Analyses Of TALE-induced Resistance And Putative Susceptibility Genes In Tomato

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Wer kennt die Welt? Wer sich selbst kennt.

(Novalis, Schriften)

CONTENTS

CONTENTS

LIST OF PUBLICATIONS

From the work presented in this thesis the following manuscript is in preparation:

Tomato CRISPR/Cas mutants lacking the TALE-sensing resistance (R) protein Bs4: a novel plant platform for discovery of TALE-induced disease phenotypes and genetic dissection of executor *R* genes

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LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

35s	Cauliflower mosaic virus 35s
aa	amino acid
AAD	Acidic activation domain
AP	Aspartic proteinase
At	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
Avr	Avirulence
AvrHah1	Avr protein homologous to AvrBs3 and Hax2 No. 1
BC	Backcross
BCP	Blue copper protein
bHLH	basic helix-loop-helix
bp	basepair(s)
Bs3	Bacterial spot disease R protein 3
Bs3p	Native Bs3 promoter
Bs4	Bacterial spot disease resistance protein 4
Bs4C	Bacterial spot disease resistance protein 4 from *Cp*
Ca	*Capsicum annuum*
Cas9	CRISPR-associated protein 9
CC	CRISPR/Cas9-engineered
cDNA	complementary DNA
CDS	Coding sequence
CFU	Colony-forming units
cm	centimetre
CNL(s)	Coiled-coil domain-containing NLR(s)
Cp	*Capsicum pubescens*
CRISPR	Clustered regularly interspaced short palindromic repeats
Cs	*Citrus sinensis*
CS-domain	CHORD-SGT1-domain
cv.	cultivar
DSB	Double-strand break
DNA	Deoxyribonucleic acid

ABBREVIATIONS

dpi	days post-infiltration
dTALE	designer TALE
e.g.	*exempli gratia*
EBE(s)	Effector-binding element(s)
ECW	Early Calwonder
EDS1	Enhanced disease susceptibility 1
EF1α	Elongation Factor 1α
EIP	Estradiol-inducible promoter
EP-domain	EDS1-PAD4-domain
ETI	Effector-triggered immunity
EtOH	Ethanol
EV	Empty vector
E. coli	*Escherichia coli*
F	Filial
FDR	False discovery rate
FMO	Flavin-containing monooxygenase
gDNA	genomic DNA
GFP	Green fluorescent protein
GOI	Gene-of-interest
GUS	β-glucuronidase
Hax	Homolog of AvrBs3 in *Xanthomonas*
HDR	Homology-directed repair
hpi	hours post-infiltration
HR	Hypersensitive response
HSP90	Heat shock protein 90
i.e.	*id est*
ICS1	Isochorismate Synthase 1
Indel	Insertion or deletion
iTALE(s)	interference TALE(s)
mRNA	messenger RNA
LB	Lysogeny Broth
LOB1	Lateral Organ Boundary 1

LRR	Leucine rich repeat
mGFP5	monomeric GFP5
MIB	Minimal Infiltration Buffer
MLA1	Mildew resistance locus a 1
MM	*S. lycopersicum* cv. Moneymaker
MtU6	*Medicago truncatula U6*
n	Numbers of independent biological replicates
NADPH	Nicotinamide adenine dinucleotide phosphate
Nb	*Nicotiana benthamiana*
NB	Nucleotide-binding
NDR1	Non-race specific disease resistance 1
NHEJ	Non-homologous end joining
NHP	N-hydroxypipecolic acid
NLR(s)	NB and LRR domains-containing protein(s)
NLS(s)	Nuclear localisation signal(s)
NOSp	*Nopaline synthase* promoter
NOS-t	*Nopaline synthase* terminator
*NPT*II	*Neomycin phosphotransferase* II
NYG	Nutrient Yeast Glycerol
OD_{600}	Optical density measured at 600 nanometres
Os	*Oryza sativa*
PBS3	AvrPphB susceptible 3
PCR	Polymerase chain reaction
PE	Pectinesterase
Pip	Pipecolic acid
PL	Pectate lyase
PR	Pathogenesis-related Protein
pv.	pathovar
qRT-PCR	quantitative real-time PCR
R	Resistance
RACE	Rapid amplification of cDNA-ends
RAR1	Required for *Mla12* resistance 1

ABBREVIATIONS

RbcS	Ribulose-1,5-bisphosphate carboxylase/oxygenase Subunit
RING	Really interesting new gene
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RT-PCR	Reverse transcription PCR
RVDs	Repeat variable di-residue(s)
RXO1	Resistance to *Xanthomonas oryzae* 1
S	Susceptibility
SA	Salicylic acid
SAR	Systemic acquired resistance
sgRNA(s)	single guide RNA(s)
SGS-domain	SGT1-specific-domain
SGT1	Suppressor of the G2 allele of *skp1*
S. lycopersicum	*Solanum lycopersicum*
SNP(s)	Single-nucleotide polymorphism(s)
Sp	*Solanum pennellii*
spp.	species
StUbi3	*Solanum tuberosum Ubiquitin 3*
sun	*Suppressor of N*
SWEET(s)	Sugars Will Eventually be Exported Transporter(s)
TAF	Transactivation function
TALE(s)	Transcription activator-like effector(s)
T-DNA	Transfer DNA
TIP41-Like	TAP42 Interacting Protein of 41 kDA-Like
TIR	Toll-interleukin1-receptor
TNL(s)	TIR domain-containing NLR(s)
TPR	Tetratricopeptide repeat
truncTALE(s)	truncated TALE(s)
TTSS	Type III secretion system
UNS1	Unique nucleotide sequence 1
UP	Unknown protein
UPA	Upregulated by AvrBs3

UTR	Untranslated region
VIGS	Virus-induced gene silencing
WT	Wild-type
Xa	*Xanthomonas R* protein
Xcc	*Xanthomonas citri* pv. *citri*
Xca	*Xanthomonas campestris* pv. *armoraciae*
Xe	*Xanthomonas euvesicatoria*
Xg	*Xanthomonas gardneri*
Xoo	*Xanthomonas oryzae* pv. *oryzae*
Xp	*Xanthomonas perforans*
XVE	Fusion protein LexA-VP16-ER
YEB	Yeast Extract Broth
zfBED	zinc-finger BED

ABSTRACT

Transcription activator-like effectors (TALEs) from *Xanthomonas* spp. interact with effector-binding elements (EBEs) in the promoter regions of their target genes to upregulate host susceptibility (*S*) genes for virulence enhancement. To counteract the pathogen, pepper and rice species have evolved so-called executor resistance (*R*) genes, which upon transcriptional activation by TALEs lead to immediate cell death and restrict bacterial growth. Additionally, plants have evolved nucleotide-binding domain leucine rich repeat-containing proteins (NLRs) that are capable of TALE recognition and mediation of cell death. While NLR-mediated immunity pathways are well-studies, executor-mediated pathway components are unknown.

Bacterial spot 3 (*Bs3*), an executor *R* gene from pepper, causes cell death upon transcriptional activation by the corresponding TALE from *Xanthomonas euvesicatoria*, *i.e.* AvrBs3, while tomato Bacterial spot 4 (Bs4) NLR protein mediates recognition of numerous TALEs. As pepper is not amenable to transformation, the genetic dissection of Bs3-mediated pathways in pepper is not straightforward. Therefore, the main aim of this work was to generate *Bs3* transgenic tomato lines lacking *Bs4* as a tool to decipher Bs3-mediated pathways.

CRISPR/Cas9-mediated *Bs4* mutagenesis yielded null alleles containing mutations within *Bs4* coding sequence (*CC-Bs4*). Phenotyping experiments revealed that tomato lines containing *CC-Bs4* alleles no longer showed TALE-dependent cell death. As the next step, transgenic tomato line containing estradiol-inducible *Bs3* was generated. Upon transcriptional activation by estradiol in this line, *Bs3* led to the cell death suggesting that Bs3-mediated pathway components are conserved between tomato and pepper. In addition, a designer TALE (dTALE) was engineered to bind the estradiol-inducible promoter upstream of the *Bs3* CDS. When delivered by *X. euvesicatoria*, this dTALE activated transcription of the *Bs3* transgene and led to a cell death phenotype. Analysis of bacterial growth showed that transcriptional activation of *Bs3* correlated with reduced *in planta* growth of dTALE-containing *X. euvesicatoria* strain. In summary, the engineered *Bs4* knockout line carrying the *Bs3* transgene provide a basis for genetic dissection of the Bs3-mediated cell death and immunity pathway.

In addition, it was tested if the knockout of *Bs4* affects TALE-dependent host gene activation. Even though Bs4 had no impact on TALE-dependent transcriptional activation of studied host genes, Bs4 was found to be epistatic to TALE-induced disease symptoms.

ZUSAMMENFASSUNG

Transkriptionsfaktor-ähnliche Effektoren (Transcription activator-like effectors, TALEs) aus *Xanthomonas* spp. binden an Effektorbindeelemente (EBEs) in den Promoterregionen pflanzlicher Zielgene (Suszeptibilitätsgene, *S*-Gene) und induzieren deren Transkription. Dadurch wird die bakterielle Virulenz begünstigt. Um dem entgegenzuwirken, haben Paprika- und Reis-Linien sogenannte Exekutor-Resistenzgene (*R*-Gene) evolviert, die nach TALE-induzierter transkriptioneller Aktivierung Zelltodreaktionen einleiten und damit das bakterielle Wachstum begrenzen. TAL-Effektoren können aber auch von *nucleotide-binding domain leucine-rich repeat*-Proteinen (NLRs) erkannt werden, wodurch ebenfalls Zelltodreaktionen ausgelöst werden. Während NLR-vermittelte Immunantworten bereits gut untersucht sind, sind die Signalwegkomponenten der Exekutor-vermittelten Abwehr noch unbekannt.

Bs3 (*bacterial spot disease resistance gene no. 3*) ist ein Exekutor-*R*-Gen aus Paprika, welches nach transkriptioneller Aktivierung durch den TAL-Effektor AvrBs3 aus *Xanthomonas euvesicatoria Z*elltodreaktionen auslöst. Bs4 (*bacterial spot disease resistance gene no. 4*) aus Tomate ist ein NLR-Protein, dass die Erkennung zahlreicher TALEs vermittelt. Da Paprikapflanzen für stabile Transformationen nicht einfach zugänglich sind, ist die genetische Aufklärung des *Bs3*-vermittelten Signalweges in Paprika schwierig. Ziel der vorliegenden Arbeit war es daher *Bs3*-transgene, *Bs4 knock out*-Tomaten als Werkzeug zur Analyse des *Bs3*-vermittelten Signalwegs zu erstellen.

Mittels CRISPR/Cas9-Technologie konnten Tomatenpflanzen mit Mutationen in der *Bs4* kodierenden Sequenz (*knock out, CC-Bs4)* generiert werden. In phänotypischen Analysen zeigten Tomatenpflanzen mit *CC-Bs4* keinen TALE-abhängigen Zelltod mehr. *CC-Bs4*-Linien dienten als Basis zur Erstellung transgener Tomaten mit Estradiol-induzierbarem *Bs3*. Die transkriptionelle Aktivierung von *Bs3* durch Estradiol resultierte in diesen Linien in einer Zelltodreaktion, was nahelegt, dass die Bs3-Signalwegkomponenten in Paprika und Tomate konserviert sind. Des Weiteren wurde ein designer TALE (dTALE) kloniert, der an den Estradiol-induzierbaren Promoter *upstream* der *Bs3* kodierende Sequenz bindet. Nach *X. euvesicatoria*-Infektion aktiviert dieser dTALE die Transkription des *Bs3*-Transgens, was in einer Zelltodreaktion resultiert. Analysen des bakteriellen Wachstums zeigten, dass die transkriptionelle Aktivierung von *Bs3* mit einem reduzierten Wachstum des dTALE-

enthaltenen *X. euvesicatoria*-Stamms korrelierte. Damit bilden die erstellten *Bs3*-transgenen, *Bs4 knock out*-Tomatenlinien die Basis zur genetischen Analyse der *Bs3*-vermittelten Immunantwort.

Zusätzlich wurde untersucht, ob der *knock out* von *Bs4* die TALE-abhängigen Aktivierungen von Wirtsgenen beeinflusst. Obwohl Bs4 keinen Einfluss auf die TALE-induzierte transkriptionelle Aktivierung der untersuchten Wirtsgene hatte, zeigte sich, dass Bs4 epistatisch über TALE-induzierte Krankheitssymptome ist.

1 INTRODUCTION

1.1 TALEs manipulate expression of host genes.

Xanthomonas euvesicatoria (*Xe*) is a common bacterial pathogen of pepper, tomato, and other solanaceous species causing defoliation and necrotic lesions on fruits (R. Cox *et al.*, 1956; J. B. Jones *et al.*, 1998; Stall *et al.*, 2009). Development of such symptoms happens in highly humid conditions after rainfalls and thus, is common within tropical and subtropical regions (R. Cox, 1966). Bacteria invade plants through storm-caused wounds, stomata, and hydathodes (Cerutti *et al.*, 2017; Stall, 1995). Successful colonisation of the intercellular space requires bacteria to inject a cocktail of so-called "effectors", pathogen-associated virulent molecules (Roux *et al.*, 2015; Toruño *et al.*, 2016), via type III secretion system (TTSS; Blocker *et al.*, 2001; Galán and Wolf-Watz, 2006; Salmond and Reeves, 1993) to promote virulence (Kay and Bonas, 2009; Qin *et al.*, 2018; L. Tan *et al.*, 2014) and to suppress host defence (J.D.G. Jones and Dangl, 2006; Schulze *et al.*, 2012; Üstün *et al.*, 2013; Üstün and Börnke, 2014).

Transcription activator-like effectors (TALEs) are a family of unique type III effectors, which manipulate host gene expression by acting like transcription factors (Boch and Bonas, 2010; K.L. Cox *et al.*, 2017; Kay *et al.*, 2007; Schornack *et al.*, 2008). TALEs contain C-terminal nuclear localisation signals (NLSs) for translocation to the host nucleus (Figure 1; Boch and Bonas,

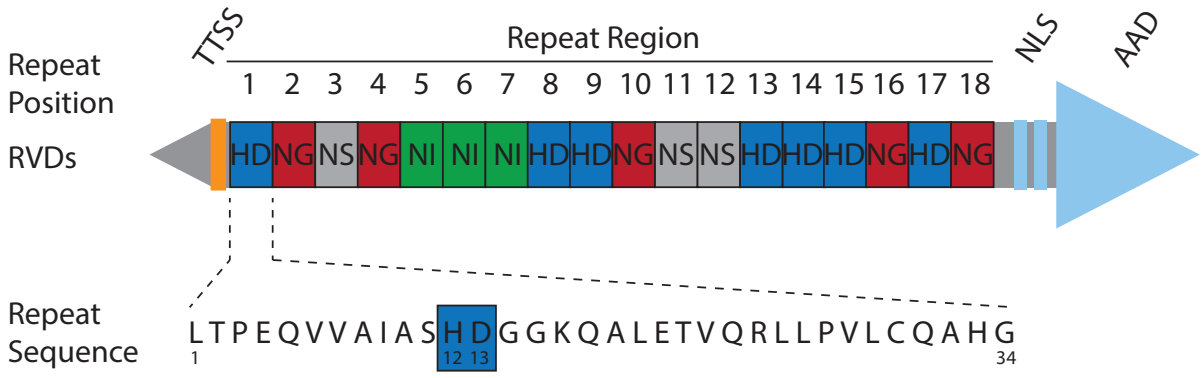

Figure 1. AvrBs3-like TALEs consist of four main structural elements. A TALE contains an N-terminal type III secretion signal for secretion via the TTSS. The C-terminal end of the TALE protein contains multiple NLSs for translocation to the nucleus and AAD for a transcriptional activation of a target gene. The central part of the TALE protein is a repeat region, which consists of an array of typically 34 aa-long tandemly arranged repeats that mediate DNA binding. AvrBs3 consists of 17,5 repeats. The individual repeats contain only a few polymorphisms. RVDs, i.e. two variable residues at positions 12 and 13 of each repeat, mediate base-specific interaction of the TALE with the EBE in the promoter region of a host target gene. The figure is adapted from Boch *et al.*, 2009.

2010). The hallmark of TALEs is the central repeat region, which consists of predominantly 34 amino acid (aa)-long repeats and mediates binding to the deoxyribonucleic acid (DNA; Figure 1; Boch and Bonas, 2010). The aa composition within the repeats is almost identical except for two variable residues at positions 12 and 13 of each repeat (Figure 1; Boch and Bonas, 2010). These repeat variable di-residues (RVDs) mediate base-specific interaction of TALEs with effector-binding elements (EBEs) in the promoter regions of host target genes (Figure 1; Boch *et al.*, 2009; Mak *et al.*, 2012; Moscou and Bogdanove, 2009). Host gene expression is manipulated via C-terminal acidic activation domain (AAD; Figure 1; Boch and Bonas, 2010).

Through knowing the TALE RVD sequence, *i.e.* the TALE-code, it is possible to identify potential EBEs within the promoters of annotated genes (Richter *et al.*, 2014; Richter *et al.*, 2016). Numerous software tools have been developed for the identification of EBEs within the promoter of putative target genes using TALE-code (Doyle *et al.*, 2012; Grau *et al.*, 2013; Pérez-Quintero *et al.*, 2013).

1.1.1 TALEs upregulate host genes to promote disease symptoms.

The first discovered TALE is AvrBs3 from *X. euvesicatoria* (Bonas *et al.*, 1989). AvrBs3 consists of 17.5 tandem 34 aa-long repeats and is known to induce *Upregulated by AvrBs3 No. 20* (*UPA20*), a gene encoding a basic helix-loop-helix (bHLH) transcription factor in *Capsicum annuum* (*Ca*; Figure 2; Kay and Bonas, 2009; Kay *et al.*, 2007). AvrBs3-upregulated *UPA20* causes cell hypertrophy which is hypothesised to benefit bacterial multiplication and spread into new tissue (Figure 2; Kay *et al.*, 2007; Marois *et al.*, 2002).

AvrBs4 from *X. euvesicatoria* is another well-studied representative of AvrBs3-like TALEs (Ballvora, Schornack*, et al.*, 2001; Schornack *et al.*, 2004), which consists of 17.5 tandem 34 aa-long repeats. However, no AvrBs4-induced susceptibility targets have been reported so far (Ballvora, Schornack*, et al.*, 2001; Schornack *et al.*, 2004; Strauß *et al.*, 2012).

AvrHah1 (Avr protein homologous to AvrBs3 and Hax2 No. 1) is an AvrBs3-like TALE from *Xanthomonas gardneri* (*Xg*; Schornack *et al.*, 2008). AvrHah1 consists of 13.5 repeat units, which have a unique architecture (Schornack *et al.*, 2008; Schwartz *et al.*, 2017). While majority of AvrBs3-like proteins consist from 34 aa-long repeats, AvrHah1 is composed of both 35 aa-long (units 1–6 and units 10–12) and 34 aa-long repeat units (units 7–9 and 13). 35 aa-long repeats of AvrHah1 contain a proline at the position 33 (Schornack *et al.*, 2008), which is

absent in 34 aa-long repeats of conventional AvrBs3-like TALEs (Ballvora, Pierre, *et al.*, 2001; Gu *et al.*, 2005; Morbitzer *et al.*, 2011; Richter *et al.*, 2014). AvrHah1 was also reported to upregulate *UPA20* in *C. annuum* (Schornack *et al.*, 2008), as well as *bHLH022* (*Solyc03g097820*), a *UPA20* orthologue from *Solanum lycopersicum* (Schwartz *et al.*, 2017). Interestingly, AvrHah1 and AvrBs3 target the same EBE within the promoter of *UPA20*, also known as *UPA* box (Schornack *et al.*, 2008). It was demonstrated that AvrHah1-mediated upregulation of *bHLH022* induces *PL* (*Solyc05g014000*), a gene encoding a pectate lyase (Schwartz *et al.*, 2017), which acts as a promoter of hypertrophy in tomato and therefore, *PL* was recognised a secondary target of AvrHah1 (Schwartz *et al.*, 2017).

Similar virulence effects were reported for other TALEs from numerous *Xanthomonas* spp. For example, PthA4, a TALE from *Xanthomonas citri* pathovar (pv.) *citri* (*Xcc*), is known to upregulate *Citrus sinensis* (*Cs*) *Lateral Organ Boundary 1* (*LOB1*) to promote citrus canker disease symptoms (Y. Hu *et al.*, 2016; Y. Hu *et al.*, 2014). Numerous TALEs from *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) upregulate expression of *Oryza sativa* (*Os*) *Sugars Will Eventually be Exported Transporter* (*SWEET*) genes: PthXo1 upregulates *OsSWEET11* (Yang *et al.*, 2006), PthXo2 targets *SWEET13* (Zhou *et al.*, 2015), while AvrXa7, PthXo3, TalC, and TalF upregulate *SWEET14* (Antony *et al.*, 2010; Streubel *et al.*, 2013; Yang and White, 2004; Yu *et al.*, 2011).

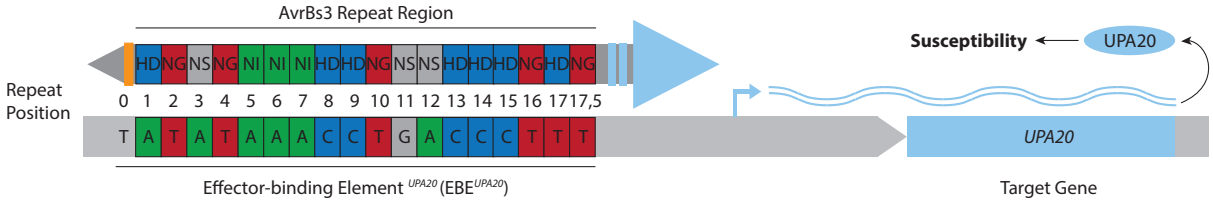

Figure 2. AvrBs3 transcriptionally upregulates *UPA20* via interaction with the EBE^{UPA20} located upstream of *UPA20* to promote disease symptoms. RVDs within the repeat region of AvrBs3 bind to the basepairs of the EBE^{UPA20} in a one-to-one fashion. Therefore, the EBE of the host target gene *UPA20* is specified by the RVD composition of AvrBs3 central repeat domain. The RVD "NI" has a high affinity towards adenine ("A"; green), "HD" to cytosine ("C"; blue), "NG" to thymine ("T"; red), and "NS" has no strong base preference (grey). Additionally, TALEs contain N-terminal non-canonical repeats which require thymine at the position "0" of any EBE for an efficient interaction. A compatible AvrBs3 – EBE^{UPA20} interaction results in transcriptional activation of the downstream host gene, *i.e. UPA20* (Kay *et al.*, 2007). UPA20 is known to cause hypertrophy of leaf tissue and these disease symptoms are hypothesised to benefit bacterial virulence and, thereby increasing host susceptibility (Kay *et al.*, 2007). The figure is adapted from Boch *et al.*, 2009.

1.1.2 Transcriptionally activated by TALEs executor resistance genes cause cell death and restrict growth of biotrophic pathogens.

Although TALEs promote virulence, they can also be avirulent factors in some hosts. TALEs are known to activate transcription of executor resistance (*R*) genes, which lead to cell death via unknown pathways (Römer *et al.*, 2007; Strauß *et al.*, 2012; Tian *et al.*, 2014; Chunlian Wang *et al.*, 2015). Promoter regions of executor *R* genes contain EBEs and serve as receptor traps of corresponding TALEs and therefore, executor *R* genes are expressed and execute cell death in the presence of their corresponding TALEs (Bogdanove *et al.*, 2010).

So far, all identified executor *R* genes have been found in *Capsicum* (Römer *et al.*, 2007; Strauß *et al.*, 2012) and *Oryza* spp. (Tian *et al.*, 2014; Chunlian Wang *et al.*, 2015). *Bacterial spot disease 3* (*Bs3*) from *Ca*, a gene encoding a flavin-containing monooxygenase (FMO), is the most well-studied executor *R* gene (Figure 3; Krönauer *et al.*, 2019; Römer *et al.*, 2007). The *Bs3* promoter contains a DNA motif of high similarity to the *UPA* box (Figure 3; Römer *et al.*, 2007). Thus, when AvrBs3 and AvrHah1 activate transcription of *Bs3*, a functional cytoplasm and nuclear-localised Bs3 protein causes rapid cell death via unknown pathways in *Capsicum* and *Nicotiana* spp. (Figure 3; Krönauer *et al.*, 2019; Römer *et al.*, 2007; Schornack *et al.*, 2008). Similarly, AvrBs4 was found to activate transcription of *Bacterial spot 4 from Capsicum pubescens* (*Bs4C*; (Strauß *et al.*, 2012). AvrXa10 and AvrXa23, two TALEs from rice pathogen *Xoo*, transcriptionally activate their corresponding executor *R* genes, *i.e. Xanthomonas R proteins 10* (*Xa10*; Tian *et al.*, 2014) and *23* (*Xa23*; Chunlian Wang *et al.*, 2015).

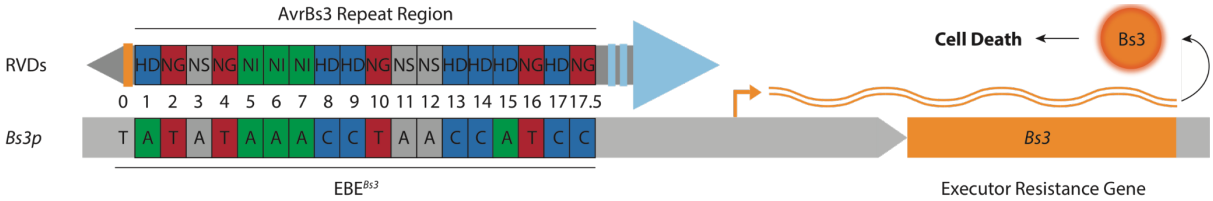

Figure 3. AvrBs3-mediated transcriptional activation of *Bs3* leads to cell death and immunity via unknown pathways. RVDs within the repeat region of AvrBs3 bind to the base pairs of EBE^{Bs3} in a one-to-one fashion. Even though there are three single nucleotide polymorphisms (SNPs) between EBE^{UPA20} and EBE^{Bs3} (positions 11, 15, and 17.5), these SNPs are tolerated by AvrBs3 RVDs. The RVD "NI" has a high affinity towards adenine ("A"; green), "HD" to cytosine ("C"; blue), "NG" to thymine ("T"; red), and "NS" has no strong base preference (grey). Additionally, TALEs contain N-terminal non-canonical repeats which require thymine at the position "0" of any EBE for an efficient interaction. AvrBs3 – EBE^{Bs3} interaction results in transcriptional activation of *Bs3* (Römer *et al.*, 2007). Bs3 is an FMO, which leads to cell death of leaf tissue and immunity via unknown pathways (Krönauer *et al.*, 2019). The figure is adapted from Römer *et al.*, 2007.

1.1.3 TALE-induced immunity is a separate case of the effector-triggered immunity.

TALE-dependent transcriptional activation of executor *R* genes is a distinct type of effector-triggered immunity (ETI). In a canonical ETI, cytosol-localised sensor nucleotide-binding (NB) domain leucine rich repeat (LRR) containing proteins (NLRs) mediate recognition of avirulence (Avr) factors, while executor *R* genes are directly transcriptionally activated by bacterial TALEs.

Commonly, NLRs are divided into two subclasses based on the differences in their N-terminal domains: toll-interleukin1-receptor (TIR) domain-containing NLRs (TNLs) and coiled-coil domain-containing NLRs (CNLs; Burdett *et al.*, 2019; J.D.G. Jones *et al.*, 2016; X. Zhang *et al.*, 2017). Both NLR types trigger transcriptional acceleration and amplification of defence pathways that lead to a hypersensitive response (HR), *i.e.* death of host cells at the infection site, restricting growth of biotrophic pathogens (Bartsch *et al.*, 2006; Jacob *et al.*, 2018; J.D.G. Jones *et al.*, 2016; Mine *et al.*, 2018).

TNLs genetically require Enhanced disease susceptibility 1 (EDS1) protein for transcriptional induction of salicylic acid (SA)-dependent and SA-independent basal defence pathways (Bartsch *et al.*, 2006; Cui *et al.*, 2018; Lapin *et al.*, 2020). CNLs generally signal via Non-race specific disease resistance 1 (NDR1), which is hypothesised to play a broad role in electrolyte release upon infection, as well as in plasma membrane - cell wall junction maintenance (Aarts *et al.*, 1998; Knepper *et al.*, 2011).

Plant immunity activation also requires the Heat shock protein 90 (HSP90) chaperone with its co-chaperones, *i.e.* Suppressor of the G2 allele of *skp1* (SGT1b) and Required for *Mla12* resistance 1 (RAR1), which play a key role in ETI (Hubert *et al.*, 2003; Y. Liu *et al.*, 2004; Takahashi *et al.*, 2003). It is hypothesised that these chaperones contribute to the assembly of NLR activation complexes and affect NLR homeostasis (Azevedo *et al.*, 2006; van Wersch *et al.*, 2020).

As TALE-dependent transcriptional activation of executor *R* genes is a unique case of ETI, it could be hypothesised that the executor proteins use canonical pathways to execute cell death and immunity. A recent study in *Nicotiana benthamiana* revealed that Bs3-dependent cell death coincides with accumulation of salicylic acid (SA) and pipecolic acid (Pip; Krönauer *et al.*, 2019). SA plays a major role in ETI and together with Pip-derived N-hydroxypipecolic acid (NHP) is the main regulator of the systemic acquired resistance (SAR;

Bernsdorff *et al.*, 2016; Hartmann and Zeier, 2019; Hartmann *et al.*, 2018). FMO1 from *Arabidopsis thaliana* (*At*) is known to catalyse Pip into NHP (Hartmann *et al.*, 2018). Since Bs3 is an FMO, one can hypothesise that Bs3 might catalyse Pip into NHP (Krönauer, 2020). However, a biochemical study revealed that Bs3 does not catalyse Pip (Krönauer, 2020). This biochemical assay was reinforced with virus-induced gene silencing (VIGS) of ETI-mediating pathway components in *N. benthamiana* with consecutive overexpression of *Bs3* (Krönauer, 2020). VIGS of *NbEDS1* did not abolish Bs3-mediated cell death, while silencing of *NbSGT1b* and *NbRAR1* abolished Bs3-mediated cell death (Krönauer, 2020). However, gene silencing efficiency was not quantified and assumption of silencing was based on changes in leaf morphology (Krönauer, 2020). Since VIGS may be incomplete (E. Liu and Page, 2008), only a knockout of these master regulators of NLR-mediated cell death and immunity would reveal their role in Bs3- and executor-mediated cell death and immunity.

1.2 *S. lycopersicum – X. euvesicatoria* is a suitable system for a comparison of NLR- and executor-mediated cell death and immunity pathways.

Since executor *R* genes were discovered in different species, a study of executor-mediated immunity pathways and comparison with NLR-mediated immunity pathways should be made within a model species that satisfies the following criteria: 1) to be a solanaceous or poaceous species, *i.e.* a suitable background for executor protein functionality (Krönauer *et al.*, 2019; Römer *et al.*, 2007; Strauß *et al.*, 2012; Tian *et al.*, 2014; Chunlian Wang *et al.*, 2015); 2) to be compliant with genetic transformation in order to engineer executor *R* gene expressing stable lines via transgenesis (Forestier *et al.*, 2021; Heidmann *et al.*, 2011; Kothari *et al.*, 2010; Sahoo *et al.*, 2011; Wittmann *et al.*, 2016); 3) to contain TNLs and / or CNLs that are able to cause cell death upon delivery of TALEs or TALE-like proteins (Read, Hutin*, et al.*, 2020; Read, Moscou*, et al.*, 2020; Schornack *et al.*, 2004); and 4) to be natural hosts of *Xanthomonas* spp. (Potnis *et al.*, 2015; Timilsina *et al.*, 2020).

1.2.1 Tomato as a playground to decipher executor-mediated pathways.

Tomato can be considered as a suitable species for comparison of NLR- and executor-mediated cell death and immunity pathways since it satisfies all afore mentioned criteria. Firstly, tomato is a solanaceous species and thus, executor proteins should remain functional in this genetic background (Krönauer *et al.*, 2019; Römer *et al.*, 2007; Strauß *et al.*, 2012).

