responded to these stimuli quite quickly. Control plants were either non-treated or sprayed with water. The detached leaves were then used for GUS staining and qRT-PCR analysis.

#### **GUS staining analysis**

For histochemical GUS analysis, plant samples were incubated in fresh GUS staining buffer overnight at 37 °C. On the next day, the GUS staining buffer was carefully removed and replaced with 70% ethanol to bleach the green chlorophyll. Several times of ethanol changes may be necessary until the tissues turned white. The stained samples were examined under a stereomicroscope (Zeiss).

**Immunodetection of recombinant proteins and detection of SOD activity**

Transient expression of the recombinant proteins in *N. benthamiana* was carried out following the protocol of Witte (Witte *et al.*, 2004). In brief, an *Agrobacterium* suspension was infiltrated from the bottom side of the leaf, and the plants were left to grow continually in the climate chamber. At 5-6 days post infiltration, leaf material was harvested and stored at -80 °C until further processing.

Total protein was extracted from ~10 mg of transformed *N. benthamiana* leaf tissue by vigorously mixing with 110 µl of extraction buffer. After centrifugation for 10 min at 13,000 rpm, 80 µl of the supernatant was supplemented with 20 µl 5x gel loading buffer and loaded on a 10% SDS-PAGE without boiling. The SDS-PAGE was run at 90 V for 60 min until the loading buffer had reached the edge of the gel. For immunodetection, proteins were blotted on a PVDF membrane and visualized with an anti-HA antibody combined with a Lumi-Light<sup>PLUS</sup> Western Blotting Kit (Rocher *et al.*) following the manufacturer's instructions. An in-gel SOD activity assay was performed following the protocol of Beauchamp and Fridovich (Beauchamp and Fridovich, 1971). The gel is incubated in 0.1% (w/v) nitro blue tetrazolium (NBT) and riboflavin with TEMED and then exposed to light. The riboflavin is first excited by photons and then reduced due to the oxidation of the electron donor TEMED. This reduced form reduces O<sub>2</sub> to superoxide anions, reducing NBT to violet formazan. As SOD can dismutate O<sub>2</sub><sup>-</sup> to H<sub>2</sub>O<sub>2</sub>, the areas of the gel where SOD is present remain yellowish transparent because NBT is not reduced there. First, the gel was shaken in 25 mM Tris-HCl (pH 7.5) with 100 mM NaCl for 10 min and washed in H<sub>2</sub>O. Next, it was shaken for 10 min in 30 ml substrate solution. After washing again with H<sub>2</sub>O, it was shaken in the dark in the reaction solution for 15 min. Most of the solution was discarded, and the gel was exposed to a neon lamp until it turned dark violet and yellow bands became visible. The remaining reaction solution was discarded and replaced with H<sub>2</sub>O, which was renewed regularly until the desired signal intensity had been obtained. The gel was stored at 4 °C overnight to increase the color intensity and scanned the next day.

**DAB staining to detect H<sub>2</sub>O<sub>2</sub> production in Arabidopsis plants**

*S. sclerotiorum* was grown on PDA at 22 °C. After three days, agar-plugs were punched from the expanding margin of the mycelium using a 6 mm cork borer and placed on fully developed leaves of 5-week-old plants with the mycelium facing the leaf surface. One day after inoculation, the leaves were detached and immersed in fresh prepared 1 mg/ml DAB staining solution at RT for overnight. On the next day, the DAB staining buffer was carefully removed and replaced with 70% ethanol to bleach the green chlorophyll. Several changes of ethanol may be necessary until the tissues turned white. The stained samples were examined under a stereomicroscope (Zeiss).

The integrated density of DAB staining was then quantified with the ImageJ Colour Deconvolution plugin choosing "H DAB" as the stain. Quantify the picture with "Colour\_2" in the title by measuring "Mean gray value", and a "Results" window will pop up with the quantification in units of intensity.

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