Secondly, tomato is not recalcitrant to genetic transformation (T. Li *et al.*, 2018; Wittmann *et al.*, 2016). Thirdly, the tomato genome contains *Bacterial spot 4* (*Bs4*) gene encoding a TNL protein that mediates recognition of TALE-like proteins (Schornack *et al.*, 2004; Schornack *et al.*, 2005; Schwartz *et al.*, 2017). Bs4 was identified as a mediator of AvrBs4 recognition via genetic mapping approach based on differential reaction of the two parental lines, *S. lycopersicum* cv. Moneymaker (MM) and *Solanum pennellii* (*Sp*) LA2963 (Ballvora, Pierre*, et al.*, 2001; Ballvora, Schornack*, et al.*, 2001; Schornack *et al.*, 2004). Bs4 causes cell death not only after delivery of AvrBs4 and its derivatives (Schornack *et al.*, 2004) or other TALEs, including AvrBs3, homologs of AvrBs3 in *Xanthomonas* No. 3 (Hax3) and 4 (Hax4; Kay *et al.*, 2005; Schornack *et al.*, 2005), but also after delivery of designer TALEs (dTALEs), *i.e.* TALEs customised to activate transcription of a specific gene-of-interest (GOI; Figure 4). In addition, Bs4 uses canonical ETI pathways; VIGS experiments in *N. benthamiana* demonstrated that Bs4-mediated cell death is EDS1- and SGT1-dependent (Schornack *et al.*, 2004).

The fourth reason to use tomato for the elucidation of executor-mediated pathways is that tomato is a natural host of *X. euvesicatoria* (Klein-Gordon *et al.*, 2020; Timilsina *et al.*, 2016; Timilsina *et al.*, 2015) and thus, establishment of the *S. lycopersicum* – *X. euvesicatoria*

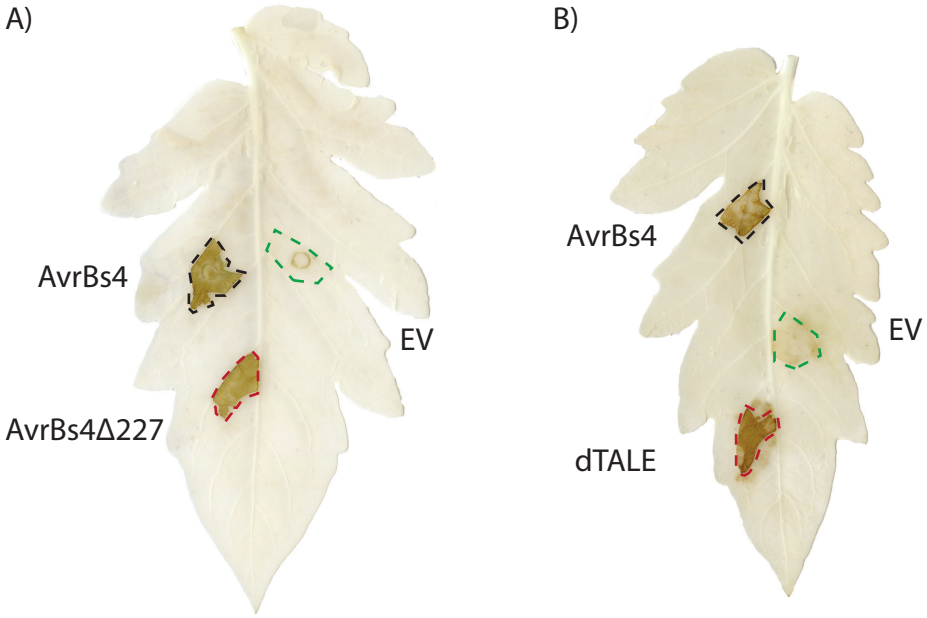

Figure 4. Bs4 mediates recognition of TALEs, their truncated derivatives, and dTALEs. A) Inocula of *Xe* 85-10 *avrBs4* (left top), *Xe* 85-10 *avrBs4Δ227* (left bottom), and *Xe* 85-10 empty vector (EV; right) with the optical density at 600 nm (OD_{600}) equal to 0.4 were infiltrated into MM tomato leaflets. AvrBs4Δ227 is the AvrBs4 derivative lacking central repeats 5,5 – 17,5, NLS, and AAD. B) *Xe* 85-10 *avrBs4* (left top), *Xe* 85-10 *dTALE* (left bottom), and *Xe* 85-10 EV (right) were infiltrated into MM tomato leaflets (OD_{600} = 0.4). *Xe* 85-10 EV served as a negative control. Phenotypes were observed 2 days post-infiltration (dpi). Leaflets were destained in 80% ethanol (EtOH) to visualise cell death. Dashed lines mark the infiltrated area.

pathosystem makes it possible to quantify executor-mediated resistance. Therefore, stable tomato transformation with the executor *R* genes should result in a set of transgenic lines that can be used for comparison of TNL- and executor-mediated immunity pathways and cell death signalling via reverse genetics. Identified putative pathway components should also be knocked out in the stable lines to test their impact of a knock out on NLR- and executor-mediated cell death and immunity.

1.2.2 An EIP and dTALE enable pathogen-free and pathogen-dependent transcriptional activation of the executor *R* genes.

Development of the executor *R* gene expressing stable tomato lines would require establishment of two methods for executor transgene activation. The first method, *i.e.* transgene activation with a chemical inducer, will enable the study of executor-mediated cell death in the absence of the *X. euvesicatoria* pathogen, thereby eliminating any pathogen-dependent virulence effect. Inducible promoters allow expression of detrimental or even lethal transgenes in a controllable fashion (Zuo *et al.*, 2000). The essential requirements for a reliable inducible system are 1) high inducibility of the transgene; 2) specificity of a transgene activation; 3) tight control over the transgene and response only to the specific inducer; and 4) absence of undesirable physiological effects on the host (Borghi, 2010; Zuo *et al.*, 2000).

The estradiol-inducible promoter (EIP) meets all afore mentioned criteria (Kubo *et al.*, 2013; Zuo *et al.*, 2000) and consists of the three modules (Figure 5; Kubo *et al.*, 2013; Zuo *et al.*, 2000). The first unit is a strong constitutive promoter G10-90 and pea *Ribulose-1,5-bisphosphate carboxylase/ oxygenase Subunit E9* terminator (*RbcS E9-t*) that control expression of the *XVE* fusion gene (Figure 5; Ishige *et al.*, 1999; Zuo *et al.*, 2000). XVE is a

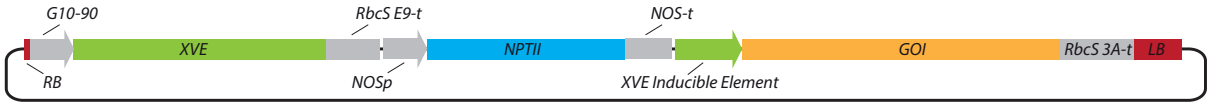

Figure 5. The estradiol-inducible promoter (EIP) provides a controlled expression of genes with detrimental or even lethal effects, such as executor *R* genes. The EIP consists of the three modules. The constitutive promoter G10-90 and pea *Ribulose-1,5-bisphosphate carboxylase/oxygenase Subunit E9* terminator (*RbcS E9-t*) control expression of the *XVE* fusion gene, which encodes a transcription activator fused to human oestrogen receptor (Ishige *et al.*, 1999; Zuo *et al.*, 2000). In the presence of β-estradiol, the XVE transcription activator binds to the XVE-inducible element to induce the downstream GOI (Zuo *et al.*, 2000). *RbcS 3A* terminator (*RbcS 3A-t*) stops transcription of GOI (Zuo *et al.*, 2000). Finally, the EIP contains *Neomycin Phosphotransferase* II (*NPT*II) used as a selection marker for a stable transformation (Zuo *et al.*, 2000). *Nopaline synthase* promoter (*NOSp*) and terminator (*NOS-t*) from *A. tumefaciens* are regulatory elements for *NTP*II. The figure is adapted from Zuo *et al.*, 2000.

chimeric transcription activator, which is composed of the DNA-binding domain of the bacterial repressor LexA, the AAD of VP16, and the human oestrogen receptor (Figure 5; Zuo *et al.*, 2000). The latter includes a binding site for oestrogen hormone and the transactivation function 2 (TAF2) domain (Zuo *et al.*, 2000). The second unit of the EIP is the LexA operator fused to the cauliflower mosaic virus minimal 35s promoter, *i.e.* XVE-inducible element (Figure 5; Zuo *et al.*, 2000). In the presence of β-estradiol, the XVE chimeric transcription activator binds to the LexA operator and recruits ribonucleic acid (RNA) polymerase II to activate transcription of the downstream GOI (Figure 5; Zuo *et al.*, 2000). *RbcS 3A* terminator (*RbcS 3A-t*) stops transcription of GOI (Zuo *et al.*, 2000). The third unit is *Neomycin Phosphotransferase* II (*NPT*II) used as a selection marker for a stable transformation (Zuo *et al.*, 2000). *Nopaline synthase* promoter (*NOSp*) and terminator (*NOS-t*) from *A. tumefaciens* are regulatory

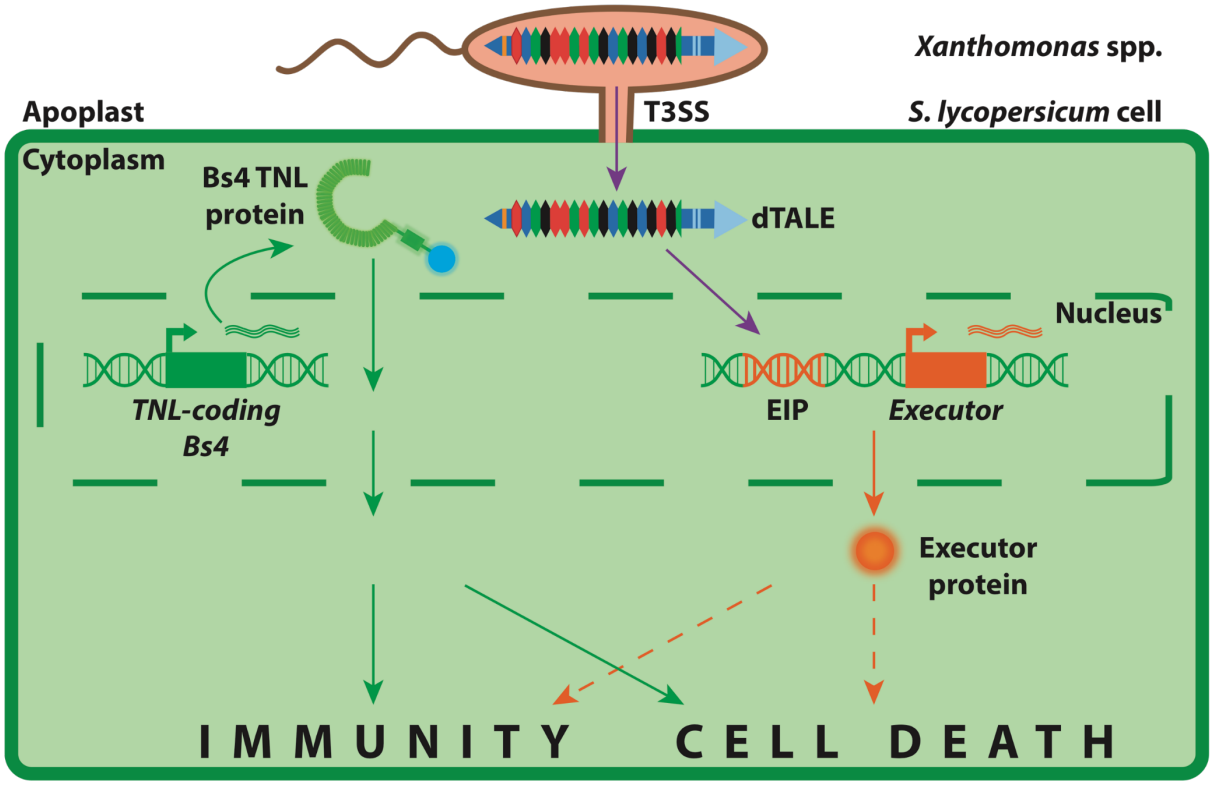

Figure 6. dTALE-mediated transcriptional activation of the executor transgene in the *Bs4* background leads to cross-activation of Bs4-mediated and executor-mediated cell death and immunity pathways. The dTALE is injected via the TTSS into the cytoplasm, where it is sensed by the cytoplasmically-localised Bs4. Bs4 will mediate signalling via known cell death and immunity pathways (straight green arrows) upon recognition of the dTALE. However, in some cells the dTALE might be translocated into the nucleus to transcriptionally activate the executor transgene. Such dTALE-triggered transcriptional activation will result in the production of a functional executor protein as well as executor-mediated cell death and immunity via unknown pathways (orange arrows with gaps). Such cross-activation of Bs4 and the executor transgene might complicate the deciphering and comparison of pathways exploited by two proteins.

elements for *NTP*II. This unit separates XVE fusion gene and XVE-inducible element units (Figure 5; Zuo *et al.*, 2000).

The second method for the executor transgene induction requires the development of a TALE-like structure, capable of binding to a user-defined sequence in the XVE inducible element unit, *i.e.* a dTALE (Figure 6; de Lange *et al.*, 2017; Morbitzer *et al.*, 2011). dTALE-dependent transcriptional activation of a transgene will help to study executor-mediated resistance to the *Xe* pathogen (Kim and Hartmann, 1985; Tian *et al.*, 2014; Chunlian Wang *et al.*, 2015; J. Wang *et al.*, 2018). Since Bs4 is capable of causing cell death upon delivery of TALEs and TALE-like structures (Figure 4; Schornack *et al.*, 2004; Schornack *et al.*, 2005), the dTALE will act as an activator of Bs4 and the executor transgenes (Figures 4 and 6; Bultmann *et al.*, 2012; de Lange *et al.*, 2017). Therefore, the *Bs4* null allele background should be used for integration of the executor transgenes to avoid dTALE-caused cross-activation of Bs4 and the executor transgene (Figure 6; Ballvora, Pierre*, et al.*, 2001; Ballvora, Schornack*, et al.*, 2001; Schornack *et al.*, 2004).

1.2.3 CRISPR/Cas9 system is a fast and reliable tool for gene knockout.

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system has revolutionised the field of genome editing (Doudna and Charpentier, 2014; Jinek *et al.*, 2012; D. Zhang *et al.*, 2021). This system is a fast, simple, and efficient method for DNA alteration and gene functional studies (Khadempar *et al.*, 2019). Gene knockout and knockdown are the main applications of CRISPR/Cas9-mediated genome editing in numerous plant species (Jacobs *et al.*, 2015; T. Li *et al.*, 2018; H.-J. Liu *et al.*, 2020; Peng *et al.*, 2017; R. Wu *et al.*, 2018; Z. Zhang *et al.*, 2019; Zsögön *et al.*, 2018).

The CRISPR/Cas9 system includes a nuclear-localised Cas9 protein with two nuclease domains and a single guide RNA (sgRNA; Jinek *et al.*, 2012). This sgRNA is a synthetic RNA molecule consisting of a 20 nucleotide-long targeting site and a hairpin structure that interacts with Cas9 (Jinek *et al.*, 2012). The Cas9/sgRNA complex scans genome for DNA sequences that complement the sgRNA target site (Jinek *et al.*, 2012). Upon successful identification of such sequences, the Cas9/sgRNA complex introduces double-strand break (DSB) to the DNA (Jinek *et al.*, 2012). The DSB is primarily followed by an imprecise reparation process known as non-homologous end joining (NHEJ; Mladenov and Iliakis, 2011). NHEJ can lead to insertion or

deletion (indel) mutations at the breakage site, which usually result in frameshifts (Mladenov and Iliakis, 2011). Therefore, the CRISPR/Cas9 system is a simple method to knock out individual genes to study their impact on TNL- and executor-mediated cell death signalling and immunity pathways.

1.3 Aims of this work.

This thesis is a compilation of two projects. The first project aimed to develop a set of tools for further comparison of TNL- and executor-mediated cell death and immunity pathways, since the events that follow transcriptional activation of the executor *R* genes and lead to cell death remain elusive (Krönauer, 2020; Krönauer *et al.*, 2019). The main focus was kept on TNL protein from *Solanum* spp. mediating recognition of TALEs and dTALEs, *i.e.* Bs4 (Figure 4; Schornack *et al.*, 2004), two executor proteins from *Capsicum* spp., *i.e.* Bs3 and Bs4C (Krönauer *et al.*, 2019; Römer *et al.*, 2007; Strauß *et al.*, 2012), as well as two executor proteins from *Oryza* spp., *i.e.* Xa10 and Xa23 (Tian *et al.*, 2014; Chunlian Wang *et al.*, 2015).

Due to the complexity of this project it was divided into numerous task blocks. The first block of tasks focused on screening *Solanum* germplasm for a *Bs4* null allele and generation of such a mutation via CRISPR/Cas9-mediated *Bs4* mutagenesis. In the second stage, a stable transformation of tomato with the executor *R* genes under the control of the EIP (Figure 5) and subsequent characterisation of stable transgenic lines exhibiting cell death upon treatment with the chemical inducer, *i.e.* liquid estradiol, had to be performed. In the third stage of the project, a combination of the *Bs4* null allele and executor transgene by crossing, followed by the selection of homozygous lines containing both traits, was planned. The forth task was to apply the dTALE technology for transcriptional activation of executor transgenes under the control of the EIP and to quantify putative executor-mediated resistance to *X. euvesicatoria*. In the later stages, it was planned to use the developed lines for knockouts of ETI master regulators, such as *EDS1* and *SGT1*, via CRISPR/Cas9-mediated mutagenesis and to test impact of these knockouts on Bs4- and executor-mediated cell death and immunity.

The second project aimed to identify tomato genes, which are upregulated by TALEs from tomato pathogenic *Xanthomonas* spp. It was planned to initially test if the *Bs4* null allele background is beneficial for the development of TALE-induced disease symptoms. Additionally, it was planned to use a set of bioinformatic tools to predict putative EBEs in

promoter regions of the annotated tomato genes (Grau *et al.*, 2013; Pérez-Quintero *et al.*, 2013). Upon the selection process, the interaction of AvrBs3-like proteins with the predicted EBEs was planned to be tested *in planta* (Mücke *et al.*, 2019; Römer *et al.*, 2010; D. Wu *et al.*, 2019). Finally, it was intended to check if AvrBs4, AvrBs3, and AvrHah1 upregulate the predicted target genes in tomato via quantitative real-time (qRT)-PCR (Mücke *et al.*, 2019; D. Wu *et al.*, 2019).

Identification of TALE-induced genes makes it possible to correlate disease progression with the expression levels of these target genes. Since AvrBs4-induced host genes with a putative susceptibility effect have not yet been reported (Strauß *et al.*, 2012), they are of an immense interest. Putative AvrBs4 targeted genes might unravel yet unknown disease scenarios. Additionally, it would be desirable to identify putative targets of AvrBs3 in tomato and to check if the same genes are also upregulated by AvrHah1 (Schwartz *et al.*, 2017).

2 RESULTS

2.1 *CC-Bs4*, a *Bs4* null mutant, enables the comparison of NLR- and executor-mediated pathways.

S. lycopersicum is a suitable species for comparison of NLR- and executor-mediated immunity pathways (Introduction, chapter 1.2.1). This chapter describes the first steps in the establishment of the *S. lycopersicum – X. euvesicatoria* pathosystem for comparison of NLR and executor-mediated cell death and immunity pathways, *i.e.* the screen of *Solanum* germplasm for *Bs4* null allele and CRISPR/Cas9-mediated *Bs4* mutagenesis.

2.1.1 *SpBs4* is a reduced function orthologue of *SlBs4*.

Previously Bs4 was identified as a mediator of AvrBs4 recognition via genetic mapping approach based on differential reactions between the two parental lines, *S. lycopersicum* cv. MM and *S. pennellii* LA2963 (Schornack *et al.*, 2004). The differences between the two plant species are well observed 2 dpi, when *X. euvesicatoria*-derived AvrBs4 and its derivative lacking central repeats 5,5 – 17,5, NLS, and AAD, *i.e.* AvrBs4Δ227, cause a strong cell death reaction in *S. lycopersicum* cv. MM, but not in *S. pennellii* LA2963 line (Figure 7A). Such a contrasting reaction led to hypothesis that *SpBs4*, a *Bs4* allele from *S. pennellii* LA2963 line, is a null mutant. However, LA2963 line exhibits a delayed cell death reaction to AvrBs4 and AvrBs4Δ227 6 dpi (Figure 7B), suggesting that *SpBs4* may be a reduced-function orthologue of *SlBs4*.

In order to clarify if SpBs4 has a reduced function or is a null allele, an experiment was initiated with a focus to study mediation of AvrBs4 recognition by genomic versions of *SlBs4* (*SlBs4*^{gDNA}) and *SpBs4* (*SpBs4*^{gDNA}) in *N. benthamiana* leaves. 35s promoter-driven *SlBs4*^{gDNA} and *SpBs4*^{gDNA} were co-expressed with 35s promoter-driven *avrBs4* or *Green fluorescent protein* (GFP) via *Agrobacterium*-mediated transfer DNA (T-DNA) delivery system. Combinations of *SlBs4*^{gDNA}/*avrBs4* and *SpBs4*^{gDNA}/*avrBs4* resulted in a cell death phenotype (Figure 8A). While combinations of *SlBs4*^{gDNA}/*GFP, SpBs4*^{gDNA}/*GFP*, and *avrBs4*/*GFP*, *i.e.* the negative controls, did not cause the cell death phenotype (Figure 8A). Thus, AvrBs4 does not trigger cell death on its own, but only when co-expressed with *SlBs4*^{gDNA} or *SpBs4*^{gDNA}. These results demonstrate that SpBs4 mediates recognition of AvrBs4.

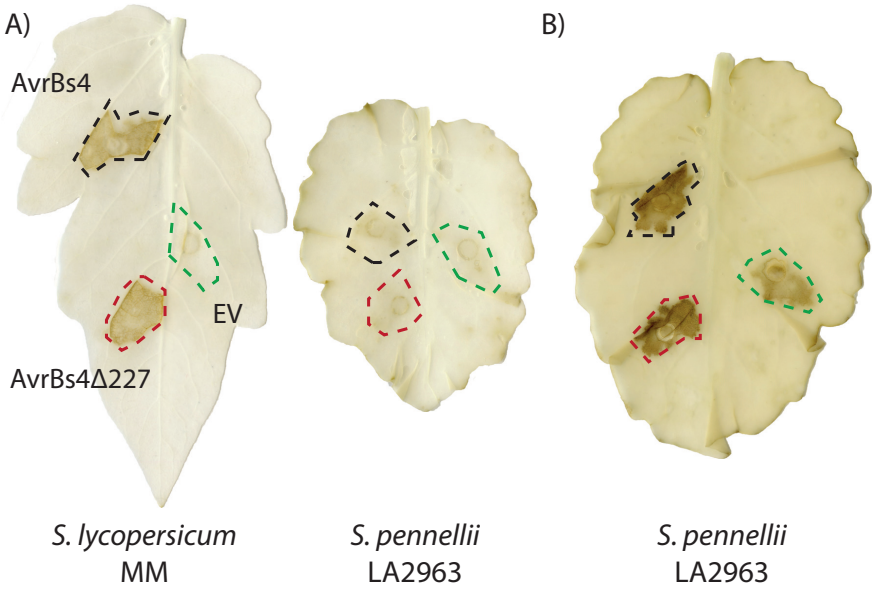

Figure 7. TALEs and truncTALEs cause cell death in *SlBs4* and *SpBs4* backgrounds. *Xe* 85-10 *avrBs4* (left top; black), *Xe* 85-10 *avrBs4Δ227* (left bottom; red), and *Xe* 85-10 EV (right; green) were infiltrated into *S. lycopersicum* cv. MM and *S. pennellii* LA2963 (OD_{600} = 0.4). AvrBs4Δ227 is the AvrBs4 derivative lacking central repeats 5,5 – 17,5, NLS, and AAD. *Xe* 85-10 EV served as a negative control. Phenotypes were observed A) 2 dpi and B) 6 dpi. All leaflets were destained in 80% EtOH to visualise cell death. Dashed lines mark the infiltrated area.

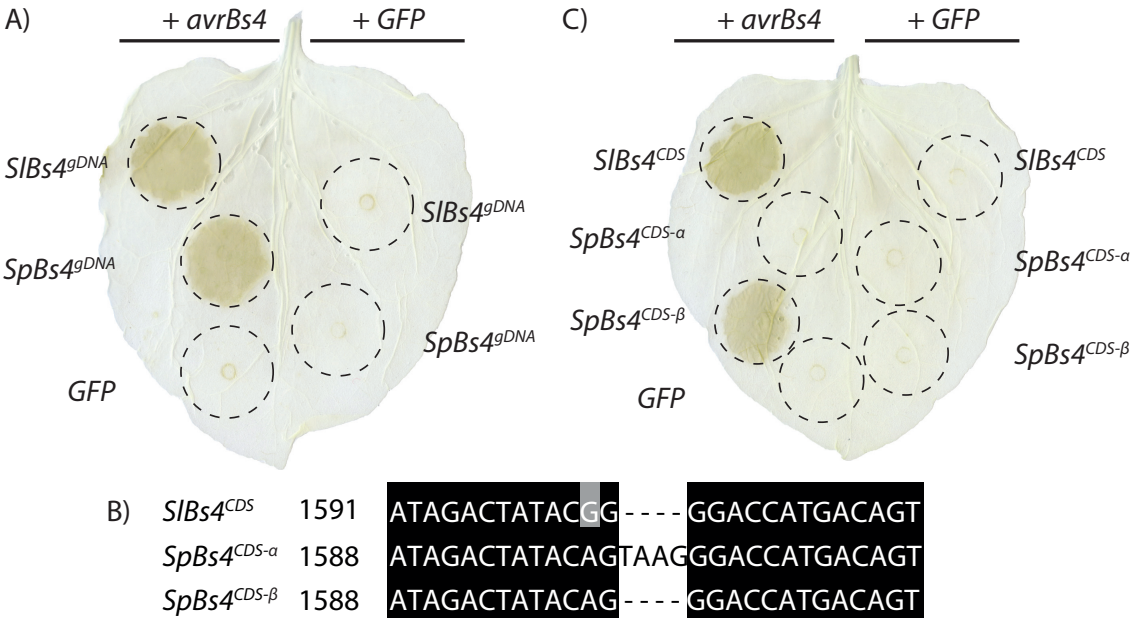

Figure 8. *SpBs4* is a functional orthologue of *SlBs4*. A) 35s-driven N-terminus-GFP-labelled genomic versions of *SlBs4* (*SlBs4*^{gDNA}) and *SpBs4* (*SpBs4*^{gDNA}) were co-infiltrated with 35s-driven *avrBs4* (left side) or *GFP* (right side) in *N. benthamiana* leaves (OD_{600} = 0.8). Co-infiltrations with 35s-driven *GFP* were used as negative controls. Phenotypes were observed 2 dpi. All leaves were destained in 80% EtOH. Dashed lines mark the infiltrated area. B) Major sequence polymorphisms within coding sequence (CDS) versions of *SlBs4* (*SlBs4*^{CDS}), *SpBs4*$^{\alpha}$ (*SpBs4*$^{CDS\text{-}\alpha}$), and *SpBs4*$^{\beta}$ (*SpBs4*$^{CDS\text{-}\beta}$). C) 35s-driven N-terminus-GFP-labelled CDS versions *SlBs4*^{CDS}, *SpBs4*$^{CDS\text{-}\alpha}$, and *SpBs4*$^{CDS\text{-}\beta}$ were co-infiltrated with 35s-driven *avrBs4* (left side) or *GFP* (right side) in *N. benthamiana* (OD_{600} = 0.8). Co-infiltrations with 35s-driven *GFP* were used as negative controls. Phenotypes were observed 2 dpi. All leaves were destained in 80% EtOH. Dashed lines mark the infiltrated area.

SpBs4 was previously found to be transcribed into different splice variants (Schornack *et al.*, 2004). Two splice variants, namely $SpBs4^{CDS\text{-}\alpha}$ and $SpBs4^{CDS\text{-}\beta}$ (Figure 8B), were re-created for a study of their functional relevance. When compared to $SlBs4^{CDS}$, $SpBs4^{CDS\text{-}\alpha}$ contains the SNP mutation G1873A and includes four consequent nucleotides, *i.e.* GTAA (1874-1877), which are annotated as a part of the second intron of $SpBs4^{gDNA}$, therefore, these additional nucleotides create a frame-shift (Figure 8B). On the other hand, $SpBs4^{CDS\text{-}\beta}$ contains only G1873A mutation (Figure 8B). 35s promoter-driven $SpBs4^{CDS\text{-}\alpha}$, $SpBs4^{CDS\text{-}\beta}$, and $SlBs4^{CDS}$, *i.e.* a positive control, were co-expressed with 35s promoter-driven *avrBs4* or *GFP* via *Agrobacterium*-mediated T-DNA delivery in *N. benthamiana* leaves. The combination $SpBs4^{CDS\text{-}\alpha}$/*avrBs4* did not cause the cell death reaction, while the combinations $SpBs4^{CDS\text{-}\beta}$/*avrBs4* and $SlBs4^{CDS}$/*avrBs4*, *i.e.* the positive control, resulted in the cell death phenotype (Figure 8C). None of the afore mentioned *SpBs4* splice variants caused cell death upon co-expression with *GFP* (Figure 8C). These results indicate that $SpBs4^{CDS\text{-}\beta}$ is able to produce a protein capable of AvrBs4 recognition, while $SpBs4^{CDS\text{-}\alpha}$ does not. Therefore, *SpBs4* is a reduced-function allele, not a null allele.

As *SlBs4* and *SpBs4* are functional alleles, screening of a collection of *S. lycopersicum* accessions for the presence of a *SlBs4* null allele was carried out (Peter, 2002). Phenotyping with *Xe* 85-10 strains expressing *avrBs4* and its truncated version *avrBs4*Δ*227* resulted in a cell death reaction in all tested cultivars (Figure 9). Leaflet parts treated with *Xe* 85-10 containing an empty vector (EV), a negative control, had no cell death reaction. This observation shows that all tested *S. lycopersicum* accessions contain functional *Bs4* alleles and, therefore, *Bs4* alleles are broadly conserved across *S. lycopersicum* accessions.

2.1.2 CRISPR/Cas9-engineered *Bs4* is a null mutant.

Study of executor-mediated cell death and resistance to *X. euvesicatoria* in tomato should be made in *Bs4* null allele background to avoid cross-activation of the executor transgenes and Bs4 by *dTALE*-expressing *X. euvesicatoria* (Figure 6; Ballvora, Pierre*, et al.*, 2001; Ballvora, Schornack*, et al.*, 2001; Schornack *et al.*, 2004). Due to the absence of a naturally occurring *Bs4* null allele in the screened *S. lycopersicum* germplasm, a null mutation in *S. lycopersicum* cv. MM background using CRISPR/Cas9 was engineered and designated as *CC-Bs4* (CRISPR/Cas9-engineered *Bs4*). It is common to see a failure of individual sgRNAs to produce

even small indel mutations (Yuen *et al.*, 2017), that is why multiple sgRNAs are generally used to secure the chances of obtaining null mutants (Jacobs *et al.*, 2015; Peng *et al.*, 2017; H. Zhang *et al.*, 2014; Z. Zhang *et al.*, 2019). Therefore, multiple sgRNAs, each targeting distinct regions of *Bs4* genomic sequence, namely TIR-domain and NB-LRR-domain encoding sequences, were used. Such a strategy maximised the chances of generating large sequence deletions between sgRNA target sites or small indels causing frameshifts.

CC-TOP software (Stemmer *et al.*, 2015) was used to design sgRNAs for CRISPR/Cas9-mediated mutagenesis of *Bs4* and to predict their specificity and efficiency *in silico*. sgRNA1 and sgRNA2 were designed to target *Bs4* sequence encoding TIR-domain (Figure 10A), while sgRNA3, sgRNA4, and sgRNA5 were designed to target *Bs4* sequence encoding NB- and LRR-domains (Figure 10A). Selected sgRNAs were assembled into two separate constructs (Figure 10B). sgRNA1 and sgRNA2 were combined in the first construct (Figure 10B), while the remaining sgRNA3, sgRNA4, and sgRNA5 were combined in the second construct (Figure 10B). These constructs were used for two stable *S. lycopersicum* cv. MM transformations.

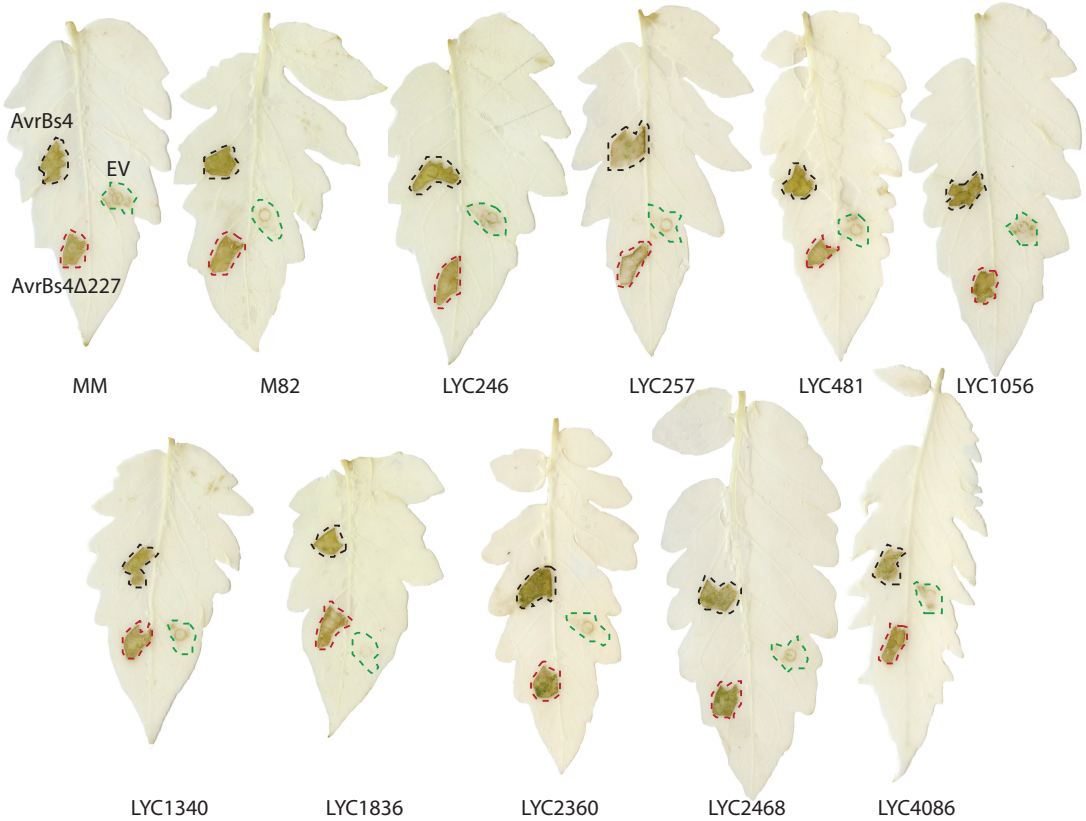

Figure 9. Functional Bs4 is abundant within *Solanum* species. Phenotyping of *S. lycopersicum* accessions from different geographic origins with *Xe* 85-10 *avrBs4* (left top; black), *Xe* 85-10 *avrBs4Δ227* (left bottom; red), and *Xe* 85-10 EV (right; green). AvrBs4Δ227 is the AvrBs4 derivative lacking central repeats 5,5 – 17,5, NLS, and AAD. *Xe* 85-10 EV served as a negative control. Phenotypes were observed 2 dpi. All leaflets were destained in 80% EtOH. Dashed lines mark the infiltrated area.

44 T0 plants representing 22 calli from the first transformation (sgRNA1 and sgRNA2) and 48 T0 plants representing 34 calli from the second transformation (sgRNA3, sgRNA4, and sgRNA5) survived stable transformation, *in vitro* propagation, and adaptation to greenhouse conditions. Putative mutants from T0 generation were genotyped for mutations in *Bs4* TIR-domain or NB-LRR-domain encoding sequences (Figure 11A). Polymerase chain reaction (PCR)-amplified genomic fragments were purified, cloned, and sequenced to reveal putative mutations introduced by sgRNAs.

Analysis of sequenced amplicons revealed the T0 plant "C" from the first transformation containing bi-allelic heterozygous mutations in the *Bs4* TIR-domain encoding sequence (Figures 11A and 11B). The *CC-Bs4* allele from the T0 plant "C", designated as C18, had a 419 basepair (bp)-long deletion spanning the *Bs4* minimal promoter, 5' untranslated region (UTR), and TIR-domain encoding sequence (Figure 11B). Since this allele lacks its N-terminus, it is unlikely to be functional. Another *CC-Bs4* allele from the T0 plant "C", designated as C39, had a 1 bp deletion (T48) within TIR-domain encoding sequence that was predicted to cause a frameshift and, therefore, a truncated protein (Figure 11B). Since these mutations are located within the target site of sgRNA1, it can be concluded that they were introduced by sgRNA1, while sgRNA2 failed to induce mutations (Figure 11B).

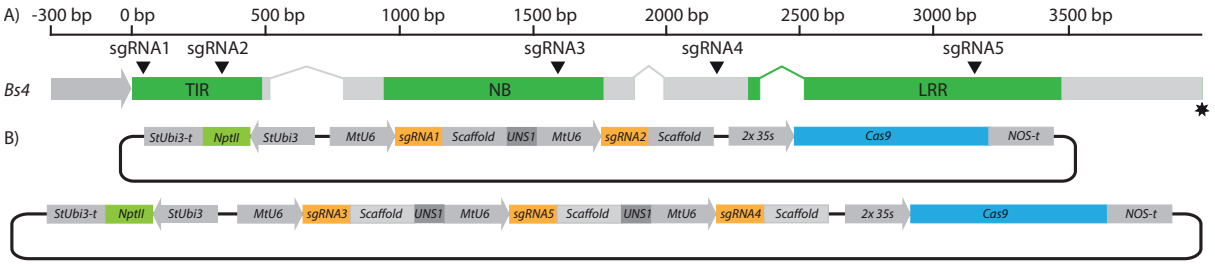

Figure 10. Five sgRNAs are used for CRISPR/Cas9-mediated *Bs4* mutagenesis. A) *Bs4* gene model and sgRNA target sites. Grey arrow represents *Bs4* minimal promoter (Schornack *et al.*, 2005). Green blocks represent sequence regions encoding TIR-, NB-, and LRR-domains. Angled lines represent introns. Black star represents the location of the annotated stop codon. sgRNA1 and sgRNA2 targeted TIR-domain encoding sequence, while sgRNA3, sgRNA4, and sgRNA5 targeted NB-, and LRR-domain-encoding sequence of Bs4. B) Assembled constructs for *Bs4* knockout. sgRNAs and scaffolds (orange and grey blocks) are driven by *Medicago truncatula U6* (*MtU6*) promoters (grey arrows). *Unique nucleotide sequence 1* (*UNS1*; dark grey blocks) is used as a spacer between sgRNAs (Jacobs *et al.*, 2015). *Cas9* (blue blocks) is driven by double 35s promoter from the cauliflower mosaic virus (grey arrows). *NOS-t* from *A. tumefaciens* (grey blocks) stops *Cas9* transcription. *NptII* from *Escherichia coli* (*E. coli*; light green blocks) is *in planta* selection marker. *NptII* is driven by *Solanum tuberosum Ubiquitin 3* (*StUbi3*) promoter (grey arrows). *StUbi3* terminator (*StUbi3-t*; grey blocks) stops *NptII* transcription.

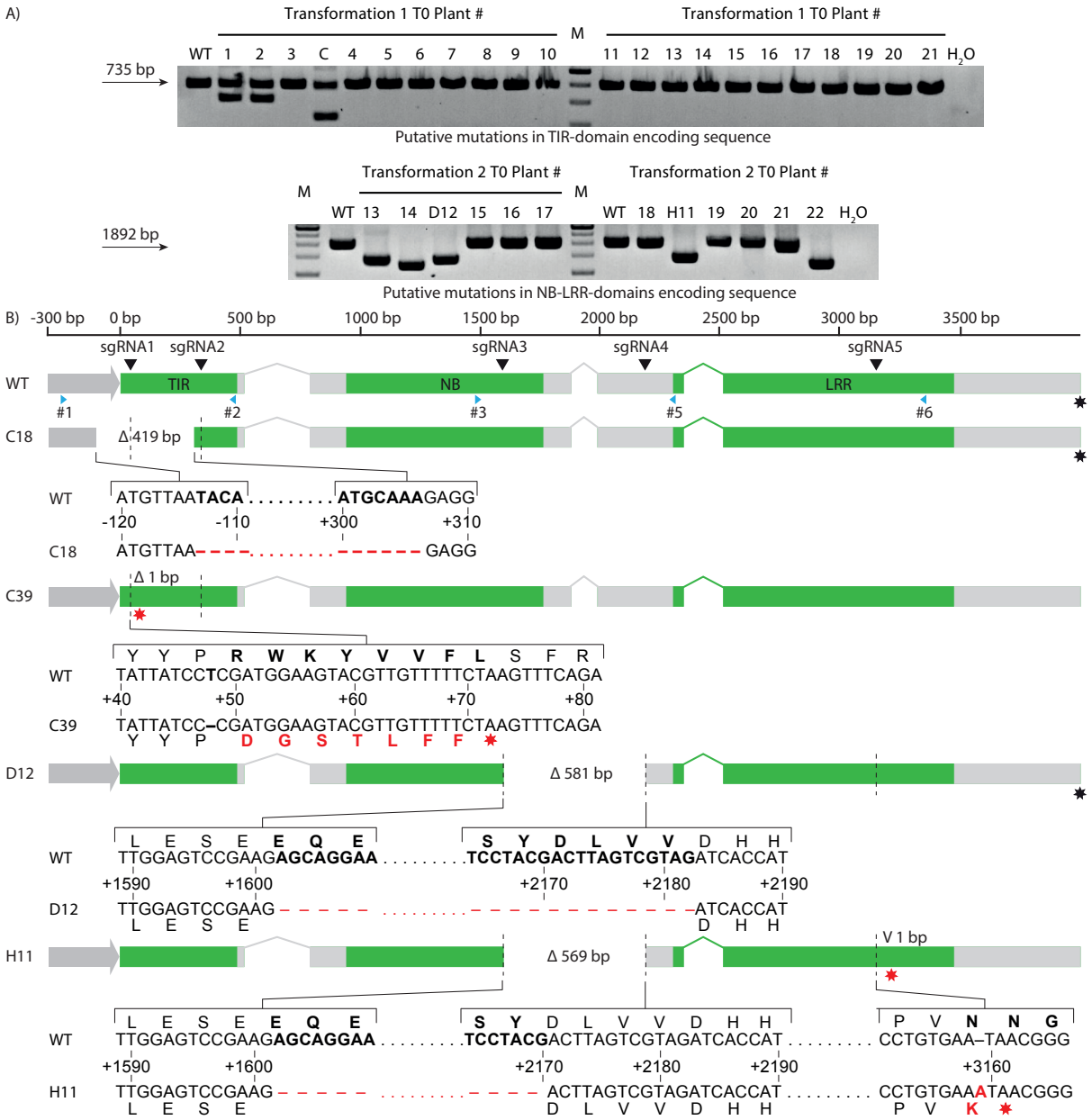

Figure 11. CRISPR/Cas9-mediated *Bs4* mutagenesis yields numerous null alleles (*CC-Bs4*). A) Genotyping of T0 plants for putative mutations in *Bs4* sequence. gDNA from the wild-type (WT) plant and H_2O served as negative and non-template controls, respectively. The T0 Plant "C" from the first transformation contained two mutated alleles, namely C18 (shorter fragment) and C39 (longer fragment), which were separated in the T1 generation. B) *Bs4* gene model and sgRNA target sites. Grey arrows represent the *Bs4* minimal promoter. Green blocks represent sequence regions encoding TIR-, NB-, and LRR-domains. Angled lines represent introns. Black and red stars represent the locations of native and premature stop codons, respectively. sgRNA1 and sgRNA2 targeted TIR-domain encoding sequence, while sgRNA3, sgRNA4, and sgRNA5 targeted NB-, and LRR-domain encoding sequence of *Bs4*. Black triangles and vertical dashed lines indicate sgRNA target sites. Blue triangles represent primers used for genotyping of putative *CC-Bs4* mutations in the T0 generation. WT *Bs4* genomic and protein sequences impacted by mutations are highlighted with bold black font. *CC-Bs4* mutations within genomic and protein sequences are marked with bold red font.

In addition, eight T0 plants from the second transformation were identified to contain large deletions within *Bs4* NB-LRR-domain encoding sequence (Figure 11A). Two of these T0 plants, namely D12 and H11, had bi-allelic homozygous mutations (Figure 11B). Both *CC-Bs4* alleles from the D12 line had identical 581 bp-long in-frame deletions located between the target sites of sgRNA3 and sgRNA4 (Figure 11B). However, no mutations were identified in the *Bs4* sequence targeted by sgRNA5 in this line. In the case of the H11 line, both *CC-Bs4* alleles had identical 569 bp-long in-frame deletions located between the target sites of sgRNA3 and sgRNA4 and one basepair insertions (3159 A 3160) within LRR-domain encoding sequence targeted by sgRNA5 (Figure 11B). These one basepair insertions (3159 A 3160) were predicted to cause a frameshift and, therefore, a truncated protein (Figure 11B).

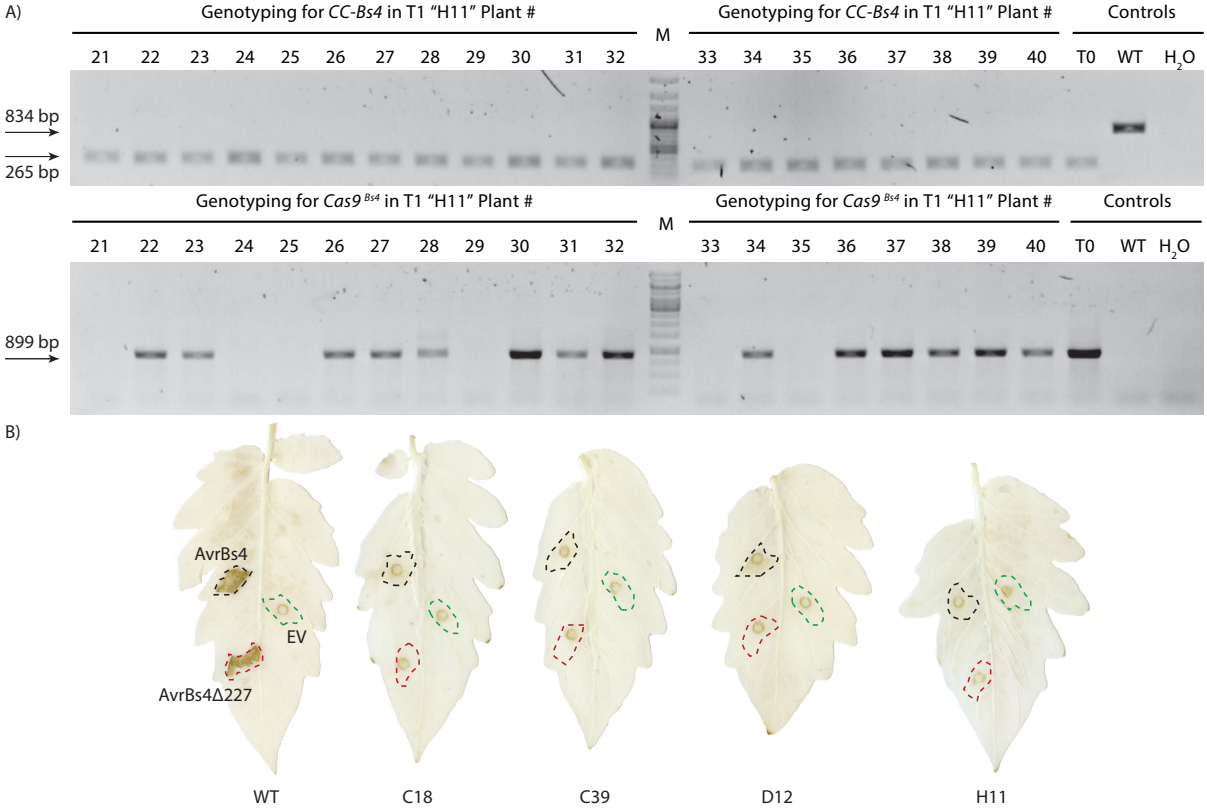

Figure 12. *CC-Bs4* alleles do not mediate AvrBs4 and AvrBs4Δ227 recognition and cell death signalling. A) Example of selected *Cas9*^{Bs4}-free *CC-Bs4* homozygous plants from the T1 generation (H11 segregating population) via genotyping for *CC-Bs4* mutation and *Cas9*^{Bs4} absence. T0 H11 gDNA served as a positive control for *CC-Bs4* and *Cas9*^{Bs4} amplification. gDNA from the WT plant and H_2O served as negative and non-template controls for *CC-Bs4* and *Cas9*^{Bs4} amplification, respectively. B) Phenotyping of WT, *i.e.* a positive control, and *Cas9*^{Bs4}-free *CC-Bs4* homozygous lines C18, C39, D12, and H11 for recognition of *Xe* 85-10 *avrBs4* (left top; black), *Xe* 85-10 *avrBs4Δ227* (left bottom; red), and *Xe* 85-10 EV (right; green). AvrBs4Δ227 is the AvrBs4 derivative lacking central repeats 5,5 – 17,5, NLS, and AAD. *Xe* 85-10 EV served as a negative control. Phenotypes were observed 2 dpi. All leaflets were destained in 80% EtOH. Dashed lines mark the infiltrated area.

To confirm that the identified mutations are in the germline, the plants from the next generation (T1) were analysed for the presence of *CC-Bs4* mutations and the absence of *Cas9^{Bs4}* transgene. In the case of C18, C39, and H11 lines, 20 plants per segregating population were genotyped to identify *Cas9^{Bs4}*-free and *CC-Bs4* homozygous plants (Figure 12A). The *Cas9^{Bs4}* transgene followed 3:1 segregation in each segregating population, which is consistent with the presence of one *Cas9^{Bs4}* transgene copy within the genomes of each T0 plant. However, within the D12 segregating population, the *Cas9^{Bs4}* transgene followed 63:1 segregation, which is consistent with the presence of three *Cas9^{Bs4}* transgene copies within the genome of the T0 D12 plant. 142 T1 plants from the D12 segregating population were genotyped and two *Cas9^{Bs4}*-free *CC-Bs4* homozygous plants were identified. As homozygous *CC-Bs4* were identified in the T1 generation in the absence of *Cas9^{Bs4}* transgene in all four lines (C18, C39, D12, and H11), these *CC-Bs4* mutations were considered to be in the germline and could therefore be propagated to further generations.

The next planned step was to determine whether or not the mutations in the identified *CC-Bs4* alleles would correlate with a loss of AvrBs4-dependent cell death in these plants. Thus, selected *Cas9^{Bs4}*-free *CC-Bs4* homozygous plants were phenotyped with *Xe* 85-10 strains expressing *avrBs4*, its truncated version *avrBs4Δ227*, and containing the EV, *i.e.* the negative control. All four mutant lines, namely C18, C39, D12, and H11, did not have cell death following delivery of AvrBs4, AvrBs4Δ227, and EV (Figure 12B). However, in the positive control, *i.e.* WT, Bs4 conferred recognition of AvrBs4 and AvrBs4Δ227, but not the EV (Figure 12B). These phenotyping results demonstrate that *CC-Bs4* alleles from the C18, C39, D12, and H11 lines are indeed loss-of-function null alleles.

In order to check if the loss of AvrBs4 and AvrBs4Δ227 recognition in these lines is due to the mutations in *Bs4*, but not in other putative off-target genes, one of the mutant lines was backcrossed to WT. Correlation between the loss-of-function phenotype and the CRISPR/Cas9-induced mutation in *Bs4* was analysed in the second backcross (BC2) generation. Since the H11 line contains the *CC-Bs4* allele with the largest (569 bp-long) in-frame sequence deletion and one bp insertion (3159 A 3160) leading to the frameshift (Figure 11B), it was selected to be backcrossed to WT (Figure 13A). 40 BC2 plants segregating for *Bs4* and *CC-Bs4* were genotyped (Figure 14B). 12 homozygous *Bs4* plants, 20 heterozygous *Bs4*/*CC-Bs4* plants, and eight homozygous *CC-Bs4* plants were identified within this BC2 population. The

genotypic data suggest that *CC-Bs4* follows 3:1 segregation (χ^2 = 0.5348; p-value = 0.4652). In addition, all 40 BC2 plants and the parental lines (WT and H11) were phenotyped with *Xe* 85-10 strains containing *avrBs4*, *avrBs4Δ227*, and the EV control (Figure 13C). All 32 plants

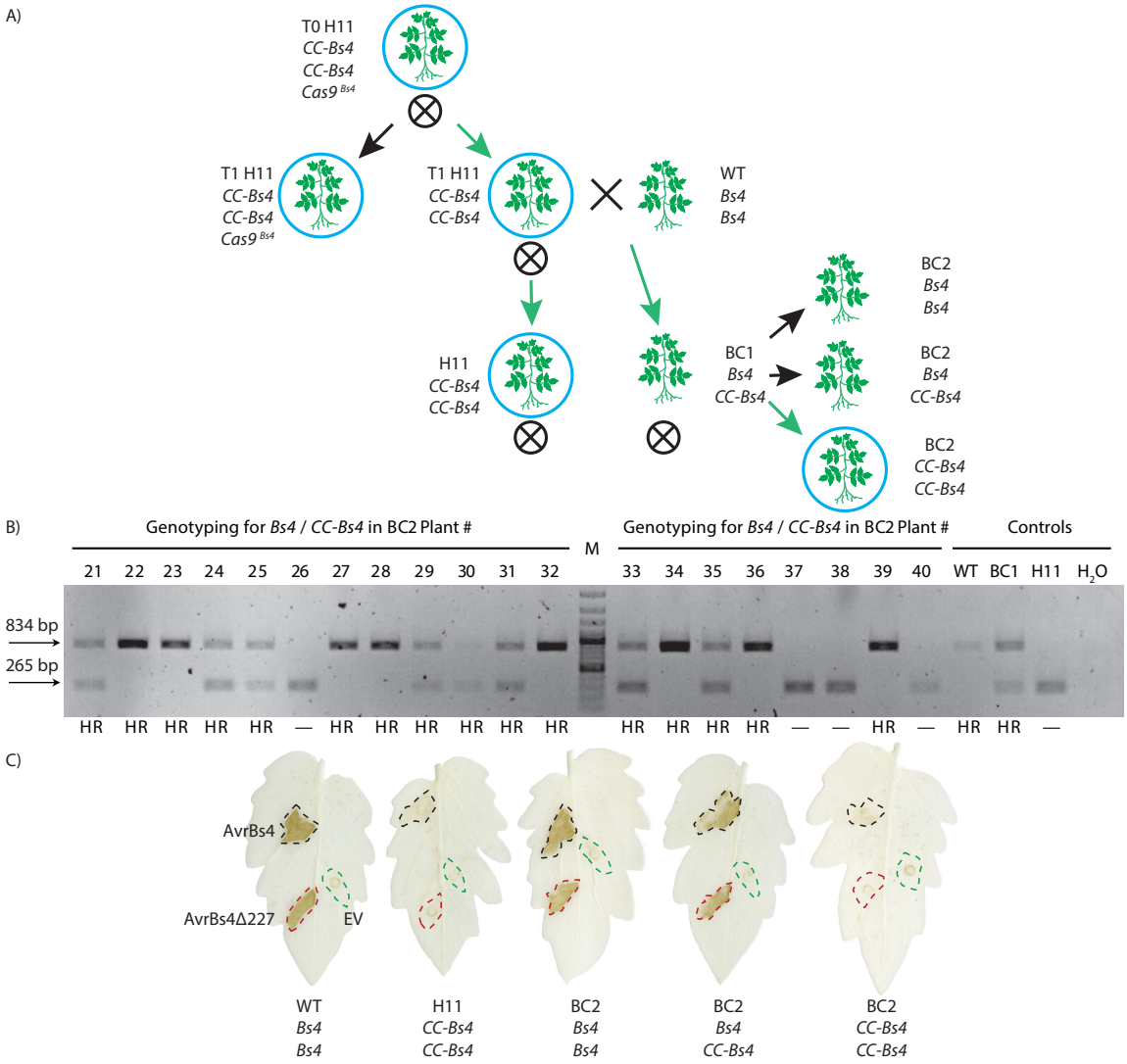

Figure 13. The loss of the cell death reaction to AvrBs4 and AvrBs4Δ227 in BC2 generation correlates with the homozygosity of the *CC-Bs4* allele. A) Schema of BC2 *CC-Bs4* line development. A *Cas9*^{Bs4}-free homozygous H11 plant from the T1 generation was backcrossed to a WT plant. Green arrows emphasise the genotypes selected for selfing and seed multiplication. × and ⊗ symbols represent crossing and selfing, respectively. Blue ring indicates that a plant is homozygous for *CC-Bs4*. B) Genotyping of BC2 population segregating for *Bs4* and *CC-Bs4*. WT and H11 gDNA served as positive controls of *Bs4* and *CC-Bs4* alleles amplification in homozygous plants, respectively. gDNA from heterozygous BC1 plant served as a positive control of *Bs4* and *CC-Bs4* alleles amplification in heterozygous plants. H_2O served as a non-template control. Presence of absence of HR reactions in each BC2 plant is indicated below the picture (HR, —). C) Phenotyping of WT, *i.e.* a positive control, H11, *i.e.* a negative control, and BC2 population for recognition of *Xe* 85-10 *avrBs4* (left top; black), *Xe* 85-10 *avrBs4Δ227* (left bottom; red), and *Xe* 85-10 EV (right; green). AvrBs4Δ227 is the AvrBs4 derivative lacking central repeats 5,5 – 17,5, NLS, and AAD. *Xe* 85-10 EV served as a negative control. Phenotypes were observed 2 dpi. All leaflets were destained in 80% EtOH. Dashed lines mark the infiltrated area.

that were homozygous or heterozygous for *Bs4* had a cell death phenotype after infiltration with *Xe* 85-10 strains containing *avrBs4* and *avrBs4*Δ*227*, but not the EV control. However, homozygous *CC-Bs4* plants in BC2 generation did not have a cell death reaction upon infiltration of *Xe* 85-10 strains containing *avrBs4*, *avrBs4*Δ*227*, or the EV control. The positive control, parental WT line, also showed cell death phenotype upon delivery of AvrBs4 and AvrBs4Δ227, but not the EV. The negative control, *i.e.* the parental H11 line, had no cell death reaction to any of the three infiltrated strains. The phenotypic data demonstrates that the absence of a cell death reaction to *Xe* 85-10 *avrBs4* or *Xe* 85-10 *avrBs4*Δ*227* also follows 3:1 segregation (χ^2 = 0.5348; p-value = 0.4652). Since the loss of AvrBs4- and AvrBs4Δ227-dependent cell death correlates with the homozygous *CC-Bs4* allele, it is conclusive that the loss of AvrBs4- and AvrBs4Δ227-dependent cell death is caused only by the mutation in *Bs4*.

An additional experiment was conducted to determine if *CC-Bs4* is a null or a reduced-function allele. 35s promoter-driven CDS version of the *CC-Bs4* allele from the H11 line, designated as *CC-Bs4*^{CDS}, was co-expressed with 35s promoter-driven *avrBs4* or *GFP* via *Agrobacterium*-mediated T-DNA delivery in *N. benthamiana* leaves (Figure 14). *SlBs4*^{CDS} was used as a positive control for mediation of AvrBs4 recognition and cell death (Figure 14). Co-infiltration of *CC-Bs4*^{CDS} and *avrBs4* did not cause a cell death phenotype, while co-infiltration of *SlBs4*^{CDS} and *avrBs4*, serving as a positive control, did cause a cell death reaction (Figure 14). The negative controls, *i.e.* combinations *SlBs4*^{CDS}/*GFP*, *CC-Bs4*^{CDS}/*GFP*, and *avrBs4*/*GFP* did not lead to a cell death reaction. These results indicate that *CC-Bs4* allele from the H11 line is a true null allele. Thus, *CC-Bs4* background could be used for integration of executor transgenes for further study of executor-mediated cell death and immunity pathways.

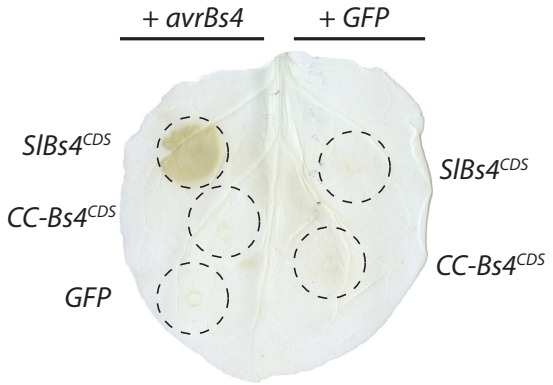

Figure 14. *CC-Bs4* allele from the H11 line does not mediate recognition of AvrBs4 in *N. benthamiana* leaves. 35s-driven N-terminus-GFP-labelled CDS versions of *SlBs4* (*SlBs4*^{CDS}) from WT or *CC-Bs4* (*CC-Bs4*^{CDS}) from the H11 line were co-infiltrated with *avrBs4* (left side) or *GFP* (right side) in *N. benthamiana* leaves (OD_{600} = 0.8). Co-infiltrations with 35s-driven *GFP* were used as negative controls. Phenotypes were observed 2 dpi. All leaves were destained in 80% EtOH. Dashed lines mark the infiltrated area.

2.2 Bs3, an executor protein from pepper, is functional in tomato.

The main focus of the project was to study *Bs3*, *Bs4C*, *Xa10*, and *Xa23*, collectively referred to as the executor *R* genes (Introduction, chapter 1.1.2), in a tomato background using two methods for their activation. The first method, *i.e.* transcriptional activation with liquid estradiol (Introduction, chapter 1.2.2), was intended to study executor-mediated cell death in the absence of the *X. euvesicatoria* pathogen. The second method, *i.e.* dTALE-mediated transcriptional activation (Introduction, chapter 1.2.3), was proposed to study executor-mediated resistance to the *X. euvesicatoria* pathogen.

2.2.1 Estradiol-inducible executor *R* genes cause cell death in *N. benthamiana*.

The CDSs of the executor *R* genes were transcriptionally fused to the EIP (Figure 15; Introduction, chapter 1.1.2). The CDSs of the executor *R* genes were translationally fused to a C-terminal triple FLAG epitope tag followed by the T2A sequence, and a GFP fluorophore (Figure 15; Material and Methods, chapter 4.2.3).

Prior to a tomato transformation, it was necessary to test inducibility of the executor *R* genes with the chemical agent, *i.e.* liquid estradiol. EIP-driven *Bs3*, *Bs4C*, *Xa10*, *Xa23*, and *GFP*, used as a negative control, were expressed via *Agrobacterium*-mediated T-DNA delivery in *N. benthamiana* leaves. Liquid estradiol (20 mM β-Estradiol dissolved in DMSO and diluted 1:1000 in water) or mock (DMSO diluted 1:1000 in water) treatments were applied to previously infiltrated areas of *N. benthamiana* leaves 24 hours post-infection and phenotyping was made 48 hours post-treatment. All four executor *R* genes caused cell death upon treatment with liquid estradiol (Figure 16A), while estradiol-induced *GFP* did not cause a cell death reaction (Figure 16A). These results demonstrated that the assembled constructs were functional and responsive to estradiol treatment in *N. benthamiana*

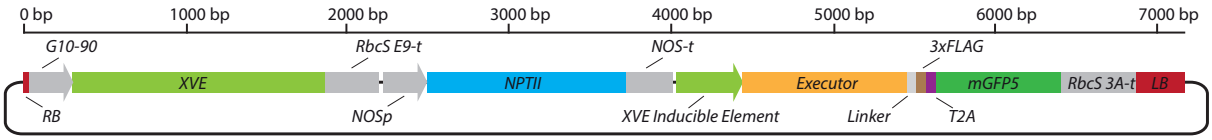

Figure 15. Structural modules of the assembled *EIP:Executor-3xFLAG-GFP* constructs. The CDSs of the executor *R* genes were transcriptionally fused to the XVE-inducible element, referred to as the EIP. The CDSs of the executor *R* genes were translationally fused to a C-terminal triple FLAG epitope tag followed by the T2A sequence and a GFP fluorophore. Address the Introduction (chapter 1.1.2) and the Material and Methods (chapter 4.2.3) for a detailed information about other modules of the EIP (Zuo *et al.*, 2000).

leaves. However, *Bs3*, *Xa10*, and *Xa23* also triggered cell death of various intensity upon mock treatment (Figure 16B), while *Bs4C* did not cause cell death upon mock treatment (Figure 16B). No cell death reaction was triggered by the *GFP*, *i.e.* the negative control, following mock treatment (Figure 16B). Collectively these results suggest that in the transient assay the EIP was leaky, thus, it was able to cause expression of the executor *R* genes in the absence of the chemical inducer.

However, transient assays do not reflect a situation in stable transgenic lines, since in a transient assay each cell has a unique transgene integration point (Kapila *et al.*, 1997). By contrast, in a stable transgenic line all cells are clonal (Müller *et al.*, 1996). Therefore, if a transgene is integrated into an appropriate genomic context, which eliminates leakiness of the promoter (van Leeuwen *et al.*, 2001; Wilson *et al.*, 1990), it is possible to select stable lines with a tight control over expression of the executor *R* genes in the absence of the chemical inducer (Holmes *et al.*, 2020).

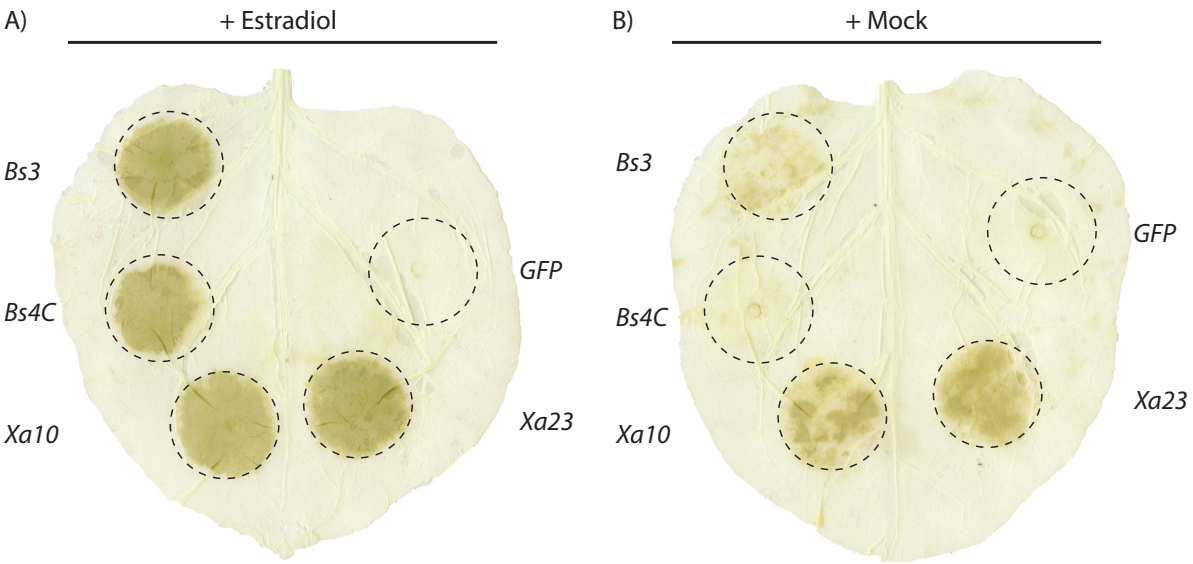

Figure 16. Estradiol-inducible *Bs3*, *Bs4C*, *Xa10*, and *Xa23* cause cell death in *N. benthamiana* leaves. The executor *R* genes under the control of EIP were expressed via *Agrobacterium*-mediated T-DNA delivery (OD_{600} = 0.4) in *N. benthamiana* leaves and treated with (A) liquid estradiol or (B) mock solution 24 hours post-infiltration (hpi) of the bacterial strains. EIP-driven *GFP* served as a negative control of the cell death phenotype after treatment with liquid estradiol. Mock treatment was used to determine if the EIP-driven executor *R* genes cause cell death phenotypes in the absence of the chemical inducer. Phenotypes were observed 2 days post-treatment. All leaves were destained in 80% EtOH. Dashed lines mark the infiltrated area.

2.2.2 Estradiol-mediated transcriptional activation of *Bs3* causes cell death in tomato.

In order to clarify if any of the estradiol-inducible executor *R* gene constructs, namely *Bs3*, *Bs4C*, *Xa10*, and *Xa23* cause cell death and confer resistance to *X. euvesicatoria* in tomato (Figure 16A), four stable tomato transformations of *S. lycopersicum* cv. MM (*Bs4* background) were initiated (Table 1). Putative T0 plants from each transformation were genotyped for the presence of the corresponding transgene and among them 32 were positive for *Bs3*, 25 for *Bs4C*, 24 for *Xa10*, and 31 for *Xa23* transgenes (Table 1). All transgene-positive plants were phenotyped via syringe infiltration of liquid estradiol or mock solutions into leaf tissue to identify T0 plants with cell death reaction to liquid estradiol treatment (Table 1). However, none of the T0 *Bs4C*, *Xa10*, and *Xa23* plants had a cell death reaction upon phenotyping with liquid estradiol and mock treatments (Table 1). Thus, all developed T0 *Bs4C*, *Xa10*, and *Xa23* plants are not estradiol-inducible.

Even though T0 *Bs4C*, *Xa10*, and *Xa23* plants showed no cell death reactions upon estradiol treatment, four T0 *Bs3* lines, designated as J8, K30, L196, and N61, showed a consistent cell death reaction to the estradiol treatment, but not to the mock treatment (Table 1). These results indicate that even though the EIP was leaky in the transient assay (Figure 16B), it was possible to generate stable lines with a tight control over expression of *Bs3* transgene in the absence of the chemical inducer (Table 1). The consistent cell death reaction to liquid estradiol treatment of the four T0 Bs3 plants demonstrated that Bs3 is functional in tomato and that Bs3-mediated cell death pathways between *Capsicum* and *Solanum* spp. are preserved.

Table 1. Summary of stable tomato transformations with the constructs containing the executor *R* genes under the control of the EIP.

Executor *R* gene	Number of transformed shoot-producing calli	Total number of identified transgene-positive plants	Number of transgene-positive plants showing cell death upon estradiol treatment
Bs3	15 out of 200	32	4
Bs4C	16 out of 200	25	0
Xa10	15 out of 200	24	0
Xa23	20 out of 200	31	0

To clarify if *Bs3* transgene remained functional in the next generation (T1) and to identify the number of transgene copies in each T0 line, 100 T1 generation plants for each line were phenotyped via syringe infiltration of liquid estradiol or mock solutions into leaf tissue. If the T1 plants from a certain line showed a cell death reaction to the estradiol treatment, all plants from this line were additionally genotyped for the transgene. In the J8 line, the cell death phenotype upon estradiol treatment did not follow a perfect 3:1 segregation based on the phenotype (χ^2 = 0.9623; p-value = 0.03767; Table 2), however the transgene followed 3:1 segregation (χ^2 = 0.1826; p-value = 0.8174; Table 2), indicating presence of potentially one functional *Bs3* copy. K30, L196, and N61 lines were also analysed in the same manner, but were neglected for further use due to the integration of numerous *Bs3* copies, some of which were not estradiol-inducible (Table 2).

Genome Walking, a PCR-based method for identification of unknown genomic sequences flanking known sequence (Cottage *et al.*, 2001; Shapter and Waters, 2014), was used to determine the insertion site of the *Bs3* transgene in the T0 J8 line. According to this approach (Materials and methods, chapter 4.2.7), the gDNA from the T0 J8 line was digested by numerous restriction enzymes that leave blunt ends and a double-strand DNA cassette, *i.e.* an adaptor, was ligated to each batch of the digested gDNA. Consequently, two PCRs were performed using the primers specific to the adaptor and to T-DNA borders. Query sequences of the purified and cloned amplicons were aligned to the *Sl*3.0 genome. All sequences were mapped to the chromosome 07 in two distinct groups. Each group was approximately one million bp away from each other, indicating the possible presence of two co-segregating

Table 2. Summary of the characterisation process of stable transgenic tomato lines containing *Bs3* under the control of the EIP.

T0 Line designation	Number of genotyped and phenotyped T1 plants	Number of T1 plants showing cell death upon estradiol treatment	Number of transgene-positive T1 plants
J8	100	66	76
K30	100	66	93
L196	100	12	N/A
N61	100	48	93

transgene copies (Figure 17A). The number and genomic location of both co-segregating *Bs3* copies were confirmed using PCR with primer pairs specific to chromosome 07 and the right or left borders of the transgene, and consecutive sequencing of PCR-amplified fragments.

The J8 plants reliably responded to estradiol treatment with a cell death reaction throughout all generations tested so far, *i.e.* T0 to T4 (Figure 17B). Transcriptional activation of *Bs3* copies 12 hours post-infiltration (hpi) with liquid estradiol was measured by quantitative real-time PCR (qRT-PCR; Figure 17C). At this timepoint *Bs3* expression upon estradiol treatment was equal to 30% of the house-keeping gene *TAP42 Interacting Protein of 41 kDA-Like* (*TIP41-Like*; *Solyc10g049850*) expression level (Figure 17C), which was significantly higher than *Bs3* expression (0%) in mock-treated samples (p-value = 0.00078 < 0.001). These results indicate a correlation between the cell death phenotype and the transcriptional activation of *Bs3* upon estradiol treatment.

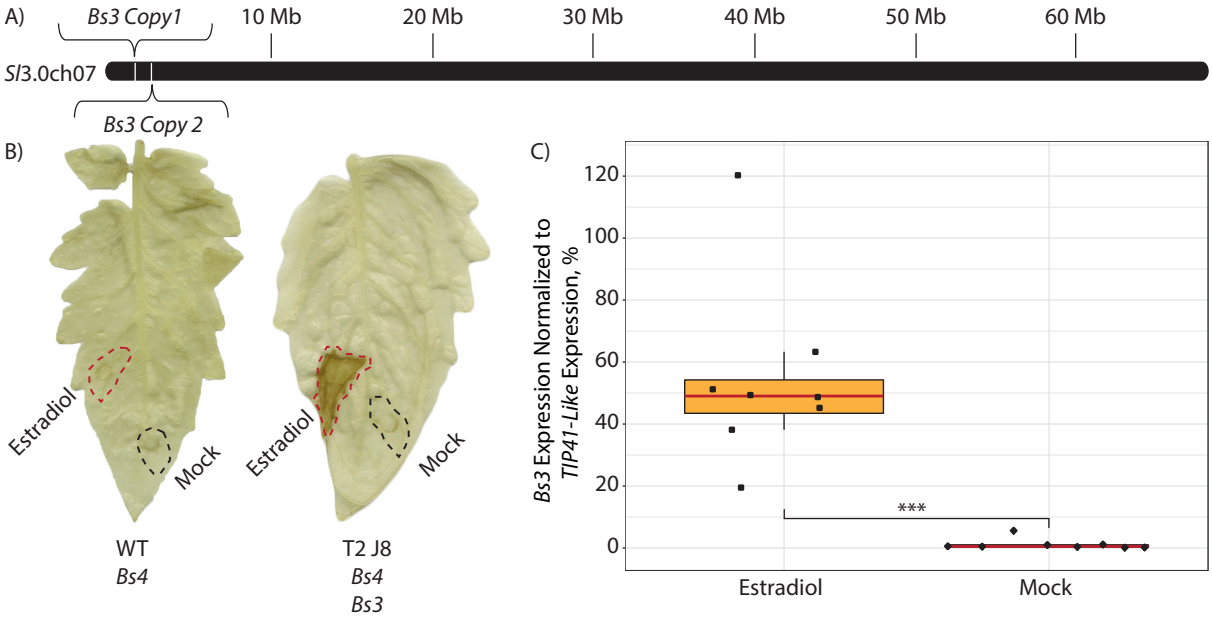

Figure 17. Estradiol-mediated transcriptional activation of *Bs3* causes cell death in tomato. A) In the J8 (*Bs4 Bs3*) line, two *Bs3* copies are integrated into chromosome 07. B) Phenotypic analysis of WT, *i.e.* a negative control, and T2 J8 leaflets with liquid estradiol (left; red) and mock (right; black) treatments. Mock treatment served as a negative control of Bs3-mediated cell death. Phenotypes were observed 2 days post-treatment. All leaflets were destained in 80% EtOH. Dashed lines mark the infiltrated area. C) *Bs3* expression in T3 J8 line 12 hpi of liquid estradiol and mock solution. Mock treatment served as a negative control of estradiol-dependent transcriptional activation of *Bs3*. *Bs3* and *TAP42 Interacting Protein of 41 kDA-Like* (*TIP41-Like*; *Solyc10g049850*) expression levels were quantified by quantitative real-time PCR (qRT-PCR). n = 8, *n* numbers of independent biological replicates. Unpaired Two-Samples Wilcoxon Test was used to calculate significant differences between groups. ***, $p \leq 0.001$.

2.2.3 dTALE34 transcriptionally activates not estradiol-inducible *Bs3* in *CC-Bs4* background.

One of the project goals was to clarify if the transcriptional activation of *Bs3* in tomato will result into Bs3-mediated resistance to *X. euvesicatoria* (Introduction, chapter 1.2.3). This goal could be achieved via deployment of dTALEs to mimic the native situation where AvrBs3 delivered by xanthomonas bacteria transcriptionally activates *Bs3* (Morbitzer *et al.*, 2011; Römer *et al.*, 2007). Utilisation of dTALEs required *Bs3* integration into tomato mutant lacking a functional copy of the *Bs4* to eliminate interference by the dTALE-activated tomato Bs4. (Figures 4 and 6; Bultmann *et al.*, 2012; de Lange *et al.*, 2017; Schornack *et al.*, 2004; Schornack *et al.*, 2005).

The tomato genotype containing the functionally-validated *Bs3* transgene, *i.e.* the J8 line (Figure 17), was crossed to the *Bs4* knockout line, *i.e.* the H11 line (Figure 12B), to identify descendants containing a functional *Bs3* transgene and a homozygous *Bs4* null allele (Figure 18A). Filial plants from the first generation (F1) which had a cell death reaction following syringe infiltrated liquid estradiol were kept for selfing (Figure 18A). In the F2 generation, homozygous *Bs4* and *CC-Bs4* plants lacking or containing *Bs3* transgene were selected by genotyping to test the ability of a dTALE to transcriptionally activate *Bs3* (Figure 18A). It was hypothesised that the *CC-Bs4 Bs3* line would show cell death reaction only upon *X. euvesicatoria*-mediated delivery of the EIP-targeting dTALE, but not AvrBs4.

dTALE34, consisting of 17,5 tandemly arranged 34 aa-long repeats, was engineered to target a 19 bp-long effector-binding element ($EBE^{dTALE34}$) within the XVE inducible element of the EIP (Figure 18B). All genotypes from the F2 generation were infiltrated with *Xe* 85-10 strains containing *avrBs4*, *dTALE34*, and EV (Figure 18C). Parental H11 (*CC-Bs4*) and J8 (*Bs4 Bs3*) lines were used as the negative and positive controls, respectively (Figure 18C). F2 *CC-Bs4* genotype did not show a cell death reaction to AvrBs4, dTALE34 or the EV (Figure 18C), which is consistent with the reaction of the parental H11 line (Figure 18C). Infiltration of AvrBs4 and dTALE34, but not the EV, into F2 *Bs4* and F2 *Bs4 Bs3* caused the cell death phenotype (Figure 18C), which is consistent with the reaction of the parental J8 line (Figure 18C). This cell death is associated with the Bs4-mediated recognition of AvrBs4 and dTALE34. F2 *CC-Bs4 Bs3* plants, hereinafter referred to as the HJ1 line, did not show a cell death reaction to AvrBs4 or EV (Figure 18C) but had a cell death phenotype upon infiltration with *Xe* 85-10 *dTALE34* (Figure

18C). Since *CC-Bs4* in the HJ1 line is a null allele, the cell death in the HJ1 line was caused by the transcriptional activation of *Bs3* by dTALE34 (Figure 18C).

To compare the efficiency of dTALE34-mediated versus liquid estradiol-mediated transcriptional activation of the *Bs3* transgene in the HJ1 (*CC-Bs4 Bs3*) line, the *Bs3* transcript

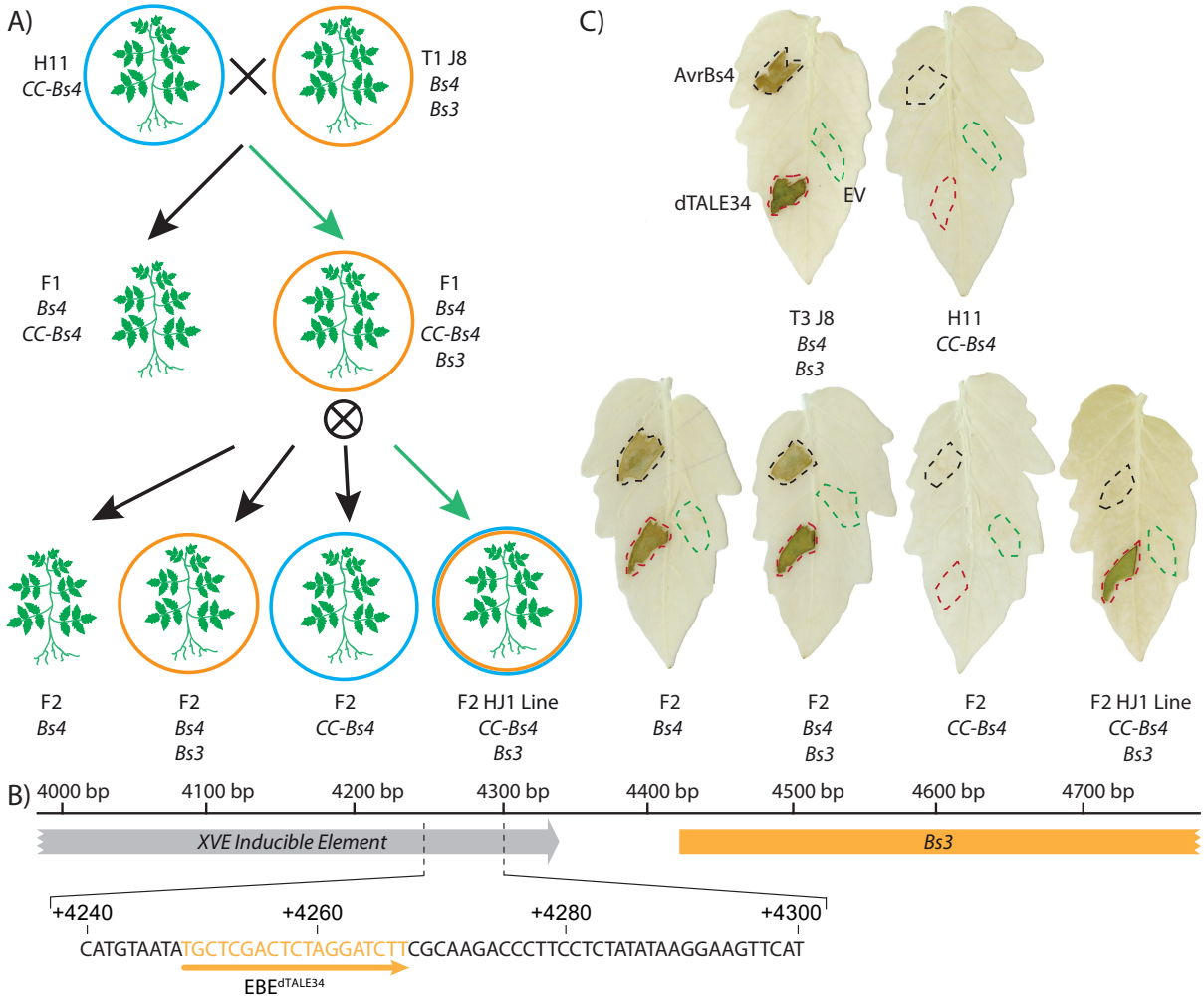

Figure 18. Delivery of dTALE34, but not AvrBs4 causes cell death in *CC-Bs4 Bs3* tomato. A) Schema of F2 *CC-Bs4 Bs3* (HJ1) line development. A *Cas9*^{Bs4}-free homozygous *CC-Bs4* (H11) plant was crossed to a heterozygous *Bs4 Bs3* (J8) plant from the T1 generation. F1 plants which had a cell death reaction after liquid estradiol infiltration were kept for selfing. Green arrows emphasise the genotypes selected for selfing and seed multiplication. × and ⊗ symbols represent crossing and selfing, respectively. Homozygous *CC-Bs4 Bs3* plants (HJ1 line) were selected in the F2 generation by genotyping. Coloured rings indicate traits: blue – homozygous *CC-Bs4* and orange – presence of *Bs3*. B) dTALE34 targets EBE^{dTALE34} within the EIP to activate transcription of *Bs3*. C) Phenotyping of F2 lines segregating for *Bs4*/*CC-Bs4*, and *Bs3* with *Xe* 85-10 *avrBs4* (left top; black), *Xe* 85-10 *dTALE34* (left bottom; red), and *Xe* 85-10 EV (right side; green). The parental J8 and H11 lines served as positive and negative controls of the Bs4-mediated cell death upon AvrBs4 and dTALE34 delivery, respectively. *Xe* 85-10 *avrBs4* served as a positive control of the Bs4-mediated cell death and as a negative control of the Bs3-mediated cell death. *Xe* 85-10 EV served as a negative control of the AvrBs4- or dTALE34-induced cell death. Phenotypes were observed 2 dpi. All leaflets were destained in 80% EtOH. Dashed lines mark the infiltrated area.

abundance was quantified by qRT-PCR 12 hpi with *Xe* 85-10 *dTALE34*, *Xe* 85-10 EV, liquid estradiol, and mock treatment. *Bs3* expression upon estradiol treatment was approximately 8% compared to the level of the house-keeping gene *TIP41-Like* expression (Figure 19), which was significantly higher than *Bs3* expression (0%) in mock treated samples (p-value = 0.00019 < 0.001; Figure 19). Moreover, *Bs3* expression in *Xe* 85-10 dTALE34-treated leaflets was on average 880% compared to the level of the house-keeping gene *TIP41-Like* expression (Figure 19), which was significantly higher than *Bs3* expression (0%) in *Xe* 85-10 EV-treated samples (p-value = 0.00019 < 0.001; Figure 19). These data indicate that the dTALE34 transcriptionally activates *Bs3* transgene strongly, while the same transgene is barely activated by the liquid estradiol treatment. In addition, this experiment shows that the dTALE34-dependent transcriptional activation of the *Bs3* transgene correlates with the cell death phenotype in the HJ1 line (Figure 18C).

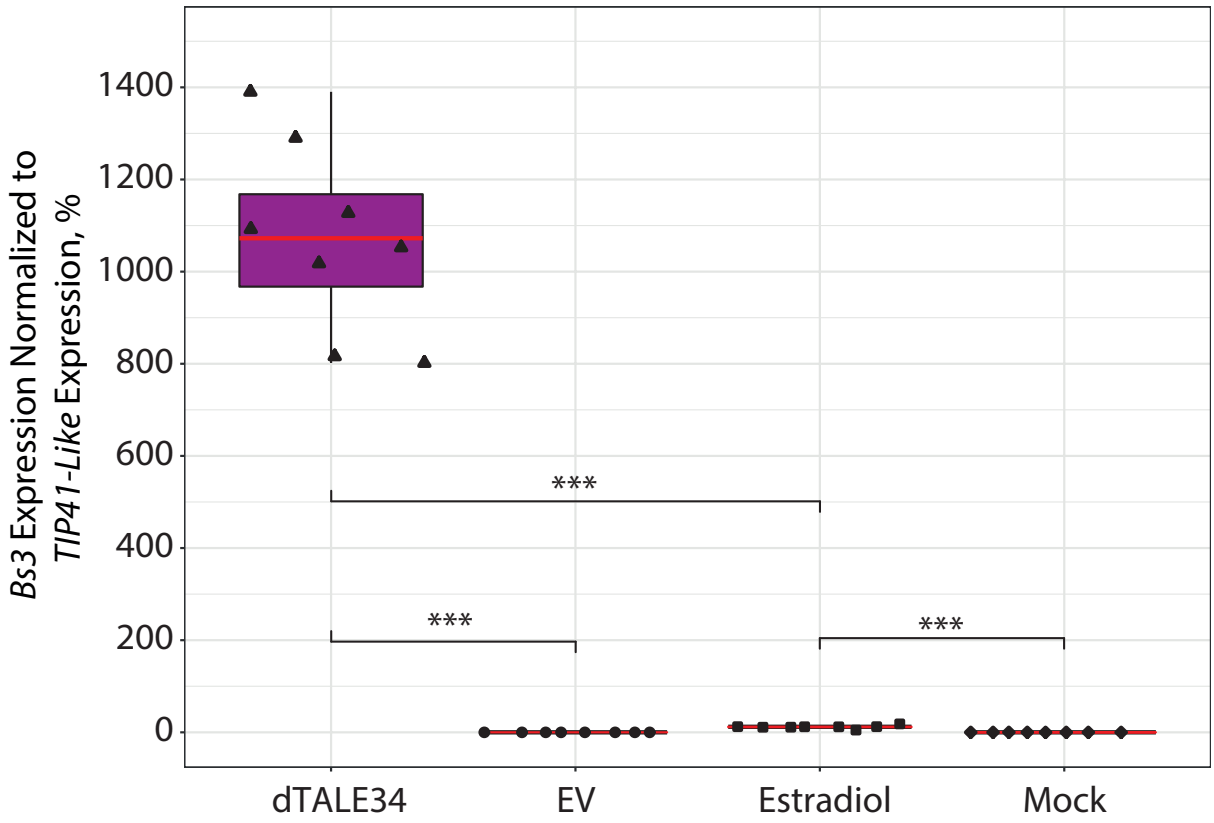

Figure 19. dTALE34 transcriptionally activates not estradiol-inducible *Bs3* transgene in *CC-Bs4* background. HJ1 (*CC-Bs4 Bs3*) leaflets were syringe infiltrated with *Xe* 85-10 *dTALE34* (OD_{600} = 0.4), *Xe* 85-10 EV (OD_{600} = 0.4), liquid estradiol, and mock solutions. Infiltrations with *Xe* 85-10 EV and mock solutions served as negative controls of dTALE34- and estradiol-dependent transcriptional activation of *Bs3*, respectively. Samples were collected 12 hpi. *Bs3* and *TIP41-Like* expression levels were quantified by qRT-PCR. n = 8, *n* numbers of independent biological replicates. Pairwise Wilcoxon Rank Sum Test and false discovery rate (FDR) p-value adjustment method for multiple comparisons were used to calculate significant differences between groups. ***, $p \leq 0.001$.

2.2.4 Bs4 has no detectable impact on dTALE34-dependent transcriptional activation of the *Bs3* transgene.

Since dTALE34 transcriptionally activated not estradiol-inducible *Bs3* transgene in the HJ1 (*CC-Bs4 Bs3*) line, it might be able to activate transcription of other not estradiol-inducible executor transgenes, namely *Bs4C*, *Xa10*, and *Xa23*, in the *Bs4* background (Table 1). To clarify if Bs4-mediated dTALE34 recognition would affect dTALE34-mediated transcriptional activation of the *Bs3* transgene, leaflets of the J8 (*Bs4 Bs3*) and HJ1 (*CC-Bs4 Bs3*) plants were infiltrated with *Xe* 85-10 *dTALE34* and *Xe* 85-10 EV. Samples were collected 24 hpi and the *Bs3* transcript abundance was quantified by qRT-PCR. *Bs3* transcript abundance upon dTALE34-mediated transcriptional activation of the *Bs3* transgene in the *CC-Bs4* background was equal to 630% of the transcript abundance level of the house-keeping gene *TIP41-Like* (Figure 20), which was significantly higher than the *Bs3* transcript abundance in EV-treated samples (0%; p-value = 0.0065 < 0.01; Figure 20). Meanwhile, *Bs3* transcript abundance upon dTALE34-

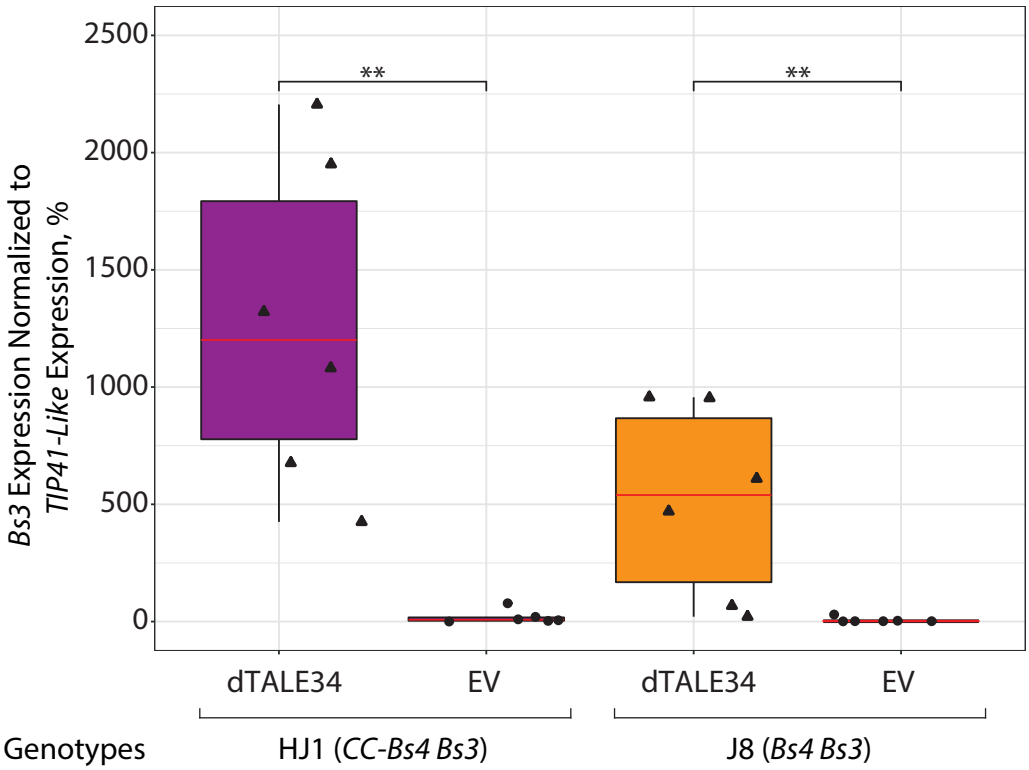

Figure 20. dTALE34 activates transcription of *Bs3* in the *CC-Bs4* and *Bs4* backgrounds. HJ1 (*CC-Bs4 Bs3*) and J8 (*Bs4 Bs3*) leaflets were syringe infiltrated with *Xe* 85-10 *dTALE34* and *Xe* 85-10 EV (OD_{600} = 0.4). Infiltrations of HJ1 and J8 leaflets with *Xe* 85-10 EV served as negative controls of dTALE34- dependent transcriptional activation of *Bs3*. Samples were collected 24 hpi. *Bs3* and *TIP41-Like* expression levels were quantified by qRT-PCR. n = 6, *n* numbers of independent biological replicates. Pairwise Wilcoxon Rank Sum Test and FDR p-value adjustment method for multiple comparisons were used to calculate significant differences between groups. **, $p \leq 0.01$.

mediated transcriptional activation of the *Bs3* transgene in the *Bs4* background was equal to 130% of the transcript abundance level of the house-keeping gene *TIP41-Like* (Figure 20), which was significantly higher than the *Bs3* transcript abundance in EV-treated samples (0%; p-value = 0.0087 < 0.01; Figure 20). Even though there was a difference in the *Bs3* transcript abundance levels upon dTALE34-mediated transcriptional activation of the *Bs3* transgene between *Bs4* and *CC-Bs4* backgrounds, this difference was not statistically significant (p-value = 0.0779 > 0.05; Figure 20). As the Bs4-mediated dTALE34 recognition did not affect dTALE34-mediated transcriptional activation of the *Bs3* transgene (Figure 20), dTALE34 could therefore be used for the transcriptional activation of the *Bs4C*, *Xa10*, and *Xa23* transgenes in the T0 lines, which did not show cell death reaction upon liquid estradiol treatment (Table 1).

2.2.5 Bs4 mediates recognition of dTALEs with 35 aa-long repeats in tomato.

Even though dTALE34-mediated transcriptional activation of the executor transgenes in the *Bs4* background (generated T0 lines; Table 1) may be measured with qRT-PCR, Bs4-mediated recognition of dTALE34 and the resulting cell death masks any executor protein-caused phenotypes. To avoid laborious and time-consuming introduction of executor transgenes to *CC-Bs4* background via crossing (Figure 18A), it was hypothesised that modifications to the dTALE architecture would prevent Bs4-mediated dTALE recognition and the resulting cell death reaction and therefore, would uncover executor protein-caused phenotypes upon transcriptional activation by a modified dTALE.

Previous studies demonstrated that AvrHah1 and Hax2, *i.e.* TALEs from *X. gardneri* and *X. campestris* pv. *armoraciae* (*Xca*), overcome Bs4-mediated recognition and cell death (Kay *et al.*, 2005; Schwartz *et al.*, 2017). It was hypothesised that these TALEs avoid Bs4-mediated recognition due to their unique architecture (Schwartz *et al.*, 2017), *i.e.* presence of proline at the position 33 in 35 aa-long repeats (Schornack *et al.*, 2008), while majority of AvrBs3-like proteins and dTALEs consist from 34 aa-long repeats, which do not contain proline at the position 33 (Ballvora, Pierre*, et al.*, 2001; Gu *et al.*, 2005; Morbitzer *et al.*, 2011; Richter *et al.*, 2014). In order to determine if Bs4 mediates recognition of dTALEs with 35 aa-long repeats, dTALE35, consisting of 17,5 tandemly arranged 35 aa-long repeats, was engineered to bind a 19 bp-long effector-binding element ($EBE^{dTALE35}$) within XVE inducible element of the EIP (Figure 21A).

The WT, H11 (*CC-Bs4*), and HJ1 (*CC-Bs4 Bs3*) lines were infected with *Xe* 85-10 strains containing *dTALE34*, *dTALE35*, *avrBs4*, or EV. Phenotyping of infected leaflets was made 3 dpi. This experiment revealed that *Xe* 85-10-mediated delivery of dTALE34 and dTALE35 into HJ1 line resulted in the cell death reaction (Figure 21B), while *Xe* 85-10 *avrBs4* and *Xe* 85-10 EV infection in the same background did not cause the cell death (Figure 21B). These results show that both dTALE34 and dTALE35 retained their full-length structure and architecture necessary for transcriptional activation of the *Bs3* transgene and its transcriptional activation resulted in a Bs3-mediated cell death reaction. Consequently, when *Xe* 85-10 strains containing *dTALE34*, *dTALE35*, *avrBs4*, and EV were infiltrated into the leaflets of the H11 plants, no cell death reaction was observed (Figure 21B). This is due to absence of the *Bs3*

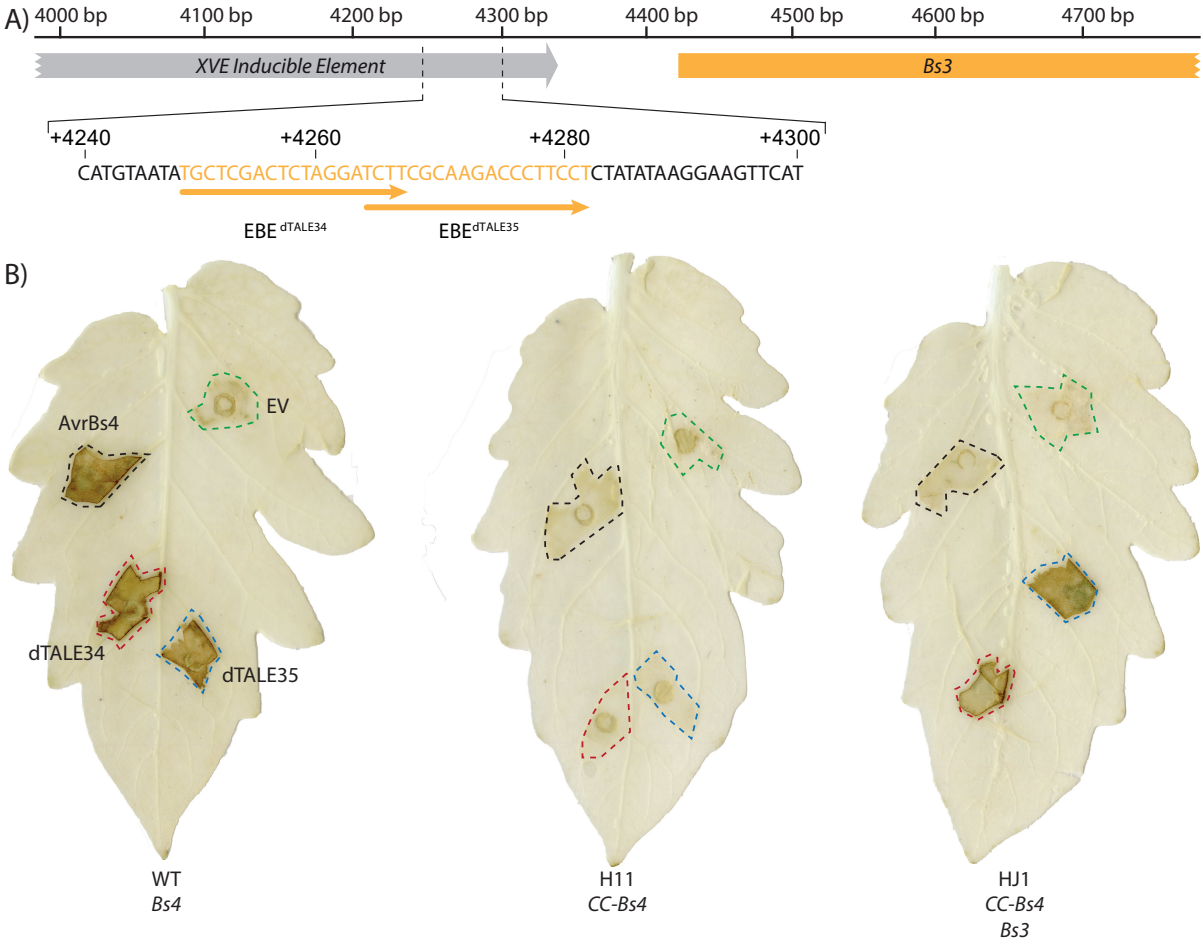

Figure 21. Bs4 mediates recognition of dTALEs with 34 and 35 amino acid-long repeats. A) Schematic location of EBEs targeted by dTALE34 and dTALE35 within the EIP of *Bs3*. B) Phenotyping of WT, H11 (*CC-Bs4*), and HJ1 (*CC-Bs4 Bs3*) lines with *Xe* 85-10 *avrBs4* (left top; black), *Xe* 85-10 *dTALE34* (left bottom; red), *Xe* 85-10 *dTALE35* (right bottom; blue), and *Xe* 85-10 EV (right top; green) at OD_{600} = 0.4. The H11 line served as a negative control of the Bs4-mediated cell death upon AvrBs4, dTALE34, and dTALE35 delivery. The HJ1 line was used to test functionality of dTALE34 and dTALE35. *Xe* 85-10 EV served as a negative control of the triggered cell death. Phenotypes were observed 3 dpi. All leaflets were destained in 80% EtOH. Dashed lines mark the infiltrated area.

transgene and presence of the *CC-Bs4* allele in the H11 line. Nevertheless, *Xe* 85-10-mediated delivery of dTALE34, dTALE35, and AvrBs4 in the WT plants led to an observable cell death reaction (Figure 21B). Since there is no *Bs3* transgene in the WT, cell death reaction on leaflets areas infected with *Xe* 85-10 *dTALE34*, *Xe* 85-10 *dTALE35*, and Xe 85-10 *avrBs4* was mediated by Bs4. Collectively, these results demonstrate that delivery of AvrBs4, *i.e.* a TALE with 34 aa-long repeats, dTALE34, *i.e.* a dTALE with 34 aa-long repeats, and dTALE35, *i.e.* a dTALE with 35 aa-long repeats, triggered Bs4-mediated cell death reaction. Thus, extended length of repeats and the presence of a proline at the position 33 within the 35 aa-long repeats did not allow dTALE35 to overcome Bs4-mediated recognition and cell death. Therefore, *CC-Bs4* background is indeed preferred for integration of not estradiol-inducible executor transgenes and consequently, their dTALE-mediated transcriptional activation, since CC-Bs4 will not mask executor protein-caused phenotypes upon transcriptional activation by a dTALE.

2.2.6 Bs3 confers resistance to *Xe* 85-10 *dTALE34* in tomato.

Previous experiments demonstrated that the transcriptionally activated *Bs3* is able to cause cell death in tomato (Figures 17B and 18C), however, the effect of *Bs3* expression on bacterial growth had not been studied. In order to test if transcriptional activation of *Bs3* has an impact on bacterial growth *in planta*, tomato *CC-Bs4* lines that either contained *Bs3* (HJ1) or lacked *Bs3* (H11) were infiltrated with a set of isogenic *Xe* 85-10 strains containing or lacking *dTALE34* targeting XVE element within the EIP of the *Bs3* transgene. Phenotypic inspection at 7 dpi revealed necrotic reactions of inoculated tissue only with the combination of *Bs3*-transgenic plants and *Xe* 85-10 expressing *dTALE34* (Figure 22A). This observation suggests that the occurrence of the cell death reaction in tomato leaflets depends on both, presence of the *Bs3* transgene in the given plant and presence of the *dTALE34* in the *Xe* 85-10 strain.

Following the phenotypic results, growth of *Xe* 85-10 *dTALE34* and *Xe* 85-10 EV strains in tomato *CC-Bs4* lines that either contained *Bs3* (HJ1) or lacked *Bs3* (H11) was quantified 7 dpi. Significantly less *Xe* 85-10 *dTALE34* colony-forming units per square centimetre (CFUs/cm^2) were detected within HJ1 line in comparison to *Xe* 85-10 EV (p-value = 0.000051 < 0.0001; Figure 22B). There were also significant differences between *Xe* 85-10 *dTALE34* growth in HJ1 and H11 lines (p-value = 0.000051 < 0.0001; Figure 22B). These results show that the reduced *in planta* growth of *Xe* 85-10 *dTALE34* correlates with the increased *Bs3* transcript levels.

This thesis describes the first transgenesis of *Bs3*, an executor *R* gene from *Capsicum* spp., to *Solanum* spp. *Bs3* under the control of the EIP was transcriptionally activated in different genetic backgrounds (*Bs4* and *CC-Bs4*) using two methods, namely activation with the liquid estradiol and with the dTALE targeting the XVE element within the EIP (Figures 17C, 19, and 20). Transcriptional activation of the *Bs3* transgene correlated with the cell death reaction (Figures 17B and 18C) and the reduced *in planta* growth of *Xe* 85-10 *dTALE34*. Collectively, engineered transgenic lines and bacterial strains represent a pathosystem that enables further dissection of the Bs3-mediated immunity pathways. Liquid estradiol-mediated transcriptional activation of the *Bs3* transgene allows for the study of Bs3-mediated cell death in absence of pathogen-associated virulence effects in the J8 (*Bs4 Bs3*) line. dTALE34-mediated

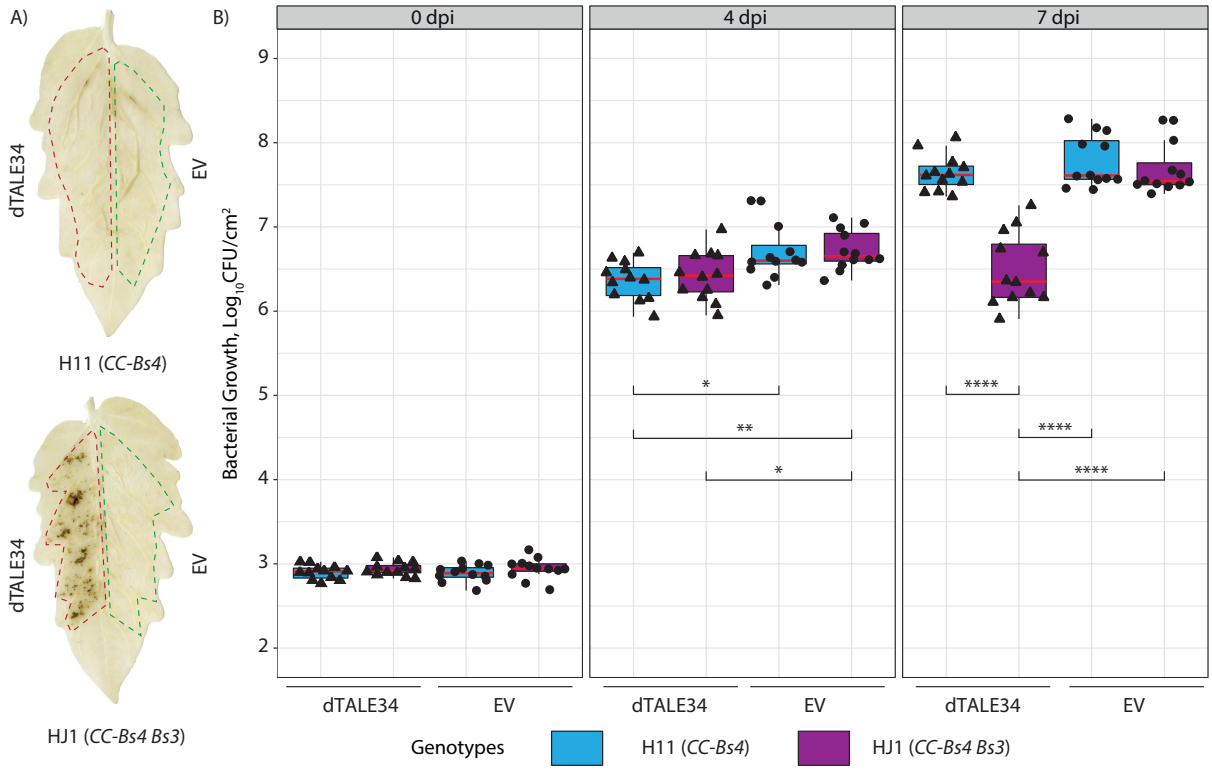

Figure 22. *Bs3* confers resistance to *Xe* 85-10 *dTALE34* in tomato 7 dpi. A) Phenotyping of H11 *(CC-Bs4*; top) and HJ1 (*CC-Bs4 Bs3*; bottom) lines with *Xe* 85-10 *dTALE34* (left; red) and *Xe* 85-10 EV (right; green) strains 7 dpi (OD_{600}=4x10^{-5}). The H11 line served as a negative control of Bs3-mediated cell death. *Xe* 85-10 EV served as a negative control of the dTALE34-mediated transcriptional activation of *Bs3*. All leaflets were destained in 80% EtOH. Dashed lines mark the infiltrated area. B) Quantification of *Xe* 85-10 *dTALE34* and *Xe* 85-10 EV growth in the H11 (*CC-Bs4*) and HJ1 (*CC-Bs4 Bs3*) lines. The H11 line served as a negative control of Bs3-mediated resistance. *Xe* 85-10 EV served as a negative control of the dTALE34-mediated transcriptional activation of *Bs3*. n = 12, *n* numbers of independent biological replicates. Colony-forming units per square centimetre (CFUs/cm^2) were counted 0, 4, and 7 dpi and log_{10}CFUs/cm^2 scores were used for the boxplot and statistical analysis. Pairwise Wilcoxon Rank Sum Test and FDR p-value adjustment method for multiple comparisons were used to calculate significant differences between groups. *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$.

transcriptional activation of the *Bs3* transgene in the HJ1 (*CC-Bs4 Bs3*) line allows the use of the established *S. lycopersicum – X. euvesicatoria* pathosystem to study Bs3-mediated resistance and the impact of putative Bs3-mediated pathway component knockouts on resistance. The afore described results are not only valuable for further discoveries in fundamental biology, but also have an applied value in agriculture. Since dTALE34-mediated transcriptional activation of the *Bs3* transgene restricts *Xe* 85-10 growth *in planta* (Figure 22B), *Bs3* under the control of a native or a synthetic promoter with EBE traps matching RVDs of the most common TALEs from *Xanthomonas* strains that are pathogenic on tomato can be a reliable source of resistance against *Xanthomonas* spp. in field conditions.

2.3 Does Bs3 exploit canonical NLR-mediated immunity pathways?

The engineered tomato *CC-Bs4* lines that either contained *Bs3* (HJ1) or lacked *Bs3* (H11) and *Xe* 85-10 strains containing *avrBs4*, *dTALE34*, and EV were used to decipher if Bs3 exploits canonical pathways to trigger a cell death reaction. As Bs3-mediated cell death and resistance are separate cases of effector-triggered immunity (Römer *et al.*, 2007), the involvement of ETI master regulators, *e.g.* EDS1 and SGT1 (Lapin *et al.*, 2020; Shirasu, 2009), into Bs3-mediated cell death was studied. Additionally, *Bs3* overexpression in *N. benthamiana* was reported to coincide with increased levels of SA and Pip (Krönauer *et al.*, 2019), suggesting putative exploitation of established immune pathways. Since Bs4 acts via EDS1 and SGT1 to mediate cell death signalling (Schornack *et al.*, 2004), the Bs4 background was ideal for CRISPR/Cas9-mediated mutagenesis of *EDS1* and *SGT1* and further validation that *CC-EDS1* and *CC-SGT1* are in fact null alleles. Consecutive crosses to the HJ1 (*CC-Bs4 Bs3*) line and selection of homozygous *CC-EDS1 CC-Bs4 Bs3* and *CC-SGT1 CC-Bs4 Bs3* lines were conducted to unravel putative roles of EDS1 and SGT1 in the Bs3-mediated cell death and immunity.

2.3.1 *Trans*-generational heritability of Cas9^{EDS1} activity facilitates *CC-EDS1 CC-Bs4 Bs3* line development.

There are two loci in tomato genome *Sl*3.0 annotated as "*EDS1*", namely *Solyc02g069400* and *Solyc06g071280*. However, only tomato *Suppressor of N 1-1* (*sun1-1*), a mutation in *Solyc06g071280*, was described to be defective in N-mediated resistance, SA accumulation and SAR (G. Hu *et al.*, 2005). Thus, a CRISPR/Cas9-mediated null mutation in *Solyc06g071280*, designated as *CC-EDS1*, was hypothesised to abort Bs4-mediated signalling. Since a functional

EDS1-PAD4 (EP) domain is required for AtEDS1-mediated cell death (Falk *et al.*, 1999; Parker *et al.*, 1996), SlEDS1$^{\Delta EP}$ mutant was preferred. Therefore, sgRNA6 and sgRNA7 were designed to target *SlEDS1* genomic sequence upstream from the encoded structural EP domain (Figure 23).

In addition to EDS1, testing the involvement of SGT1b, an NLR co-chaperone that positively regulates ETI (Austin *et al.*, 2002; Azevedo *et al.*, 2006), in Bs3-mediated cell death and immunity was also important. Mining for AtSGT1a (At4g23570) and AtSGT1b (At4g11260) homologous protein sequences in tomato revealed SlSGT1a (Solyc06g036410) and SlSGT1b (Solyc03g007670). Percent identity matrix generated with Clustal 2.1 Omega algorithm revealed that SlSGT1a shared 64,47% homology with AtSGT1a and 63.31% with AtSGT1b at the protein level, while SlSGT1b shared 68,1% homology with AtSGT1a and 68.17% with AtSGT1b at the protein level. As either any of these two tomato SGT1 proteins could be involved into Bs4-mediated cell death, both were knocked out independently. Thus, three sgRNAs, namely sgRNA8, sgRNA9, and sgRNA10, were designed to target the *SGT1a* genomic sequence upstream of the encoded CHORD-SGT1 (CS) and SGT1-specific (SGS) domains (Figure 23). Three other sgRNAs, namely sgRNA11, sgRNA12, and sgRNA13, were designed to target the *SGT1b* genomic sequence upstream of the encoded tetratricopeptide repeat (TPR) and SGS domains (Figure 23).

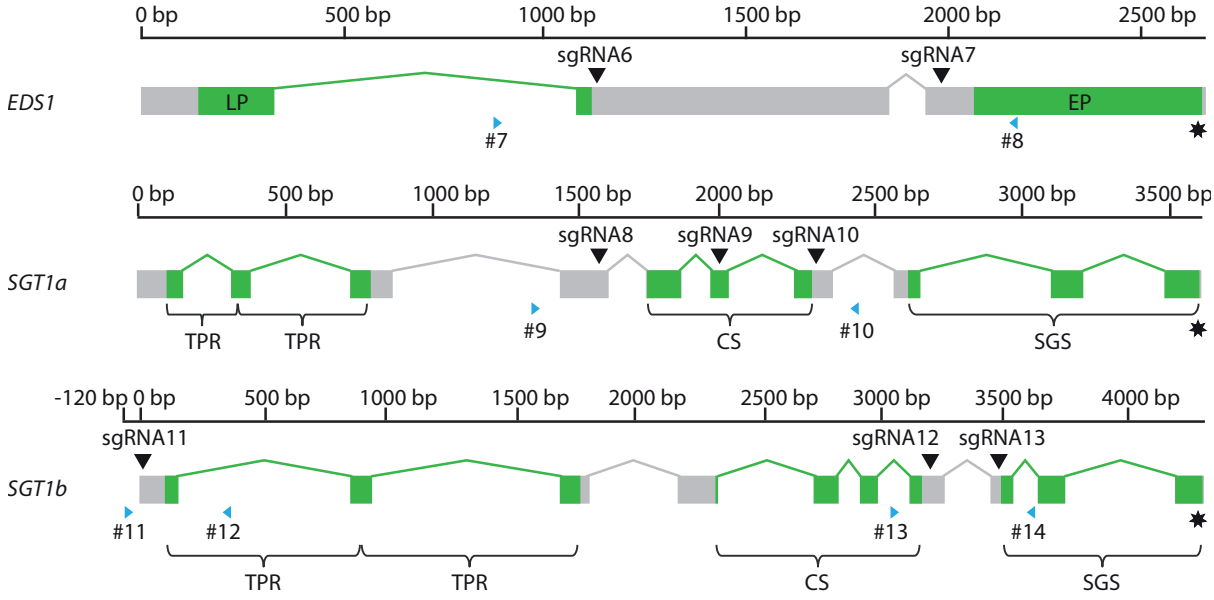

Figure 23. sgRNA designed for CRISPR/Cas9-mediated *EDS1*, *SGT1a*, and *SGT1b* mutagenesis in tomato. Black triangles indicate sgRNA target sites. Green blocks represent sequence regions encoding functional domains. Black stars represent the location of annotated stop codons. Blue triangles represent primers used for genotyping of putative mutants in the T0 generation.

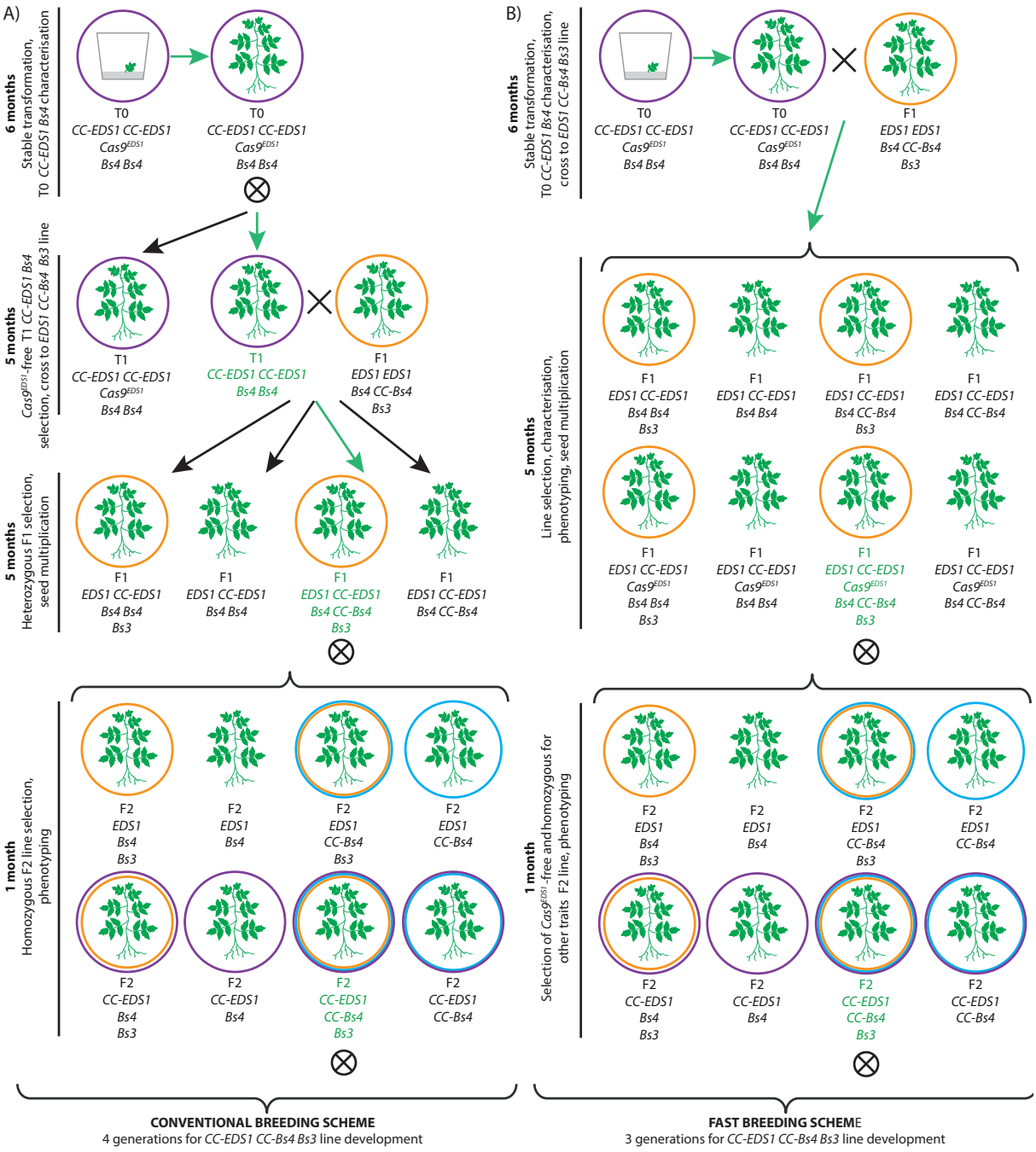

Figure 24. A cross between *Cas9*^{EDS1}-containing T0 *CC-EDS1 Bs4* plant and *EDS1 CC-Bs4 Bs3* line facilitates F2 *CC-EDS1 CC-Bs4 Bs3* line development. A) Conventional breeding scheme for multiple trait combination includes stable transformation, T0 *CC-EDS1 Bs4* identification, selection of *Cas9*^{EDS1}-free homozygous *CC-EDS1 Bs4* plant, cross to *CC-Bs4* and *Bs3* donor line, selection of F1 *EDS1*/*CC-EDS1 Bs4*/*CC-Bs4 Bs3* plants, selection and phenotyping of homozygous F2 *CC-EDS1 CC-Bs4 Bs3* plants. B) Fast breeding scheme for multiple trait combination includes transformation, *Cas9*^{EDS1}-positive T0 *CC-EDS1 Bs4* identification and cross to *CC-Bs4* and *Bs3* donor line, selection of F1 *EDS1*/*CC-EDS1 Bs4*/*CC-Bs4 Bs3* plants, selection and phenotyping of homozygous F2 *CC-EDS1 CC-Bs4 Bs3* plants. Coloured rings indicate traits: purple – homozygous *CC-EDS1*, blue – homozygous *CC-Bs4*, and orange – presence of *Bs3*. Green coloured arrows and text emphasise the genotypes selected for selfing and seed multiplication. × and ⊗ symbols represent crossing and selfing, respectively.

However, the conventional process of CRISPR/Cas9-mediated mutant line development and combination of the mutant trait with other traits by crossing is laborious and time consuming (Figure 24A). For example, stable *Agrobacterium*-mediated transformation of MM tomato calli for *EDS1* mutagenesis, identification and characterisation of T0 mutants, selection of *Cas9*^{EDS1}-free homozygous *CC-EDS1 Bs4* mutant plants in the T1 generation, *CC-EDS1* allele combination with *CC-Bs4* allele and *Bs3* transgene by crossing, and consecutive selection of *CC-EDS1 CC-Bs4 Bs3* genotype in F2 generation was estimated to take four generations. As this conventional process requires so much time, a shortcut for *CC-EDS1 CC-Bs4 Bs3* line development was used. The step involving *Cas9*^{EDS1}-free T1 *CC-EDS1 Bs4* line development was skipped and *Cas9*^{EDS1}-positive T0 *CC-EDS1 Bs4* plant was crossed with the *CC-Bs4* and *Bs3* donor line (Figure 24B; Rodríguez-Leal *et al.*, 2017). This approach, hereinafter referred to as the fast breeding scheme, was based on *trans*-generational heritability of *Cas9*^{EDS1} activity and

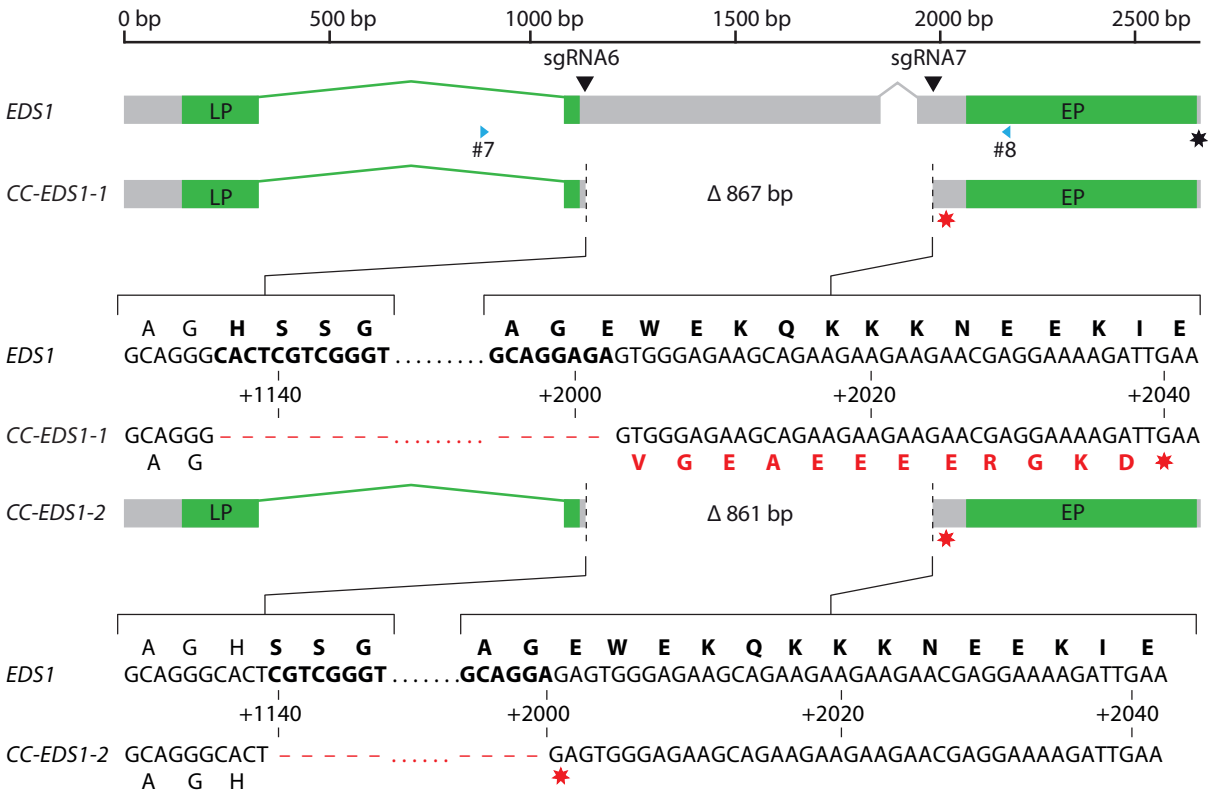

Figure 25. CRISPR/Cas9-mediated *EDS1* mutagenesis and gene models of T0 *CC-EDS1* mutants. Green blocks represent sequence regions encoding functional domains. Black triangles and vertical dashed lines indicate the sgRNA6 and sgRNA7 target sites. Blue triangles represent primers used for genotyping. WT *EDS1* genomic and protein sequences impacted by mutations are highlighted with bold black font. *CC-EDS1* mutations within genomic and protein sequences are marked with bold red font. Black and red stars represent the locations of native and premature stop codons, respectively.

was intended to save one generation in F2 *CC-EDS1 CC-Bs4 Bs3* line development (Figure 24B; Rodríguez-Leal *et al.*, 2017). However, the fast breeding scheme was expected to require extensive selection for the *CC-EDS1* and *CC-Bs4* alleles and the *Bs3* transgene in F1 and F2 generations.

Individual stable transformations of WT tomato were performed to knock out *EDS1*, *SGT1a*, and *SGT1b* for further testing for an impact of such mutations on Bs3-mediated cell death and immunity. Two T0 lines were identified to contain *CC-EDS1* alleles with large sequence deletions between LP-domain-encoding and EP-domain-encoding sequences (Figure 25). *CC-EDS1-1* allele had an 867 bp-long sequence deletion, while *CC-EDS1-2* allele had an 861 bp-long sequence deletion (Figure 25). Both mutations were predicted to cause frameshifts and therefore, truncated proteins lacking C-terminal EP-domains, namely CC-EDS1-1 and CC-EDS1-2 (Figure 25).

In addition to aforementioned T0 *CC-EDS1* lines, two T0 lines containing *CC-SGT1a* alleles with large genomic sequence deletions were identified (Figure 26). *CC-SGT1a-1* allele had a 744 bp-long sequence deletion between TPR-domain-encoding and SGS-domain-encoding sequences, while *CC-SGT1a-2* allele had a 165 bp-long sequence deletion between TPR-domain-encoding and CS-domain-encoding sequences (Figure 26). Both mutations were predicted to cause frameshifts and therefore, truncated CC-SGT1a-1 and CC-SGT1b-2 proteins lacking C-terminal domains (Figure 26).

In the case of the *SGT1b* knockout, identification of putative T0 lines containing mutations in *SGT1b* was complicated due to low transformation rates or, probably, by lethality of *SGT1b* knock out. Consequently, only one T0 line containing a *CC-SGT1b* allele with a 246 bp-long genomic sequence deletion between CS-domain-encoding and SGS-domain-encoding sequences was identified and this allele was named *CC-SGT1b-1* (Figure 26). However, this mutation did not introduce frameshifts and did not affect any sequences encoding functional domains and therefore, CC-SGT1b-1 protein is likely to be functional (Figure 26).

Following the fast breeding scheme (Figure 24B; Rodríguez-Leal *et al.*, 2017), the *CC-Bs4* allele and the *Bs3* transgene-containing line was crossed to *Cas9*-positive T0 *CC-EDS1-1*, *CC-SGT1a-2*, and *CC-SGT1b-1* lines and F1 seeds were collected. The selection process prioritised only *CC-EDS1 CC-Bs4 Bs3* line development due to time limitations. In the F1

generation, only one plant heterozygous for the *Bs4* and *CC-Bs4* alleles and hemizygous for the *Bs3* transgene was identified. In addition, this F1 plant was also *Cas9*^{EDS1}-positive. To clarify if the selected F1 plant contained *EDS1* and *CC-EDS1-1* alleles from its parental plants, the *EDS1* genomic sequence targeted by sgRNA6 and sgRNA7 was PCR-amplified, purified, cloned, and sequenced (Figure 27). Analysis of Sanger sequencing reads failed to detect neither *EDS1* nor *CC-EDS1-1* alleles from the parental plants, however, four unique and previously unidentified *CC-EDS1* alleles, namely *CC-EDS1-3*, *CC-EDS1-4*, *CC-EDS1-5*, and *CC-EDS1-6*, were found (Figure 27). Each of these *CC-EDS1* alleles had sequence deletions of various length between LP-domain-encoding and EP-domain-encoding sequences (Figure 27). All of these mutations were predicted to cause frameshifts and therefore, truncated EDS1 proteins lacking C-terminal EP-domains (Figure 27).

The selected F1 plant was kept for selfing and seed multiplication. In the F2 generation all plants were genotyped for the *Cas9*^{EDS1} transgene. All *Cas9*^{EDS1}-free plants were further genotyped for *Bs4* and *CC-Bs4* alleles and for the *Bs3* transgene. To clarify if the *Cas9*^{EDS1}-free F2 plants contained *EDS1* and previously identified *CC-EDS1* alleles, the *EDS1* genomic sequence targeted by sgRNA6 and sgRNA7 was PCR-amplified, purified, cloned, and sequenced (Figure S1). Analysis of Sanger sequencing reads revealed *EDS1* WT allele, as well as 13 unique *CC-EDS1* alleles (Figure S1). Only one of these 13 unique *CC-EDS1* alleles, namely *CC-EDS1-4*, was previously identified in the parental F1 plant (Figure 27; Figure S1). Majority of the newly identified *CC-EDS1* alleles had sequence deletions of various length between LP-domain-encoding and EP-domain-encoding sequences (Figure S1). These mutations were predicted to cause frameshifts and therefore, truncated EDS1 proteins lacking C-terminal EP-domains (Figure S1). Only *CC-EDS1-14* allele had a three bp-long deletion within the target site of the sgRNA6 and this mutation was predicted not to cause a frameshift. Therefore, CC-EDS1-14 protein likely was functional (Figure S1).

As *CC-EDS1* alleles were cloned from *Cas9*^{EDS1}-free F2 plants, all identified *CC-EDS1* mutations must have been present already in the *Cas9*^{EDS1}-positive parental F1 plant. In this case, the *CC-EDS1* allele from the *Cas9*^{EDS1}-positive T0 *CC-EDS1 Bs4* parent could have been used as a template for the homology-directed DNA repair (HDR) upon *Cas9*^{EDS1}-caused double-stranded break in the *EDS1* allele inherited from the *CC-Bs4* and *Bs3* donor parent (Figure 24B). Thus,

the fast breeding scheme stimulated the production of new *CC-EDS1* alleles *in planta* by CRISPR/Cas9-mediated mutagenesis of *EDS1* allele present in the F1 plant (Figure S1). This simple approach is based on *trans*-generational heritability of *Cas9*^{EDS1} activity and provides a possibility to create new mutant alleles without the need to repeat laborious transformation and tissue culture (Hunter, 2021; Rodríguez-Leal *et al.*, 2017). Additionally, it might be applied for efficiency improvement of precise CRISPR/Cas9-mediated knock-ins of GOI via HDR (Movahedi *et al.*, 2021; M. Wang *et al.*, 2017; J.-P. Zhang *et al.*, 2017).

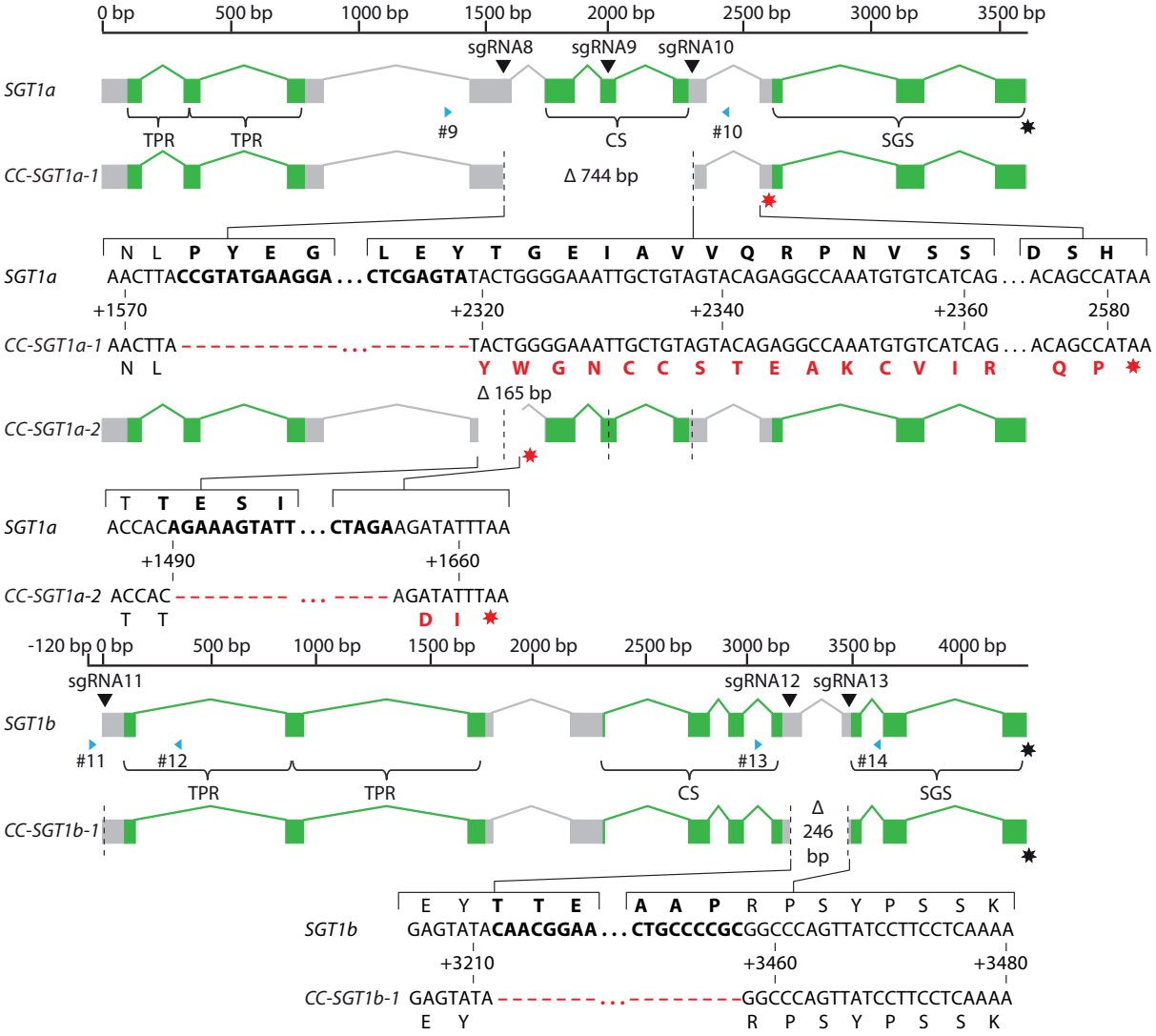

Figure 26. CRISPR/Cas9-mediated *SGT1a* and *SGT1b* mutagenesis and gene models of T0 *CC-SGT1a* and T0 *CC-SGT1b* mutants. Green blocks represent sequence regions encoding TPR, CS, and SGS domains. Black triangles and vertical dashed lines indicate sgRNA target sites. Blue triangles represent primers used for genotyping of T0 plants for putative *CC-SGT1a* and *CC-SGT1b* mutations. WT *SGT1a* and *SGT1b* genomic and protein sequences impacted by mutations are highlighted with bold black font. *CC-SGT1a* and *CC-SGT1b* mutations within genomic and protein sequences are marked with bold red font. Black and red stars represent the locations of native and premature stop codons, respectively.

Even though the fast breeding scheme saved one generation in the development of F2 *CC-EDS1 CC-Bs4 Bs3* plants in comparison to the conventional breeding scheme (Figure 24B), the abundance of numerous newly evolved *CC-EDS1* alleles complicated the analysis of mutations and selection of F2 plants for future phenotyping experiments.

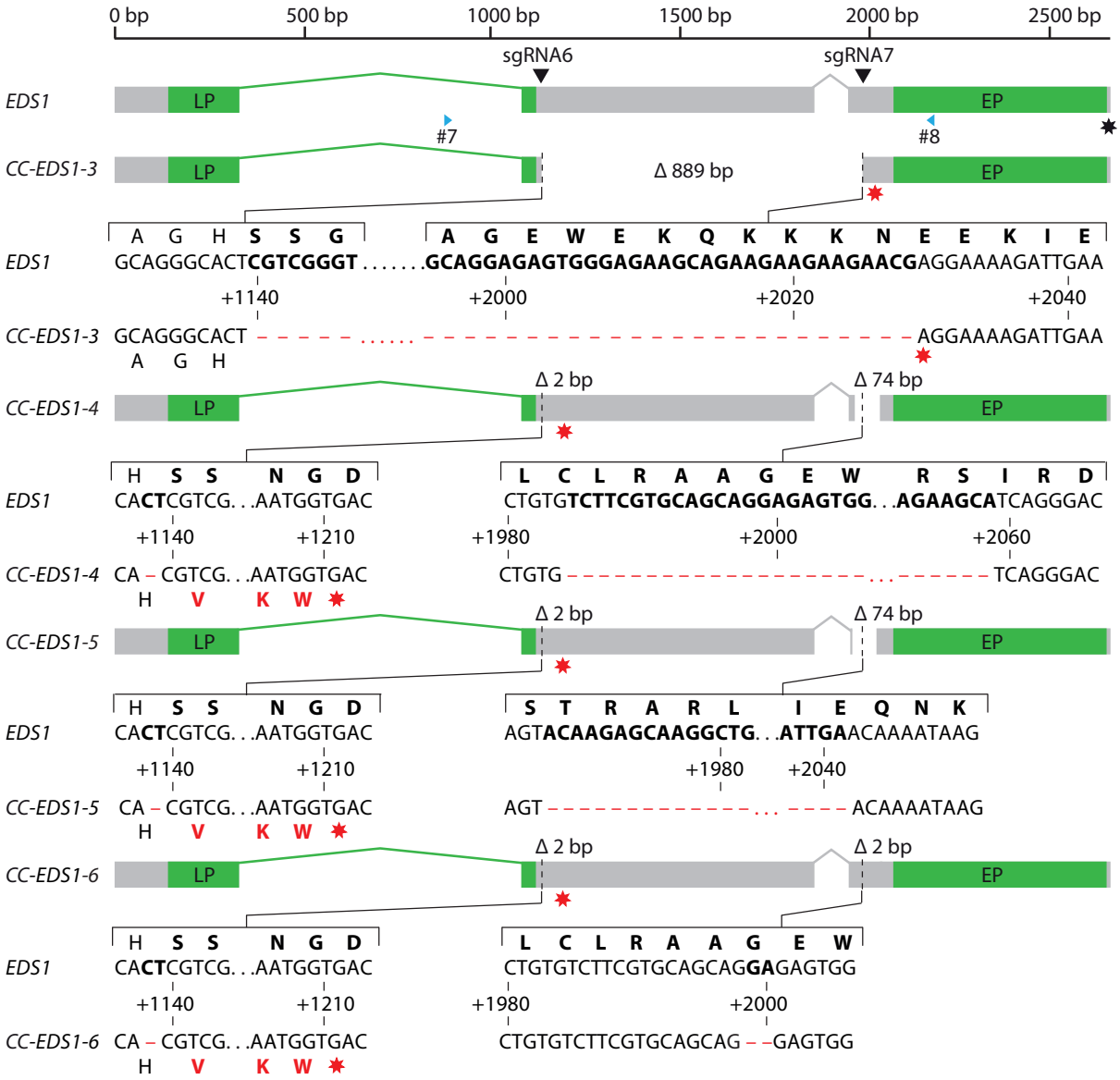

Figure 27. CRISPR/Cas9-mediated *EDS1* mutagenesis and gene models of four *CC-EDS1* alleles identified in F1 generation. Green blocks represent sequence regions encoding functional domains. Black triangles and vertical dashed lines indicate the sgRNA6 and sgRNA7 target sites. Blue triangles represent primers used for genotyping. WT *EDS1* genomic and protein sequences impacted by mutations are highlighted with bold black font. *CC-EDS1* mutations within genomic and protein sequences are marked with bold red font. Black and red stars represent the locations of native and premature stop codons, respectively.

2.3.2 CC-EDS1 does not abolish Bs3-mediated cell death in tomato.

In order to clarify if knock out of *EDS1* would abolish Bs3-mediated cell death, all *Cas9^{EDS1}*-free plants in the F2 generation were split into eight classes based on the combinations of *EDS1*/*CC-EDS1* and *Bs4*/*CC-Bs4* with the presence or absence of the *Bs3* transgene. The F2 plants representing *EDS1 Bs4 Bs3*, *EDS1 CC-Bs4 Bs3*, *CC-EDS1 Bs4 Bs3*, and *CC-EDS1 CC-Bs4 Bs3* genotypes and the controls, namely WT (*EDS1 Bs4*), HJ1 (*EDS1 CC-Bs4 Bs3*), H11 (*EDS1 CC-Bs4*), and E26 (*CC-EDS1-2 Bs4*) lines, were used in a phenotyping experiment (Figure 28). The selected plants were infiltrated with *Xe* 85-10 *avrBs4*, *Xe* 85-10 *dTALE34*, and *Xe* 85-10 EV and the phenotypes were observed 2 dpi (Figure 28). Since EDS1 is required for Bs4-mediated cell death signalling (Schornack *et al.*, 2004), the loss of AvrBs4 or dTALE34 recognition in *CC-EDS1 Bs4* background would indicate that the corresponding *CC-EDS1* allele is null.

Indeed, the reaction of the controls to infiltration with the *Xe* 85-10 strains was consistent with previous experiments. *Xe* 85-10 *dTALE34* caused Bs3-mediated cell death in the HJ1 (*EDS1 CC-Bs4 Bs3*) line (Figure 28). In the WT (*EDS1 Bs4*) line, AvrBs4 and dTALE34 triggered Bs4-mediated cell death, which was abolished in the H11 (*EDS1 CC-Bs4*) line (Figure 28). In addition, Bs4-mediated cell death upon delivery of AvrBs4 and dTALE34 was abolished in the E26 (*CC-EDS1-2 Bs4*) line (Figure 28), indicating that the *CC-EDS1-2* allele was null. Since *CC-EDS1-2* allele from the E26 (*CC-EDS1-2 Bs4*) line was predicted to produce a truncated protein lacking the functionally relevant EP-domain (Figure 25; Falk *et al.*, 1999; Parker *et al.*, 1996), other *CC-EDS1* alleles with the mutations that were predicted to cause a frameshift and proteins lacking an intact EP-domain were expected to be null (Figure S1).

Additional controls, *i.e.* the F2 plants #42 (*EDS1 Bs4 Bs3*) and #78 (*EDS1 CC-Bs4 Bs3*), resembled the same phenotypes as the WT (*EDS1 Bs4*) and HJ1 (*EDS1 CC-Bs4 Bs3*) lines, respectively (Figure 28). F2 plants #79 and #24, representing *CC-EDS1 Bs4 Bs3* and *CC-EDS1-4 CC-Bs4 Bs3* genotypes, did not show a cell death reaction upon infiltration of *Xe* 85-10 *avrBs4* (Figure 28). The loss of AvrBs4-triggered cell death reaction in the F2 plant #79 is due to the presence of *EDS1* null alleles (*CC-EDS1-7* and *CC-EDS1-8*) which produce truncated EDS1 proteins lacking EP-domain and are therefore deficient in mediation of downstream signalling from Bs4. In case of the F2 plant #24, it contains *Bs4* and *EDS1* null alleles (*CC-Bs4* and *CC-EDS1-4*) that are deficient in mediation of AvrBs4 recognition and downstream signalling. However, these genotypes (*CC-EDS1 Bs4 Bs3* and *CC-EDS1-4 CC-Bs4 Bs3*) showed a cell death

reaction upon infiltration of *Xe* 85-10 *dTALE34* (Figure 28). Collectively, these results indicate that *EDS1* knockout does not abolish Bs3-mediated cell death and therefore, Bs3 does not exploit EDS1-mediated signalling for the cell death execution.

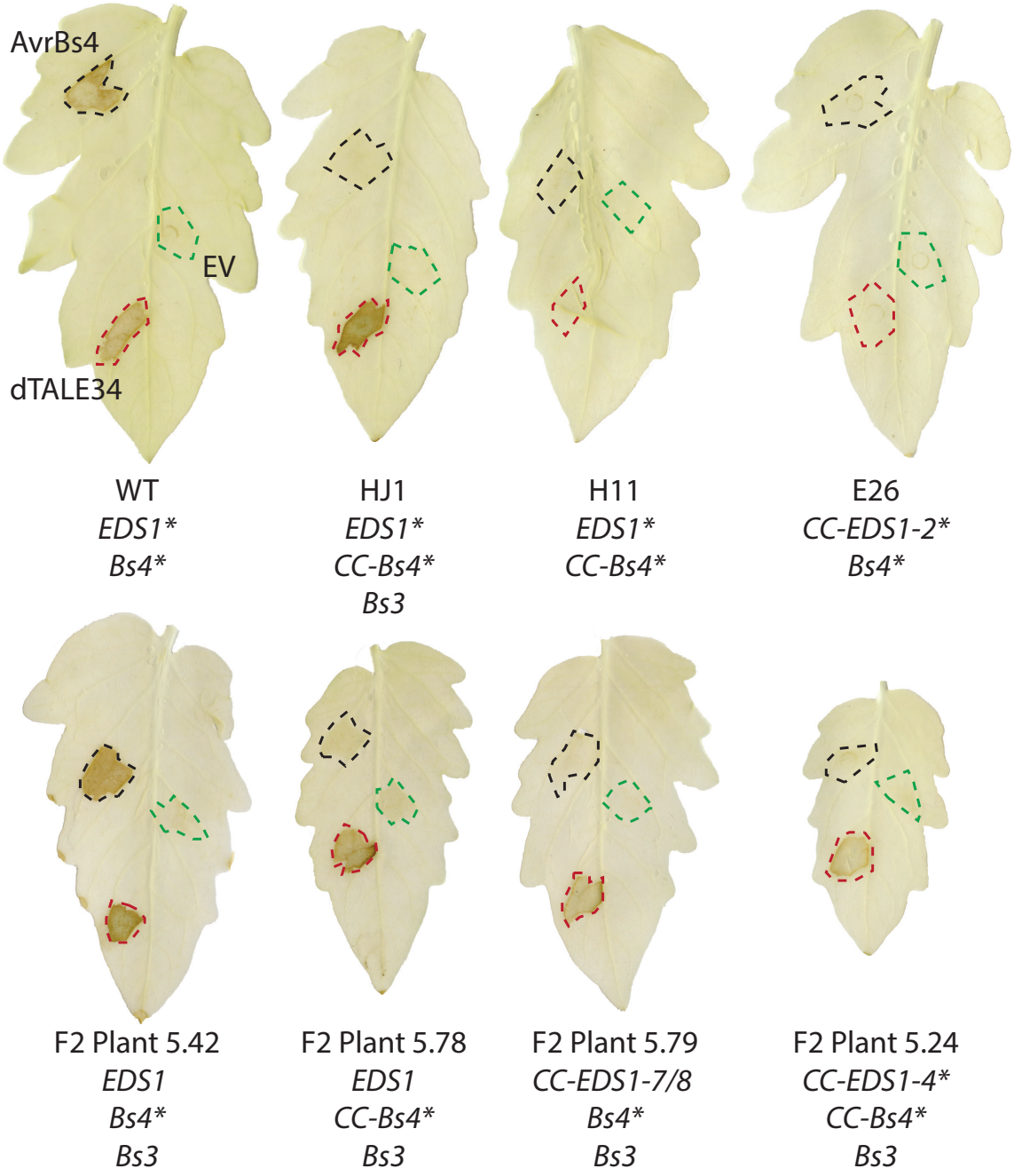

Figure 28. CC-EDS1 does not abolish Bs3-mediated cell death. All F2 lines were developed via the fast breeding scheme for multiple trait combination. WT (*EDS1 Bs4*), HJ1 (*EDS1 CC-Bs4 Bs3*), H11 (*EDS1 CC-Bs4*), and E26 (*CC-EDS1-2 Bs4*) lines served as the controls of Bs4-mediated AvrBs4 and dTALE34 recognition, EDS1-mediated downstream signalling, and Bs3-mediated cell death. F2 plants #42 and #78 served as positive controls for Bs3-mediated cell death reaction in *EDS1* background. Asterisk (*) indicates that the plant is homozygous for the given allele. All plants were infiltrated with *Xe* 85-10 *avrBs4* (left top; black; Bs4 activator), *Xe* 85-10 *dTALE34* (left bottom; red; Bs4 activator and *Bs3* transcriptional activator), and *Xe* 85-10 EV (right; green; negative control) at OD_{600} = 0.4. Phenotypes were observed 2 dpi. Leaflets were destained in 80% EtOH. Dashed lines mark the infiltrated area.

2.4 AvrBs3 manipulates the expression of tomato host genes.

Previous experiments demonstrated that Bs4 mediates recognition of AvrBs4, its derivative AvrBs4Δ227 lacking central repeats 5,5 – 17,5, NLS, and AAD (Figure 12B), and dTALEs with different repeat architecture, namely dTALE34 and dTALE35 (Figure 21). Therefore, it was hypothesised that Bs4 could mediate recognition of native TALEs with different repeat architecture, such as AvrBs3 and AvrHah1 from *Xanthomonas* spp. pathogenic on tomato and pepper.

2.4.1 Bs4 is epistatic to disease symptoms induced by AvrBs3 and AvrHah1 in tomato.

In order to test if Bs4 mediates recognition of AvrBs3 and AvrHah1, leaflets of WT (*Bs4*) and H11 (*CC-Bs4*) plants were infiltrated with *Xe* 85-10 strains containing *avrBs4*, *avrBs3*, *avrHah1*, and EV. In the *Bs4* background, AvrBs4 caused the cell death reaction, while AvrBs3 and AvrHah1 did not (Figure 29A). On the other hand, AvrBs3 and AvrHah1 caused mild hypertrophy in the *Bs4* background (Figure 29A). AvrBs4 did not cause any cell death reaction and any disease phenotype in the *CC-Bs4* background (Figure 29B), however, AvrBs3 and AvrHah1 caused severe hypertrophy and water-soaking in the *CC-Bs4* background. The disease symptoms caused by AvrBs3 and AvrHah1 in the *CC-Bs4* background were much stronger than in the *Bs4* background (Figures 29A and 29B). Infiltration with *Xe* 85-10 EV caused neither cell death reaction nor disease symptoms in both genetic background (Figures 29A and 29B). These experimental outcomes lead to the following conclusions: 1) AvrBs3 and AvrHah1 promote disease symptoms, such as hypertrophy and water-soaking in tomato; 2) Bs4 supresses disease symptoms caused of AvrBs3 and AvrHah1; 3) AvrBs4 does not promote any observable disease symptoms in tomato.

Despite the similarity of the repeat length between AvrBs4 (34 aa-long) / dTALE34 (34 aa-long) / AvrBs3 (34 aa-long) and between dTALE35 (35 aa-long) /AvrHah1 (mixed 34-long and 35 aa-long), it is unclear why in the *Bs4* background AvrBs4, dTALE34, and dTALE35 trigger Bs4-mediated recognition and cause cell death (Figure 21; Schornack *et al.*, 2004), while AvrBs3 causes cell death inconsistently (Figure 29A; Schornack *et al.*, 2004; Schwartz *et al.*, 2017) and AvrHah1 does not cause cell death (Figure 29A; Schwartz *et al.*, 2017).

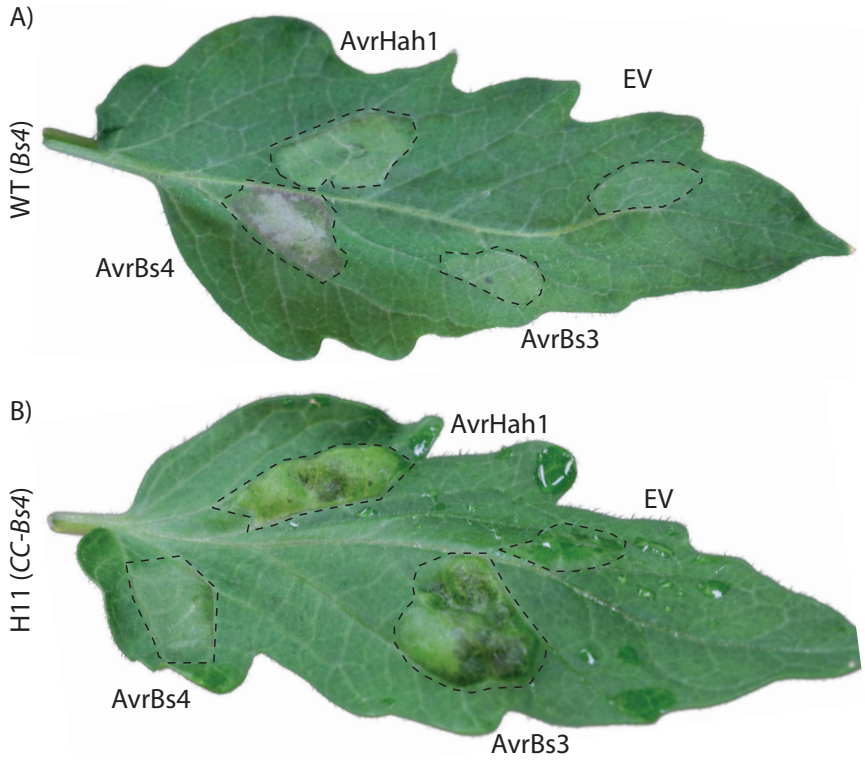

Figure 29. AvrBs3 and AvrHah1 cause severe hypertrophy and water-soaking in the *CC-Bs4* background. A) WT (*Bs4*) and B) H11 (*CC-Bs4*) leaflets were infiltrated with *Xe* 85-10 *avrBs4*, *Xe* 85-10 *avrBs3*, *Xe* 85-10 *avrHah1*, and *Xe* 85-10 EV strains (OD_{600} = 0.4). *Xe* 85-10 EV strain served as a negative control of TALE-induced disease symptoms. Plants were kept in high humidity conditions (≈96%). Phenotypes were observed 3 dpi. Dashed lines mark the infiltrated area.

2.4.2 Putative Bs4-mediated resistance to *Xe* 85-10 *avrBs4* was not quantified in the leaflet infiltration assay.

As delivery of AvrBs4 did not cause a cell death reaction (Figure 29B) and delivery of AvrBs3 and AvrHah1 resulted in severe disease symptoms in the *CC-Bs4* background (Figure 29B), it was assumed that the H11 (*CC-Bs4*) line should be more susceptible to TALE-encoding genes expressing *Xe* 85-10 strains than the WT (*Bs4*) line. To test this hypothesis, growth of *Xe* 85-10 *avrBs4* and *Xe* 85-10 EV strains in leaflets of *Bs4* and *CC-Bs4* plants was quantified at 0, 4, 7, and 9 dpi. At 9 dpi, there were no statistically significant differences in bacterial growth between the tested genotypes and strains ($p > 0.05$; Figure 30). These data demonstrate that the H11 (*CC-Bs4*) line is as susceptible to *Xe* 85-10 *avrBs4* as the WT (*Bs4*) line (Figure 30). In addition, the WT (*Bs4*) line did not show a Bs4-mediated resistance effect to *Xe* 85-10 *avrBs4* (Figure 30). These results are contradictory to the expectation that Bs4-mediated AvrBs4 recognition should restrict growth of the biotrophic *Xe* 85-10 *avrBs4* strain, therefore, these results and the experimental setting should be taken with caution.

The data for the aforementioned experiment were collected after growing total bacteria extracted from the tissue samples on medium containing only rifampicin, which selected for all *Xe* 85-10 CFUs. Therefore, there was no strict selection for pDSK602 *avrBs4* or pDSK602 *EV* containing *Xe* 85-10 CFUs on rifampicin and spectinomycin. It was assumed that there might be differences in the number of *Xe* 85-10 CFUs grown on rifampicin-containing medium, which included or excluded spectinomycin. Upon quantification of *in planta* growth of *Xe* 85-10 *avrBs4* and *Xe* 85-10 EV strains 9 dpi, there were 5 times fewer CFUs on rifampicin and spectinomycin-containing medium in comparison to medium containing only rifampicin (Figures 31A and 31B); these differences were statistically significant ($p \leq 0.01$; $p \leq 0.0001$). Even though this assay conditions did not reflect the natural infection scenario, its experimental outcomes indicate that not all of the *Xe* 85-10 CFUs propagated pDSK602 *avrBs4* and pDKS602 EV plasmids during their growth *in planta*. The loss of pDSK602 *avrBs4*

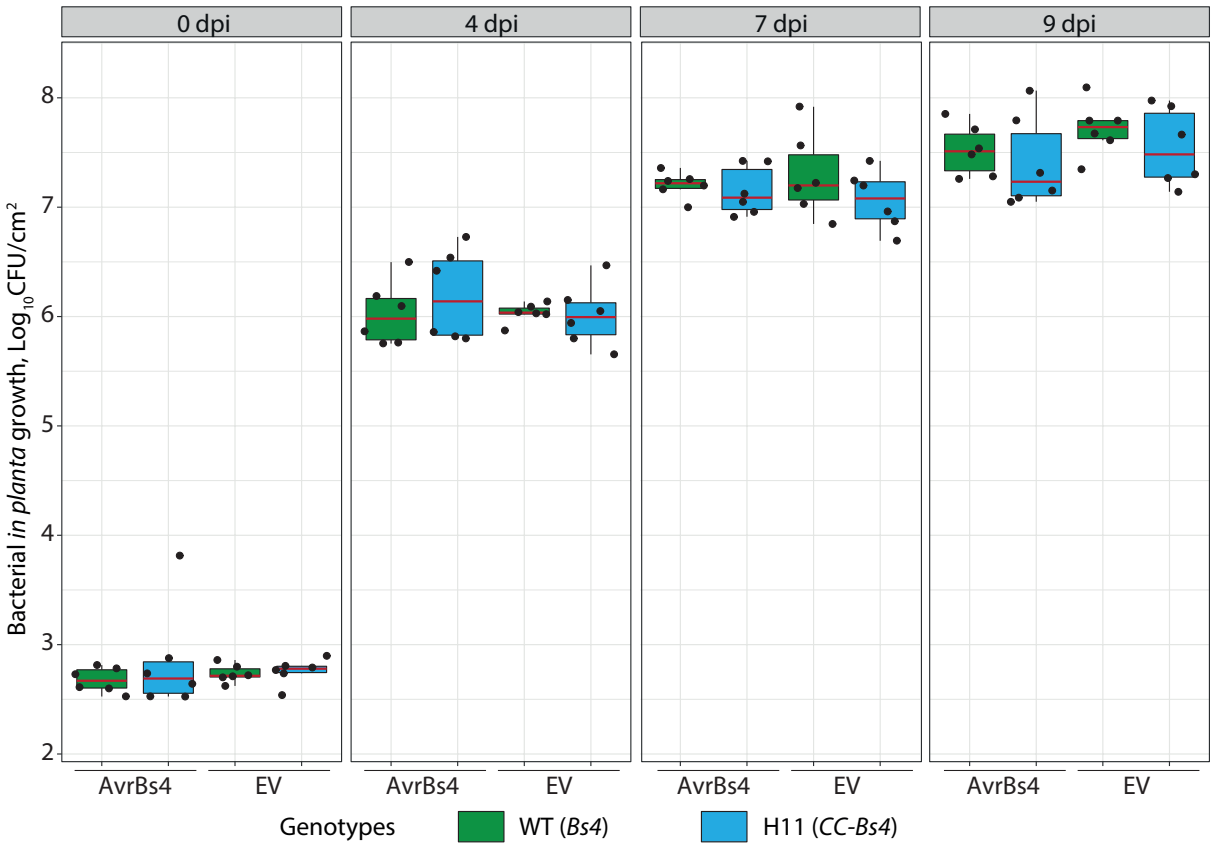

Figure 30. *Bs4* does not confer resistance to *Xe* 85-10 *avrBs4* in tomato 9 dpi. *Xe* 85-10 *avrBs4* and *Xe* 85-10 EV were infiltrated into WT (*Bs4*) and H11 (*CC-Bs4*) leaflets (OD_{600} = 4x10^{-5}). The WT line served as a positive control of Bs4-mediated resistance. *Xe* 85-10 EV served as a negative control of the AvrBs4-triggered Bs4 activation. Number of colony-forming units per square centimetre (CFUs/cm^2) was quantified 0, 4, 7, and 9 dpi and log_{10}CFUs/cm^2 scores were used for the boxplot and statistical analysis. n=6, *n* numbers of independent biological replicates. Pairwise Wilcoxon Rank Sum Test and FDR p-value adjustment method for multiple comparisons were used to calculate significant differences between groups ($p > 0.05$).

propagation would lead to lower rate of Bs4 activation and therefore, to impossibility to quantify Bs4-mediated resistance. Thus, a more stable vector system and infection methods mimicking the natural infection scenario, *e.g.* dipping of leaflets into an inoculum, should be used to enable reliable quantification of bacterial growth *in planta*.

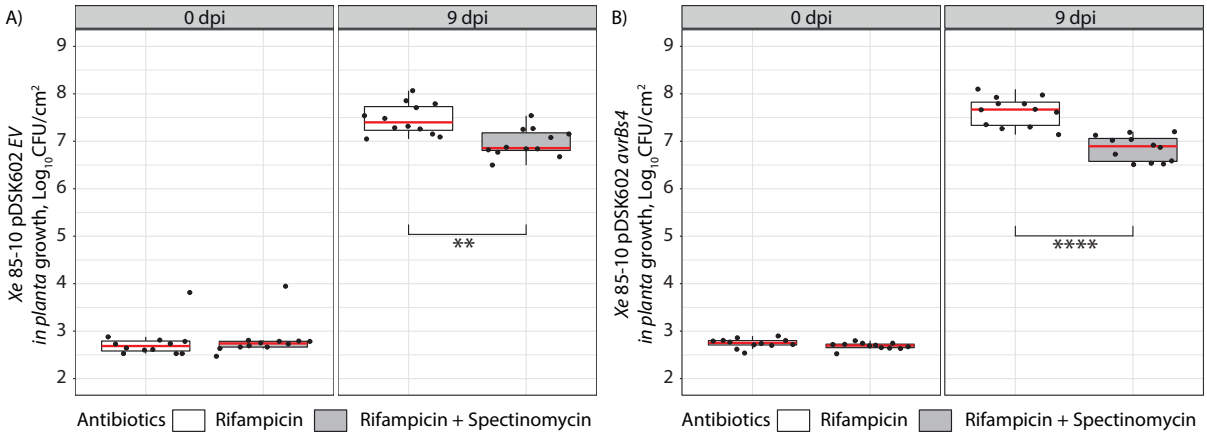

Figure 31. *Xe* 85-10 discard their pDSK602 *avrBs4* and pDSK602 EV plasmids *in planta*. *Xe* 85-10 *avrBs4* and *Xe* 85-10 EV were infiltrated in WT (*Bs4*) and H11 (*CC-Bs4*) leaflets (OD_{600} = $4x10^{-5}$). The medium containing only rifampicin was used to select total amount of *Xe* CFUs. The medium containing rifampicin and spectinomycin was used to select only pDSK602 plasmid carrying *Xe* CFUs. Bacterial growth quantification was made 0 and 9 dpi. n=12 (6 from WT and 6 from H11), *n* numbers of independent biological replicates. Number of colony-forming units per square centimetre ($CFUs/cm^2$) were counted 0 and 9 dpi and $log_{10}CFUs/cm^2$ scores were used for the boxplots and statistical analysis. Pairwise Wilcoxon Rank Sum Test and FDR p-value adjustment method for multiple comparisons were used to calculate significant differences between groups. **, $p \leq 0.01$; ****, $p \leq 0.0001$.

2.4.3 AvrBs3 upregulates transcription of *bHLH022* to cause hypotrophy and water soaking in tomato.

Even though the afore mentioned assay focusing on quantification of the bacterial growth *in planta* was not suitable for observation of the putative Bs4-mediated resistance in the *Bs4* background (Figure 30) and a putative virulence activity of AvrBs4 in the *CC-Bs4* background, differences in the phenotypes caused by AvrBs4, AvrBs3, and AvrHah1 in the *Bs4* and *CC-Bs4* backgrounds were remarkable (Figure 29B). It was assumed that the analysis of putative AvrBs4, AvrBs3, and AvrHah1 targets in tomato might clarify differential phenotypes caused by these TALEs.

bHLH022 (*Solyc03g097820*), a gene encoding a basic helix-loop-helix transcription factor 022, was previously described to be directly upregulated by AvrHah1 in tomato (Schwartz *et al.*, 2017). AvrHah1-mediated up-regulation of *bHLH022* was demonstrated to upregulate *PL*

(*Solyc05g014000*), a gene encoding a pectate lyase (Schwartz *et al.*, 2017). Therefore, *PL* was recognised a secondary target of AvrHah1 (Schwartz *et al.*, 2017). However, putative targets of AvrBs4 and AvrBs3 in tomato have not been reported so far. Tomato genome mining for the presence of putative EBEs targeted by AvrBs4 and AvrBs3 in promoter and 5' UTR regions of annotated tomato genes revealed putative TALE targets. The top 100 predicted EBEs for each TALE were processed to visualise consensus EBE sequences using sequence logos (Figure 32). In order to shortlist the potential target genes of AvrBs4 and AvrBs3, strict selection criteria were introduced to the EBE location in the promoter regions of putative targets (Boch *et al.*, 2009; Grau *et al.*, 2013; Pérez-Quintero *et al.*, 2013). Firstly, an EBE of a putative target should start with "T" at position "0" (Boch *et al.*, 2009). Secondly, an EBE should be in the forward orientation towards the predicted transcription start site of a putative target (Boch *et al.*, 2009; Grau *et al.*, 2013; Pérez-Quintero *et al.*, 2013). Thirdly, an EBE should be located

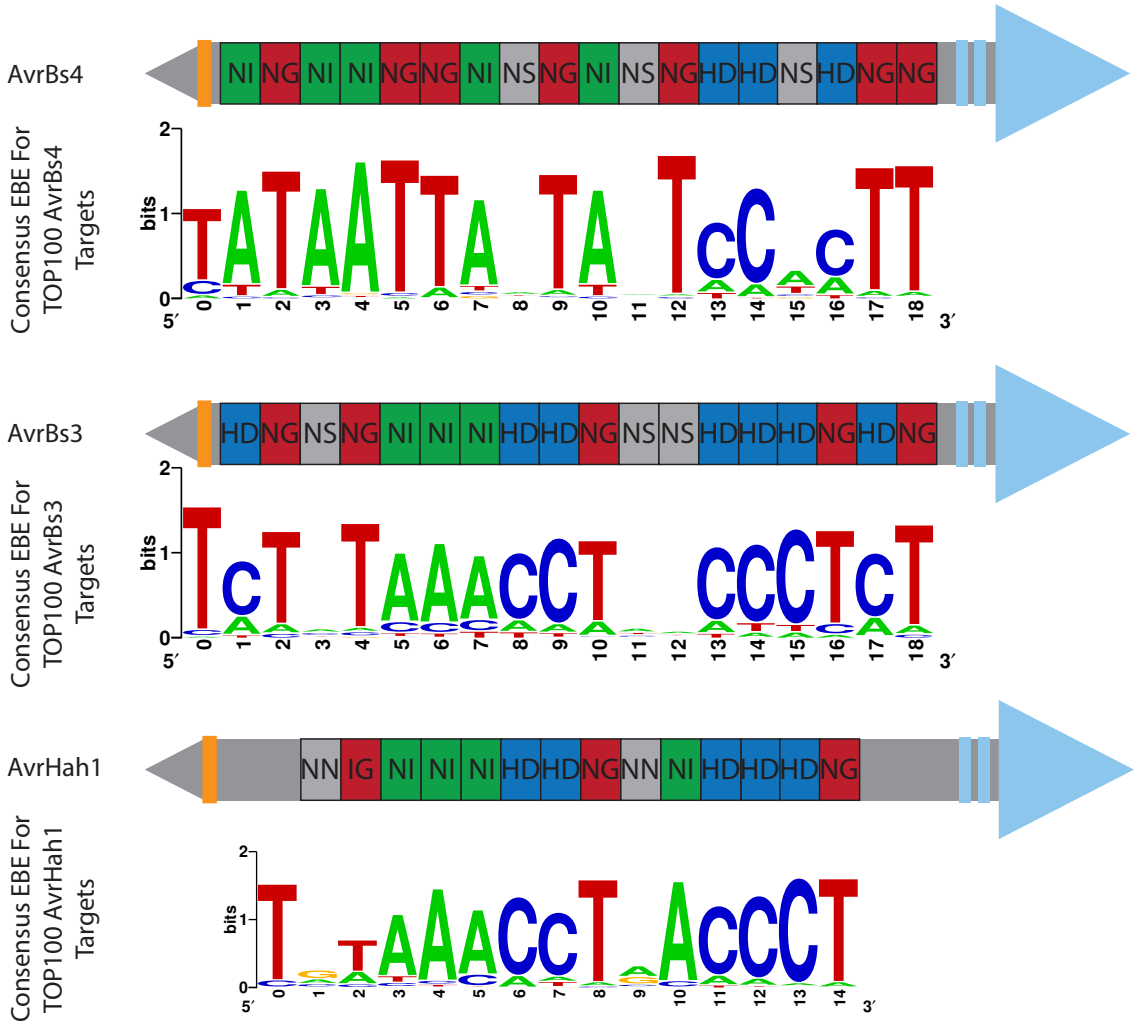

Figure 32. RVD composition of AvrBs4, AvrBs3, and AvrHah1 TALEs and sequence logos of their top 100 predicted targets in the tomato genome. RVD composition of the TALEs, *Sl*3.0 version of the tomato genome, and TALgetter software (Grau *et al.*, 2013) were used for the prediction of EBEs within promoter and 5' UTR sequences of annotated genes.

approximately 50 – 300 bp upstream a predicted start codon of a putative target (Grau *et al.*, 2013; Pérez-Quintero *et al.*, 2013).

Application of these criteria let to select four loci as putative AvrBs4 targets (Table 3), namely *Solyc00g050430* (*bHLH073*) encoding a basic helix-loop-helix transcription factor 073, *Solyc04g018050* (*UP*) encoding unknown protein, *Solyc10g009483* (*RING*) encoding E3 ligase, and *Solyc11g012130* (*BCP*) encoding blue copper protein. Since AvrBs3 and AvrHah1 target the same EBEs of *Bs3* and *UPA20* in *Capsicum* spp. (Schornack *et al.*, 2008), both TALEs may upregulate the same genes in tomato. Following this hypothesis, predicted putative target genes that were common for both TALEs were selected for further analysis (Table 3). The selected putative AvrBs3 and AvrHah1 targets were *Solyc01g057220* (*PE*) encoding a pectinesterase 4-related protein, *Solyc02g083450* (*AP*) encoding aspartic proteinase-like protein, and *Solyc03g097820* (*bHLH022*) encoding basic helix-loop-helix transcription factor 022.

Table 3. Known and selected putative AvrBs4, AvrBs3, and AvrHah1 targets with corresponding EBE sequences. EBE sequences in the promotor regions of the corresponding putative target genes were predicted with TALgetter (Grau *et al.*, 2013). Red-coloured nucleotides are not preferred base pairs for the optimal binding of corresponding RVDs.

TALEs	Crop	(Predicted) EBE Sequence	Locus	Gene ID	Protein Function
AvrBs4	*C. pubescens*	TATAAAAAATAGTCCTCTC	*AFW98885*	*Bs4C*	Putative TM Protein
AvrBs4	*S. lycopersicum*	TTTAATTATTAATCCACTT	*Solyc00g050430*	*bHLH073*	Transcription Factor
AvrBs4	*S. lycopersicum*	TACAACTACTAATCCCCTT	*Solyc04g018050*	*UP*	Unknown Protein
AvrBs4	*S. lycopersicum*	TATATTTAGTACTCCTCTT	*Solyc10g009483*	*RING*	RING/U-box Protein
AvrBs4	*S. lycopersicum*	TATAATTATTAATTCACTT	*Solyc11g012130*	*BCP*	Blue Copper Protein
AvrBs3/AvrHah1	*C. annuum*	TATATAAACCTAACCATCC	*Ca02g00940*	*Bs3*	FMO-like Protein
AvrBs3/AvrHah1	*C. annuum*	TATATAAACCTGACCCTTT	*Ca03g22700*	*UPA20*	Transcription Factor
AvrBs3/AvrHah1	*S. lycopersicum*	TATGTACACCTCCCCCTCT	*Solyc01g057220*	*PE*	Pectinesterase
AvrBs3/AvrHah1	*S. lycopersicum*	TCTGTAAACCTAACCCAAT	*Solyc02g083450*	*AP*	Aspartic Proteinase
AvrBs3/AvrHah1	*S. lycopersicum*	TATATAAACCTGACCCTTT	*Solyc03g097820*	*bHLH022*	Transcription Factor

To clarify if the TALEs interact with the predicted EBEs from the shortlisted putative TALE target genes from the tomato genome *in planta* (Table 3), the CDS of the *β-glucuronidase (GUS)* reporter gene from *E. coli* was transcriptionally fused to the native pepper *Bs3* promoter (*Bs3p*) however, in this *Bs3p* the native pepper EBE^{Bs3} was substituted with the EBEs of the predicted TALE targets from the tomato genome (Table 3). These constructs were

co-expressed with individual C-terminus GFP-labelled TALE-encoding genes under the transcriptional control of the *35s* promoter in *N. benthamiana* leaves (Figure 33A). Consecutively, infiltrated and harvested leaf disks were incubated in the 2% X-Gluc solution and destained in 80% EtOH to visualise enzymatic activity of β-Glucuronidase. In this experimental setting, any interaction between a TALE and an EBE would result in the transcriptional activation of the *GUS* reporter and therefore, the enzymatic activity of β-Glucuronidase could be detected. If the TALE would not interact with the EBE, the *GUS* reporter would not be transcriptionally activated and the enzymatic activity of β-Glucuronidase could not be detected.

This experiment revealed a strong enzymatic activity of β-Glucuronidase upon co-expression of *avrBs4-GFP* with the reporter constructs containing EBEs of *UP*, *RING*, and *BCP* within the *Bs3p* (Figure 33B), while only weak enzymatic activity of β-Glucuronidase was detected upon co-expression of *avrBs4-GFP* with the reporter construct containing $EBE^{bHLH073}$ within the *Bs3p* (Figure 33B). In addition, a strong enzymatic activity of β-Glucuronidase was observed upon co-expression of *avrBs4-GFP* with the *Bs3pEBE^{Bs4C}:GUS* reporter construct, which served as a positive control in this assay, since EBE^{Bs4C} is the confirmed binding site for AvrBs4 in *Capsicum* spp. (Strauß *et al.*, 2012). No enzymatic activity of β-Glucuronidase was observed upon co-expression of *avrBs4-GFP* with the negative controls, *i.e.* the reporter constructs containing EBEs of *Bs3* and other tomato genes predicted as AvrBs3 and AvrHah1 targets, namely *PE*, *AP*, and *bHLH022* (Figure 33B). These data demonstrate that AvrBs4 might interact with the EBEs of its four predicted target genes from tomato genome *in planta*.

In case of *avrBs3-GFP* and *avrHah1-GFP*, a strong enzymatic activity of β-Glucuronidase was detected upon co-expression of each of these TALE-encoding genes with the reporter constructs under the transcriptional control of the *Bs3p* containing EBEs of *PE*, *AP*, and *bHLH022*, as well as the EBE of *Bs3*, which served as a positive control in this assay, since EBE^{Bs3} is the confirmed binding site for AvrBs3 and AvrHah1 in *Capsicum* spp. (Figure 33B; Römer *et al.*, 2007; Schornack *et al.*, 2008). No enzymatic activity of β-Glucuronidase was detected upon co-expression of *avrBs3-GFP* and *avrHah1-GFP* with the reporter constructs containing EBEs of tomato genes predicted as AvrBs4 targets (Figure 33B). These data demonstrate that AvrBs3 and AvrHah1 might interact with the EBEs of their three predicted target genes from tomato genome *in planta*.

The performed *GUS* reporter assay demonstrated that AvrBs4, AvrBs3, and AvrHah1 might interact with the EBEs of their predicted target genes from tomato genome *in planta* and therefore, influence enzymatic activity of β-Glucuronidase (Figure 33B), however, such interactions might not result in a transcriptional upregulation of the predicted TALE target genes in tomato. In order to test if AvrBs4, AvrBs3, and AvrHah1 cause transcriptional upregulation of their predicted target genes in tomato, C-terminus GFP-labelled TALE-encoding genes under the transcriptional control of the *35s* cauliflower mosaic virus promoter were expressed via *Agrobacterium*-mediated T-DNA delivery in leaflets of C18 (*CC-Bs4*) and

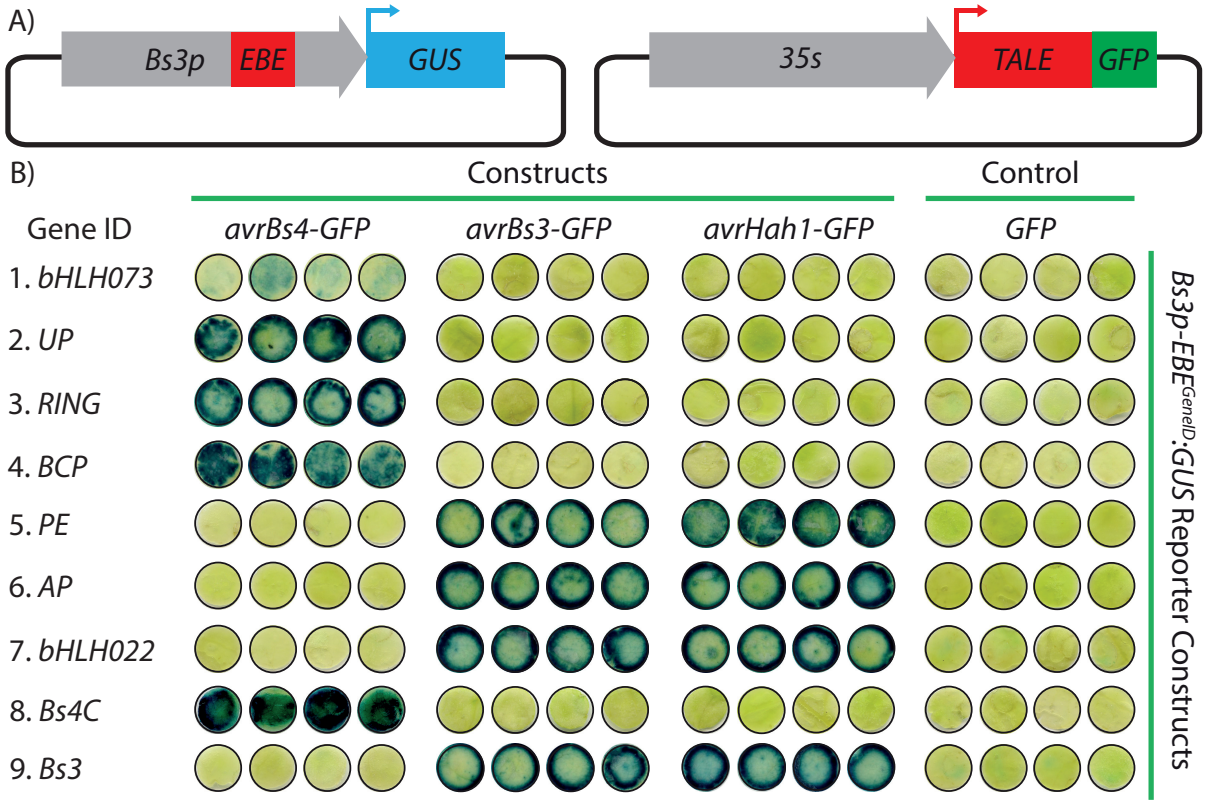

Figure 33. AvrBs4, AvrBs3, and AvrHah1 interact with the respective EBEs of their predicted tomato target genes *in planta*. A) Schematic of the constructs that were prepared for transient co-expression in *N. benthamiana* leaves. TALE-encoding genes were transcriptionally fused to the *35s* cauliflower mosaic virus promoter. TALE-encoding genes were translationally fused to C-terminal GFP fluorophore. The CDS of the *β-glucuronidase (GUS)* reporter gene from *E. coli* was transcriptionally fused to the native pepper *Bs3* promoter (*Bs3p*), however, in this *Bs3p* the native pepper EBE^{Bs3} was substituted with the EBEs of the predicted TALE targets from the tomato genome. B) *A. tumefaciens* GV3101 strains containing afore mentioned constructs were co-infiltrated into *N. benthamiana* leaves (OD_{600} = 0.6). *Bs3pEBE*^{Bs4C}*:GUS* served as a positive control for AvrBs4-dependent reporter transcriptional activation and as a negative control for AvrBs3 and AvrHah1. *Bs3pEBE*^{Bs3}*:GUS* served as a positive control of the transcriptional activation by AvrBs3 and AvrHah1 and as a negative control by AvrBs4. *35s* promoter-driven *GFP* served as a negative control for TALE-dependent transcriptional activation of the *GUS* reporter. Samples were collected 36 hpi. Leaf disks were stained in 2% X-Gluc solution for 24 hours and destained in 80% EtOH to visualise enzymatic activity of β-Glucuronidase.

WT (*Bs4*) plants. *35s* promoter-driven *GFP* was used as a negative control for TALE-dependent transcriptional upregulation of tomato genes. Samples for the semi-quantitative reverse transcription PCR (RT-PCR) analysis were harvested 48 hpi. Analysis of the amplified cDNA fragments revealed that none of the four predicted AvrBs4 targets were transcriptionally upregulated by AvrBs4, AvrBs3, AvrHah1, and GFP 48 hpi, neither in the *CC-Bs4* nor in *Bs4*

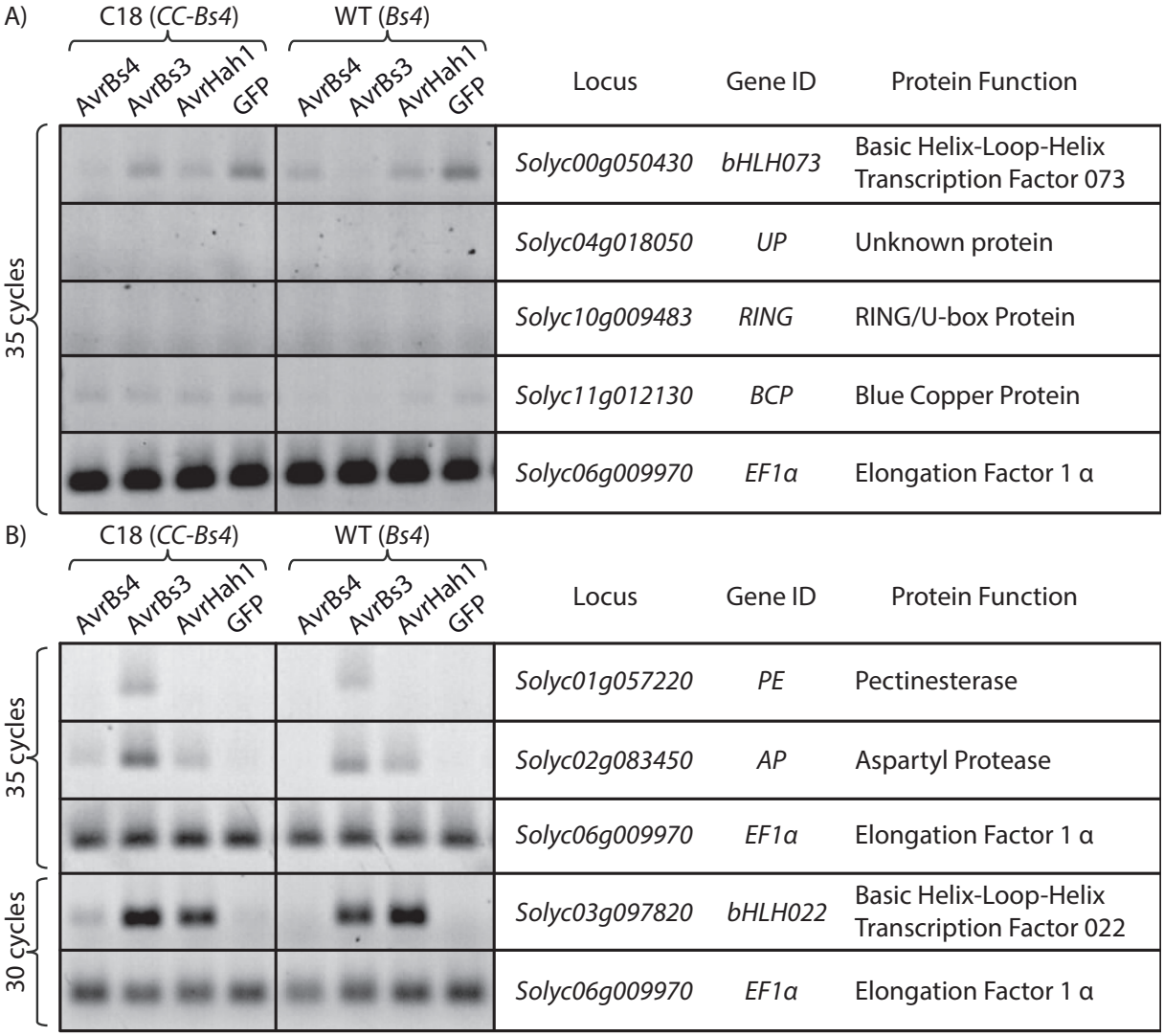

Figure 34. AvrBs3 transcriptionally upregulates all three tomato genes predicted as its putative targets. Semi-quantitative reverse transcription PCR (RT-PCR) was used to amplify transcripts of putative A) AvrBs4 and B) AvrBs3 and AvrHah1 tomato target genes in the C18 (*CC-Bs4*) and WT (*Bs4*) lines. Leaflets were inoculated with the indicated *A. tumefaciens* strains expressing C-terminus GFP-labelled TALE-encoding genes or only *GFP* under the transcriptional control of the *35s* cauliflower mosaic virus promoter (OD_{600} = 0.4). AvrBs4 served as a negative control for AvrBs3- and AvrHah1-dependent transcriptional upregulation of their predicted target genes. AvrBs3 and AvrHah1 served as negative controls for AvrBs4-dependent transcriptional upregulation of its predicted target genes. GFP served as a negative control of TALE-dependent transcriptional upregulation of any predicted target gene. Samples were collected 48 hpi. *EF1α* (*Elongation Factor 1α*; *Solyc06g009970*), a house-keeping gene, served as a positive control for amplification of transcript fragments of tomato genes.

backgrounds (Figure 34A). These results are in consent with the absence of visible AvrBs4-induced disease symptoms in the *CC-Bs4* background (Figure 29B). *PE* was exclusively upregulated by AvrBs3 in the *CC-Bs4* and in *Bs4* backgrounds, but not by AvrHah1, AvrBs4, and GFP (Figure 34B). *AP* and *bHLH022* were upregulated by both AvrBs3 and AvrHah1, but not by AvrBs4 and GFP in the *CC-Bs4* and in *Bs4* backgrounds (Figure 34B). These data indicate that despite high similarities within RVD composition of AvrBs3 and AvrHah1 (Schornack *et al.*, 2008), these TALEs have individual and common targets. In addition, the experiment demonstrated that the TALE-dependent transcriptional upregulation of the tomato genes happens in the *Bs4* and the *CC-Bs4* backgrounds.

Since Bs4 is epistatic to disease symptoms induced by TALEs from *Xanthomonas* spp. (Figure 29A), it was assumed that the epistatic effect of the *Bs4* background could be detected on transcriptional level and therefore, quantification of TALE-mediated upregulation of target genes and their secondary targets was made in *Bs4* and *CC-Bs4* backgrounds via qRT-PCR. For the purposes of this experiment, AvrBs3 was selected as an inducer, *bHLH022* as its target gene, and *PL* as a secondary target of AvrBs3. AvrHah1 and AvrBs4 were used as positive and negative controls, respectfully. *Xe* 85-10 strains expressing *avrBs4*, *avrBs3*, and *avrHah1* were syringe-infiltrated into leaflets of the WT (*Bs4*) and H11 (*CC-Bs4*) lines. Abundance of *bHLH022* and *PL* transcripts was quantified 24 hpi via qRT-PCR. The results showed that *bHLH022* was significantly upregulated by AvrBs3 and AvrHah1 but not by AvrBs4 ($p = 0.0041 < 0.01$; Figure 35A). There were no significant differences in the levels of AvrBs3- and AvrHah1-dependent transcriptional upregulation of *bHLH022* between the *Bs4* and the *CC-Bs4* backgrounds ($p = 0.18 > 0.05$; $p = 0.56 > 0.05$; Figure 35A). Quantification of *PL* transcripts revealed that this gene was significantly upregulated in the samples infiltrated with *avrBs3*- and *avrHah1*-expressing *Xe* 85-10 strains but not in the *Xe* 85-10 *avrBs4*-treated samples ($p = 0.0046 < 0.01$; $p = 0.014 < 0.05$; Figure 35B). Additionally, there were no significant differences in the levels of AvrBs3- and AvrHah1-dependent transcriptional upregulation of *PL* between the *Bs4* and the *CC-Bs4* backgrounds ($p = 0.1981 > 0.05$; $p = 0.8182 > 0.05$; Figure 35B).

These results indicate that Bs4 is epistatic only to AvrBs3- and AvrHah1-induced disease symptoms but not to the transcriptional upregulation of *bHLH022* and *PL* (Figures 29A, 29B, 35A, and 35B). In addition, this data demonstrates that AvrBs3 and AvrHah1 have a common primary target not only in pepper genome (*UPA20*), but also in tomato (*bHLH022*; Figure 35A).

Moreover, the EBE^{bHLH022} and EBE^{UPA20} targeted by AvrBs3 and AvrHah1 are identical (Table 3) and therefore, a further study of their flanking sequences may explain why these elements are conserved.

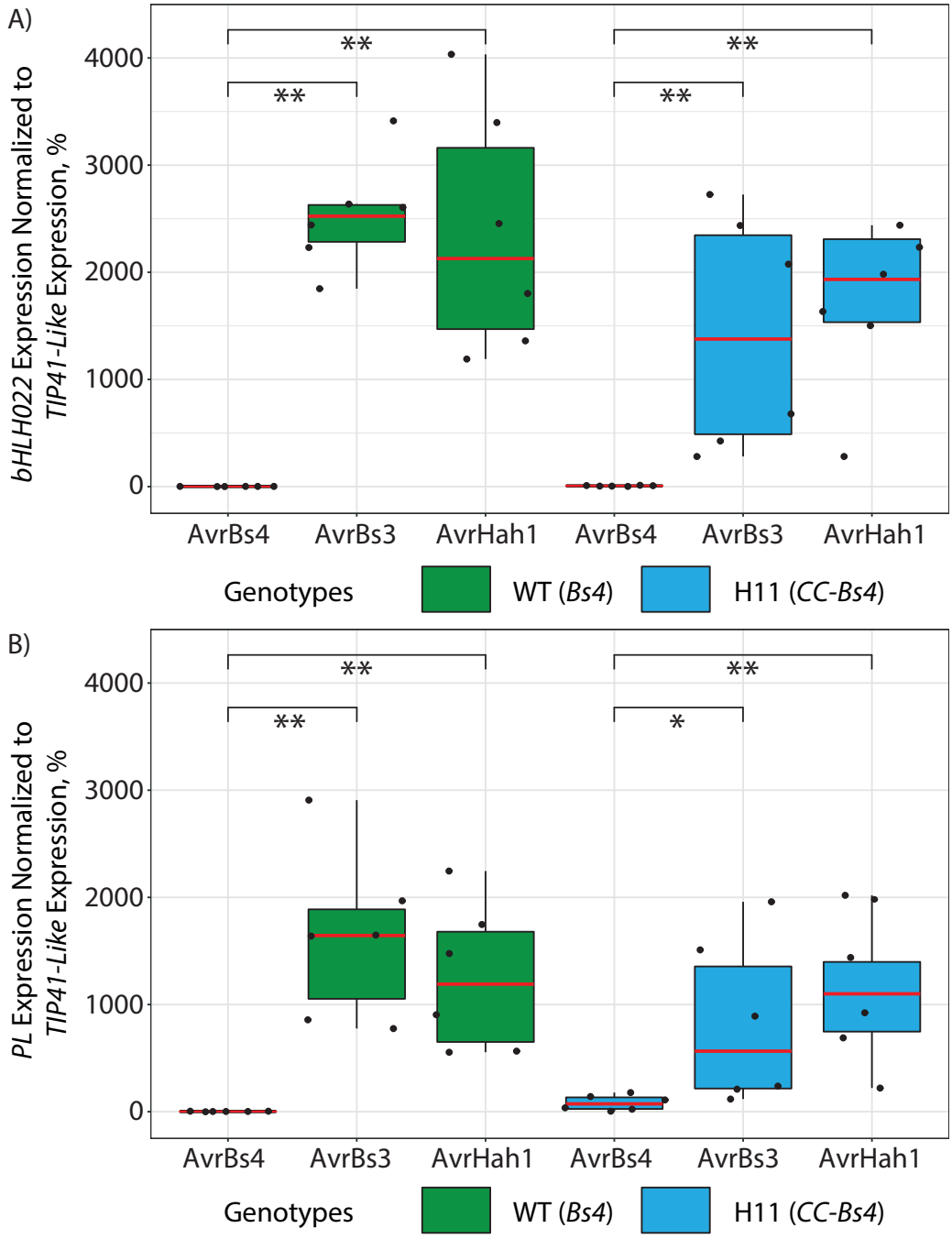

Figure 35. AvrBs3 and AvrHah1 transcriptionally upregulate *bHLH022* and *PL* in *Bs4* and *CC-Bs4* backgrounds. Transcript abundance analysis of A) *bHLH022* and B) *PL* in WT (*Bs4*) and H11 (*CC-Bs4*) leaflets infected with *avrBs4*, *avrBs3*, and *avrHah1* expressing *Xe* 85-10 (OD_{600} = 0.4). AvrBs4 served as a negative control of TALE-dependent transcriptional upregulation of *bHLH022* and *PL*. AvrHah1 served as a positive control of TALE-dependent transcriptional upregulation of *bHLH022* and *PL*. Samples were collected 24 hpi. n=6, *n* numbers of independent biological replicates. Pairwise Wilcoxon Rank Sum Test and FDR p-value adjustment method for multiple comparisons were used to calculate significant differences between groups. *, $p \leq 0.05$; **, $p \leq 0.01$.

2.4.4 The *UPA* box targeted by AvrBs3 and AvrHah1 and its flanking sequences are conserved within the 5' UTRs of *bHLH022*-like genes from solanaceous species.

EBE^{bHLH022} and EBE^{UPA20}, *i.e.* the *UPA* (*Upregulated by AvrBs3*) boxes identified upstream of tomato *bHLH022* and pepper *UPA20* start codons, were found to be identical (Table 3). Therefore, AvrBs3 and AvrHah1 could transcriptionally upregulate *bHLH022* and *UPA20* in both species (Figure 35A; Gürlebeck *et al.*, 2009; Kay *et al.*, 2007; Schornack *et al.*, 2008; Schwartz *et al.*, 2017). Moreover, AvrBs3 and AvrHah1 were previously reported to cause hypertrophy not only on tomato and pepper, but also on other solanaceous species (Kay *et al.*, 2007; Marois *et al.*, 2002). It was assumed that the genomes of all solanaceous species might contain *bHLH022*-like and *UPA20*-like genes with the *UPA* boxes, *i.e.* EBEs matching RVDs of AvrBs3 and AvrHah1, upstream of their start codons.

The genomic sequence of tomato *bHLH022* (*Solyc03g097820*) was used as a query for mining of *bHLH022*-like gene sequences within the genomes of other solanaceous species. Tomato *bHLH022* orthologues were identified in the genomes of *S. tuberosum* (*St*), *S. melongena* (*Sm*), *N. benthamiana* (*Nb*), *N. tabacum* (*Nt*), *N. attenuata* (*Nt*), *P. axillaris* (*Pa*), and *P. inflata* (*Pi*; Supplementary Information 5.2). Alignment of the sequence fragments located 300 upstream of the predicted or annotated start codons of *bHLH022*-like genes revealed presence of the 63 bp-long sequence which was highly-conserved among the genomes of all afore mentioned

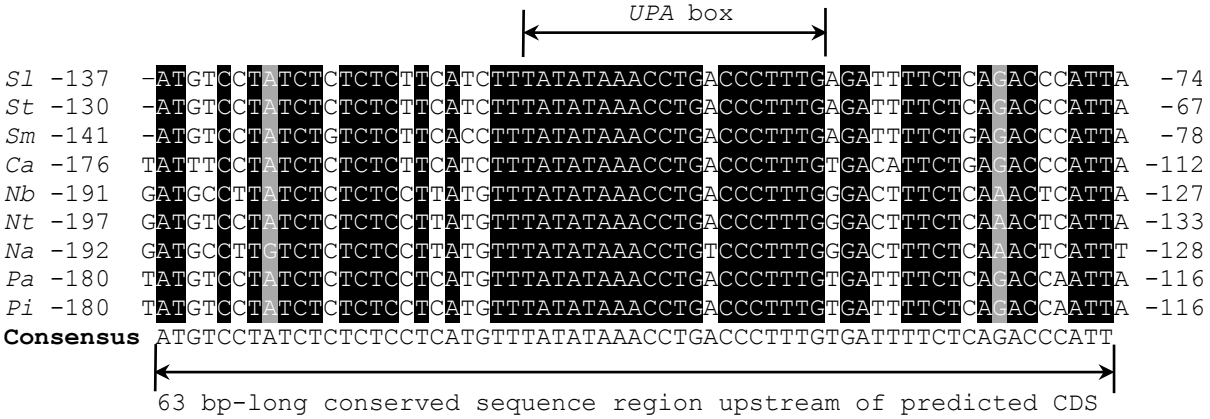

Figure 36. The *UPA* box targeted by AvrBs3 and AvrHah1 is a part of the 63 bp-long sequence which is conserved within the genomes of solanaceous species. Alignment of sequence fragments upstream of start codon of *bHLH022*-like genes from genomes of solanaceous species, namely *S. lycopersicum* (*Sl*), *S. tuberosum* (*St*), *S. melongena* (*Sm*), *C. annuum* (*Ca*), *N. benthamiana* (*Nb*), *N. tabacum* (*Nt*), *N. attenuata* (*Nt*), *P. axillaris* (*Pa*), *P. inflata* (*Pi*). 303 bp-long sequences (300 bp upstream and 3 bp representing the start codon) were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004). The most conserved nucleotides were annotated with the BoxShade algorithm. Shaded (black and grey) nucleotides represent the most conserved sequence fragments.

species including *S. lycopersicum* and *C. annuum* (Figure 36; Figure S2). Moreover, this 63 bp-long fragment contained the *UPA* box, *i.e.* 19 bp-long sequence which was identical to EBE^{bHLH022} and EBE^{UPA20} (Figure 36; Figure S2). These results indicate that the *UPA* box is highly conserved within sequence regions located upstream of the predicted or annotated start codons of *bHLH022*-like genes within the genomes of the inspected solanaceous species.

Such level of conservation of the *UPA* box and its flanking sequences suggested their putative role as *cis*-regulatory elements of *bHLH022*-like genes. Further mining of transcriptome reference sequences of solanaceous species revealed that the identified 63 bp-long sequence fragment containing the *UPA* box is a part of the transcribed RNA in *Nicotiana*, *Petunia*, and *Capsicum* spp. (Supplementary Information 5.3; Kay *et al.*, 2007). These results suggest that the identified sequence fragment containing the *UPA* box, *i.e.* EBE for AvrBs3 and AvrHah1, is a part of 5' UTR and might be important for regulation of translation of *bHLH022*-like genes. Therefore, AvrBs3 and AvrHah1 exploit a conserved element upstream of *bHLH022*-like genes to transcriptionally upregulate them and to promote disease symptoms in numerous solanaceous species.

In summary, tomato genes that might be upregulated by AvrBs4 were not identified in this study (Figure 34A), however, AvrBs3 and AvrHah1 were found to transcriptionally upregulate *bHLH022* in a direct manner and *PL* in an indirect manner (Figures 35A and 35B). Transcriptional upregulation of tomato *bHLH022* and *PL* by AvrBs3 and AvrHah1 was found to correlate with the AvrBs3- and AvrHah1-caused disease symptoms, *i.e.* hypertrophy and watersoaking (Figure 29B). In addition, the EBE^{bHLH022} was found to be identical to the EBE^{UPA20} (Table 3; Figure 36). Moreover, these *UPA* boxes and their flanking sequences are conserved within the upstream sequences of *bHLH022*-like genes from numerous solanaceous species suggesting their putative role as *cis*-regulatory elements of *bHLH022*-like genes (Figure 36; Supplementary Information 5.2). These results provide an explanation to why AvrBs3 and AvrHah1 cause similar disease symptoms in numerous solanaceous species.

In addition to the afore mentioned results, Bs4 was found to be epistatic to TALE-caused disease phenotypes in tomato (Figure 29A), however, Bs4 had no detectable impact on AvrBs3- and AvrHah1-dependent transcriptional upregulation of *bHLH022* and *PL* (Figures 35A and 35B). It is still unknown if the transcriptional upregulation of *bHLH022* and *PL* by AvrBs3 and AvrHah1 might affect bacterial growth *in planta*. Therefore, a modification to the

pDSK602 plasmid system for TALE-encoding gene expression or a genomic integration of TALE-encoding genes to the *Xe* 85-10 strain as well as deployment of assays mimicking the natural infection scenario, *e.g.* dipping of leaflets into an inoculum, should be used to enable reliable quantification of bacterial growth *in planta* and testing of assumptions regarding TALE-caused disease symptoms and their putative effect on bacterial growth *in planta*.

3 DISCUSSION

3.1 Bs4 should play a crucial role in immunity of *Solanum* spp.

Previous investigations identified two *S. pennellii* accessions, namely LA2963 and LA1282, which had no cell death reaction 2 dpi with *avrBs4* expressing *X. euvesicatoria* strain (Ballvora, Pierre*, et al.*, 2001). However, testing of *Xe* 85-10 *avrBs4* recognition in LA2963 line revealed visible cell death reaction 6 dpi suggesting presence of a protein that mediates AvrBs4 recognition (Figure 7B). Co-expression of $SpBs4^{gDNA}$ and *avrBs4*, as well as $SpBs4^{CDS\text{-}\beta}$ and *avrBs4* under the control of the 35s promoter in *N. benthamiana* resulted in a cell death phenotype (Figures 8A and 8C), indicating that *SpBs4* is not a null allele, but rather a reduced function allele and is capable of TALE, truncated TALE (truncTALE), and dTALE recognition in the *S. pennellii* accession LA2963 and *S. lycopersicum* MM^{SpBs4}-BC4 line.

The reduced functionality of SpBs4 might be caused by one or more of 74 identified nucleotide polymorphisms when compared to SlBs4 (Schornack *et al.*, 2004). These evolutionary changes in *SpBs4* could have been influenced by ecologic adaptation of *S. pennellii* to growth in arid areas in the Andes mountains (Correll, 1958; Hardon, 1967). These extreme environmental conditions are not favourable for *in planta* growth of *Xanthomonas* spp. Thus, there is no evolutionary pressure on *S. pennellii* plants to maintain a fully functional orthologue of *SlBs4*.

In order to identify a *Bs4* null allele, numerous *S. lycopersicum* accessions from different geographic origins were screened, however functional *Bs4* alleles were found in all of them (Figure 9; Peter, 2002). Moreover, *Bs4* orthologues were reported to be found within the genomes of other *Solanum* spp. , namely *S. demissum* and *S. tuberosum* (Schornack *et al.*, 2005). Prevalence of *Bs4* orthologues within the genomes of so many *Solanum* spp. suggests that it has an important biological function. Therefore, Bs4 might provide solanaceous plants with a basic defence against *Xanthomonas* spp. with rapidly evolving TALEs and their derivatives. Bs4 was described to mediate recognition of Hax3, Hax4 (Kay *et al.*, 2005), AvrBs3 (Schwartz *et al.*, 2017), AvrBs4 and its numerous C-terminus truncated versions ranging from AvrBs4Δ215 lacking NLS and AAD to AvrBs4Δ230 lacking central repeats 3,5 – 17,5, NLS and AAD (Schornack *et al.*, 2004), consequently, Bs4-mediated cell death should restrict growth of the biotrophic pathogen. However, quantification of the bacterial growth in *Bs4* and *CC-Bs4* backgrounds did not demonstrate the Bs4-mediated resistance to the *Xe* 85-10 *avrBs4* strain

(Figure 30). Similar results were previously reported by Bonas *et al.* (1993). This study demonstrated that Bs4-mediated cell death restricted *in planta* growth of *Xe* 82-8 strain which naturally contains *avrBs4* gene, but failed to restrict *in planta* growth of *Xe* 75-3 *avrBs4* strain and other *Xe* 75-3 strains carrying numerous *avrBs4* derivatives. The most probable explanation to the inconsistency of Bs4-mediated resistance is the deployment of the infection method that does not mimic the natural infection scenario, *i.e.* infiltration with a blunt-end needless syringe infiltration (Figure 30; Bonas *et al.*, 1993). Therefore, a deployment of an alternative infection assay, *e.g.* swabbing of carborundum-containing inoculum on the abaxial side of the tomato leaflet or dipping of leaflets into inoculum (Canteros *et al.*, 1991; Cerutti *et al.*, 2017; Luneau *et al.*, 2021), might help to quantify Bs4-mediated resistance to *Xe* 85-10 *avrBs4* strain in the *Bs4* background or the putative loss of Bs4-mediated resistance to this strain in the *CC-Bs4* background.

The hypothesis that Bs4 provides solanaceous plants with a basic defence against *Xanthomonas* spp. with rapidly evolving TALEs may be supported by T3SS effectome profiling of strains pathogenic on *Solanum* and *Capsicum* spp. The screens of *Xanthomonas* strains collected from *S. lycopersicum* do not report any strain that harbours *avrBs4* or *avrBs3*, since these genes can only be found in strains pathogenic in *Capsicum* spp. (Canteros *et al.*, 1991; Stall *et al.*, 2009). Lack of *avrBs4* and *avrBs3* within the effectome of strains pathogenic in tomato might be explained by a severe Bs4-mediated selection pressure. Since these TALE-encoding genes are plasmid born (Stall *et al.*, 2009), loss of the plasmid allows bacterial strains to remain pathogenic in tomato.

Several studies on tomato bacterial spot report structural shifts in the species composition of *Xanthomonas* populations that are stimulated by three main factors: a) the uniformity of the global seed market with the prevalence of a few cultivars (Timilsina *et al.*, 2015); b) the use of intensive agronomic practices for tomato fruit production, *e.g.* high plant densities, over-head irrigation, and high humidity, and temperatures (Abrahamian *et al.*, 2020; Klein-Gordon *et al.*, 2020). The third factor is a recombination between different *Xanthomonas* spp. via horizontal gene transfer (Timilsina *et al.*, 2015).

Currently *X. euvesicatoria* is being quickly replaced by *X. perforans* (*Xp*) in Florida (Klein-Gordon *et al.*, 2020). In addition, *X. gardneri* was found to be highly prevalent in tomato production facilities in different regions of the world (Potnis *et al.*, 2015). These pathogens

are associated with two TALEs, namely AvrHah1 and PthXp1, which could be acquired via horizontal gene transfer between different *Xanthomonas* spp. It has also been reported that *X. perforans* populations demonstrate genotypic and phenotypic differences, despite lacking a clear selection pressure (Klein-Gordon *et al.*, 2020). However, since Bs4 is epistatic to the disease symptoms caused by AvrHah1 and AvrBs3 (Figures 29A and 29B), it might also limit the activity of PthXp1, which remains to be uncovered.

Another TALE that does not cause cell death upon expression in the *Bs4* background is Hax2, a TALE from *Xca* (Kay *et al.*, 2005; Schornack *et al.*, 2008). Both AvrHah1 and Hax2 are the most distant from other AvrBs3-like proteins and share a unique structure of their tandemly arranged 35 amino acid-long repeat units, which is hypothesised to help AvrHah1 and Hax2 to avoid recognition by Bs4 (Schornack *et al.*, 2008; Schwartz *et al.*, 2017). However, dTALE35, a dTALE with tandemly arranged 35 aa-long repeats, was recognised by Bs4 and triggered the Bs4-mediated cell death (Figure 21B). Thus, it remains unclear why the 35 aa-long repeats-containing TALE proteins, namely AvrHah1 and Hax2, do not trigger Bs4-mediated cell death. Nevertheless, TALE co-immunoprecipitation would be an alternative method to determine if Bs4 binds to tagged AvrHah1, Hax2, and PthXp1, and, therefore, confers their direct recognition (Read, Hutin*, et al.*, 2020).

3.2 Does AvrBs4 transcriptionally upregulate tomato host genes?

Due to its central role in mediation of TALE recognition, Bs4 is epistatic to TALE-induced disease symptoms (Figures 29A and 29B). CRISPR/Cas9-engineered *Bs4* null allele (*CC-Bs4*) does not mediate recognition of TALEs delivered by *Xanthomonas* spp. and thus, unmasks TALE-caused phenotypes. It is remarkable that AvrBs4 does not cause any visible disease symptoms in the *CC-Bs4* background (Figure 29B). Lack of visible AvrBs4-induced disease symptoms can be explained either by the absence of AvrBs4 targets within the tomato genome or by the impossibility to detect these symptoms on a leaflet surface.

AvrBs4 has previously been reported to transcriptionally activate *Bs4C*, an executor *R* gene from *Cp* (Strauß *et al.*, 2012), while no other targets in pepper or other species were described. Overexpression of *avrBs4*, but not of *avrBs4Δ227* lacking central repeats 5,5 – 17,5, NLS, and AAD, was found to lead to formation of catalase-derived rhomboid crystals in peroxisomes of *N. benthamiana* cells (Gürlebeck *et al.*, 2009). It was hypothesised that AvrBs4-induced

catalase accumulation might inhibit plant defences via detoxification of hydrogen peroxide (Gürlebeck *et al.*, 2009).

avrBs4 was originally isolated from pepper pathogenic *Xe* 82-8 strain (Table 4; Bonas *et al.*, 1993). To clarify if AvrBs4 might target any EBEs within the promoters of annotated tomato genes, the tomato genome was checked for presence of such putative EBEs with TALgetter (Grau *et al.*, 2013) *In silico* analysis revealed *bHLH073* (*Solyc00g050430*) to be the most probable AvrBs4 target (Table 3). Testing the binding ability of AvrBs4 to the predicted $EBE^{bHLH073}$ in the reporter assay *in planta* resulted into weak enzymatic activity of β-Glucuronidase indicating that the interaction between AvrBs4 and $EBE^{bHLH073}$ was weak (Figure 33B). This result is consistent with the previous study which demonstrated that AvrBs4 induces less transcription than AvrBs3 (Gürlebeck *et al.*, 2009). Since *bHLH073* encodes a transcription factor, even small changes in its expression may significantly contribute to the development of disease symptoms.

Table 4. Original *Xanthomonas* strains and TALE-encoding genes isolated from them.

TALE-encoding gene	Bacterial strain (source)	Host (isolated on)	References
avrBs4	*Xe* 82-8	Pepper	Bonas *et al.* (1993)
avrBs3	*Xe* 71-21	Pepper	Bonas *et al.* (1989)
avrHah1	*Xg* 444	Tomato	J B Jones *et al.* (2000); Schornack *et al.* (2008)

Analysis of the $EBE^{bHLH073}$ and RVD composition of AvrBs4 revealed possible technical reasons for the weak interaction and consequently, the absence of putative visible disease symptoms. One of these reasons is the mismatch in the first position of the $EBE^{bHLH073}$ (Figure 37), which is expected to prevent AvrBs4 binding. “NI”, the first RVD of the N-terminal repeat of AvrBs4, should preferably binds to “A” (Boch *et al.*, 2009; Miller *et al.*, 2015), while $EBE^{bHLH073}$ contains “T” at this position (Figure 37). The second reason that could explain the absence of putative visible disease symptoms upon delivery of AvrBs4 in tomato is the location of the $EBE^{bHLH073}$ 510 bp upstream of the predicted translation start site of *bHLH073*. In the case that *bHLH073* is weakly upregulated by AvrBs4 in tomato, 5’ extension of the messenger RNA (mRNA)

transcript of *bHLH073* would occur, which might trigger degradations via nonsense mediated decay or translated into non-functional protein.

C-terminus AvrBs4 truncations ranging from AvrBs4Δ215 lacking NLS and AAD and to AvrBs4Δ230 lacking central repeats 3,5 – 17,5, NLS and AAD are known to trigger Bs4-mediated cell death (Schornack *et al.*, 2004). Since all tested AvrBs4 derivatives contained "NI" RVD of the N-terminal repeat at the position "1" and lacked the activation domain (Schornack *et al.*, 2004), they could not transcriptionally upregulate *bHLH073*. Substitution of "NI" (binding to "T"), the first N-terminal repeat of AvrBs4, to "NG" (binding to "T") may overcome these binding limitations. Alternatively, a dTALE targeting EBE^{bHLH073} or any other part of the *bHLH073* native promoter could be designed to activate transcription of *bHLH073*. Thus, an AvrBs4$^{1\text{-NI>NG}}$ mutant or a dTALE targeting EBE^{bHLH073} might be able to transcriptionally upregulate *bHLH073* and to cause putative disease symptoms in the *CC-Bs4* background.

It still remains unknown if *X. euvesicatoria*-mediated delivery of AvrBs4 leads to formation of catalase-derived rhomboid crystals in peroxisomes of tomato cells and if this phenotype can be caused by putative transcriptional upregulation of *bHLH073* (Gürlebeck *et al.*, 2009). To find the answers to these questions, an RNA sequencing (RNA-Seq) approach on tomato tissue infected with *Xanthomonas* spp. strains expressing *avrBs4* and *avrBs4Δ227* lacking central repeats 5,5 – 17,5, NLS, and AAD, should be used to identify host genes that are directly or indirectly upregulated by AvrBs4 (Schwartz *et al.*, 2017).

3.3 AvrHah1 and AvrBs3 target a putative *cis*-regulatory element within 5'UTR of *bHLH022*-like genes.

Afore mentioned RNA-Seq approach was used for the identification of direct and indirect targets of AvrHah1 from *X. gardneri* in the tomato genome (Schwartz *et al.*, 2017). The study

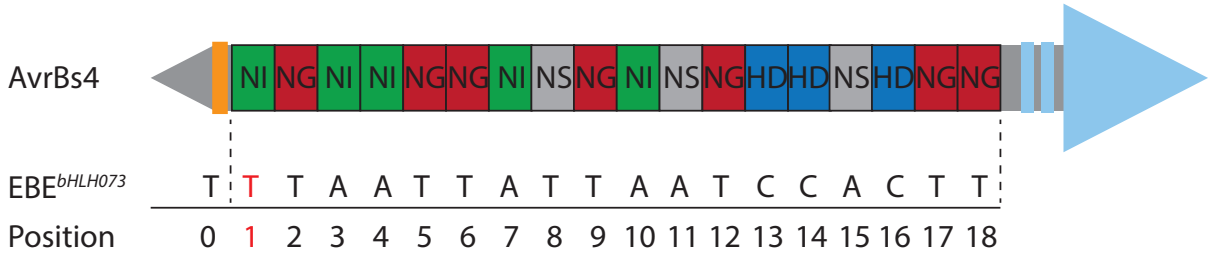

Figure 37. The mismatch at position "1" may prevent AvrBs4 binding to EBE^{bHLH073}. AvrBs4 RVD structure and corresponding nucleotides within EBE^{bHLH073} are annotated. Each RVD is coloured according to its optimal binding preferences: green = "A", red = "T", grey = "N", and blue = "C".

reported that AvrHah1 indirectly targets a *Pectate Lyase* (*Solyc05g014000*) via transcriptional upregulation of *bHLH022* (*Solyc03g097820*) and *bHLH048* (*Solyc06g072520*). However, no tomato genes were reported as targets of AvrBs3.

avrBs3 was originally isolated from pepper pathogenic *Xe* 71-21 strain (Table 4; Bonas *et al.*, 1989), while *avrHah1* was identified in tomato pathogenic *Xg* 444 strain (Table 4; JB Jones *et al.*, 2000; Schornack *et al.*, 2008). Interestingly, AvrBs3 and AvrHah1 have a similar RVD compositions (Schornack *et al.*, 2008), which enables both TALEs to transcriptionally upregulate *UPA20* (*Ca03g22700*) in *Capsicum* spp. (Kay *et al.*, 2007; Schornack *et al.*, 2008). *UPA20* encodes a basic helix-loop-helix transcription factor which induces hypertrophy (Kay *et al.*, 2007). It was previously demonstrated that AvrHah1-induced hypertrophy leads to water soaking in tomato and encourages intake of surface localised bacteria into *N. benthamiana* leaves (Schwartz, 2016; Schwartz *et al.*, 2017). In addition, AvrBs3 and AvrHah1 bind to EBE^{Bs3} and activate transcription of *Bs3* (*Ca02g00940*) in *Capsicum* spp. (Römer *et al.*, 2007; Schornack *et al.*, 2008). Thus, one might hypothesise that AvrBs3 and AvrHah1 would also target the identical EBEs and the same genes in tomato (Schornack *et al.*, 2008). This hypothesis is supported by 1) similarity between AvrBs3 and AvrHah1-caused disease phenotypes in the *CC-Bs4* background (Figure 29B); 2) binding of AvrBs3 and AvrHah1 to the same predicted EBEs *in planta* (Figure 33B); 3) identity of tomato $EBE^{bHLH022}$ and pepper EBE^{UPA20}, also referred to as the *UPA* box (Table 3; Boch *et al.*, 2009; Kay *et al.*, 2007); 4) a high homology between tomato *bHLH022* and pepper *UPA20* on CDS (87%) and protein (83%) levels; and 5) transcriptional upregulation of *bHLH022* and indirect induction of *PL* by AvrBs3 and AvrHah1 in tomato (Figures 35A and 35B).

Moreover, AvrBs3 was previously reported to cause hypertrophy not only on tomato and pepper, but also on other solanaceous species (Kay *et al.*, 2007; Marois *et al.*, 2002). Mining of genomes of *Solanum*, *Capsicum*, *Nicotiana*, and *Petunia* species revealed presence of *bHLH022*-like genes with the *UPA* box located upstream of the predicted start codons (Supplementary Information 5.2). The *UPA* box was found to be a part of the 63 bp-long sequence which was highly-conserved among the genomes of the inspected solanaceous species (Figure 36; Figure S2). Interestingly, this 63 bp-long sequence fragment was found to be transcribed in *Nicotiana*, *Petunia*, and *Capsicum* spp. suggesting that the *UPA* box and its flanking sequences might form a *cis*-regulatory element of *bHLH022*-like genes

(Supplementary Information 5.3; Kay *et al.*, 2007). In summary, AvrBs3 and AvrHah1 exploit a conserved element within genomes of solanaceous species to transcriptionally upregulate *bHLH022*-like genes and to promote disease symptoms.

Similar pathogenesis strategy is followed by *Ralstonia solanacerum*. A recent study demonstrated that *R. solanacerum* utilises Brg11, a TALE-like effector, to upregulate transcription of tomato *arginine decarboxylase* (*ADC*) genes (D. Wu *et al.*, 2019). Brg11 was found to target an EBE within a conserved 50 bp-long sequence, *i.e.* the *ADC* box, located upstream of *ADC* genes. Brg11 induces truncated ADC mRNAs lacking the *ADC* box and therefore, bypasses the *ADC* box-mediated translational control to boost polyamine levels.

Further study of the *UPA* box and its flanking sequences might reveal their roles in translational regulation of *bHLH022*-like genes in solanaceous species. In addition, it remains to be determined if the upregulation of *bHLH022*-like genes and putative secondary targets results in a higher susceptibility of a host to *avrBs3* and *avrHah1*-expressing *X. euvesicatoria* strains via quantification of bacterial growth *in planta*.

3.4 AvrHah1- and AvrBs3-induced disease symptoms may abolish Bs4-mediated cell death.

So far it is unknown why delivery of AvrBs4 and its C-terminus lacking derivatives consistently triggers cell death in the *Bs4* background, while delivery of AvrBs3 inconsistently triggers Bs4-mediated cell death (Schornack *et al.*, 2004; Schornack *et al.*, 2008; Schwartz *et al.*, 2017). Moreover, AvrHah1 does not trigger Bs4-mediated cell death (Schornack *et al.*, 2008; Schwartz *et al.*, 2017). One might assume that lack of cell death reaction to AvrBs3 and AvrHah1 is dependent on successful transcriptional upregulation of their respective targets in tomato genome.

β-Glucuronidase enzymatic activity assay demonstrated that AvrBs3 and AvrHah1 transcriptionally activated *GUS* reporter under control of the promoter containing the $EBE^{bHLH022}$ (Figure 33B). In tomato, the interactions between $EBE^{bHLH022}$ and AvrBs3 / AvrHah1 result into transcriptional upregulation of *bHLH022*, *i.e.* a cell size master regulator, and *PL* (Figure 35A, 35B, and 38), which leads to hypertrophy of mesophyll cells and water soaking (Kay *et al.*, 2007; Marois *et al.*, 2002). It was previously demonstrated that the cell death and desiccation of the leaf tissue restricts pathogen growth (Wright and Beattie, 2004). Moreover,

both Cf-4/Avr4- and Cf-9/Avr9-dependent cell death in tomato are suppressed under high humidity (95%; C. Wang *et al.*, 2005). Therefore, it can be hypothesised that transcriptional upregulation of tomato *bHLH022* and *PL* as well as induction of water soaking by AvrBs3 and AvrHah1 suppresses Bs4-mediated cell death and prevents desiccation of the leaflet tissue. This hypothesis is supported by the observation that delivery of dTALEs targeting *bHLH022* and *PL* does not lead to cell death in tomato (Schwartz, 2016), while AvrBs4, dTALE34, and dTALE35, which do not target *bHLH022* or any of its pathway components, trigger cell death in *Bs4* background (Figure 21B).

In order to clarify if the disease symptoms caused by AvrHah1 and AvrBs3 abolish Bs4-mediated cell death, AvrHah1 and AvrBs3 derivatives lacking activation domains (AvrHah1$^{\Delta AD}$ and AvrBs3$^{\Delta AD}$) or nucleus localisation signals (AvrHah1$^{\Delta NLS}$ and AvrBs3$^{\Delta NLS}$) should be tested in the *Bs4* background. The truncated derivatives of AvrBs3 and AvrHah1 would not be able to upregulate transcription of *bHLH022* and instead, might trigger Bs4-mediated cell death similarly to AvrBs4 derivatives (Schornack *et al.*, 2004). Previously, a similar experiment was conducted and reported that AvrHah1$^{\Delta AD}$ did not cause Bs4-mediated cell death in Heinz tomato cultivar 2 dpi (Schwartz *et al.*, 2017). However, this study was made under low humidity conditions and with low density bacterial inoculum (OD_{600} = 0.1), which might influence the speed of Bs4-mediated cell death development. Since Bs4-mediated cell death upon delivery of dTALE35 consisting of 35 aa-long repeats only became visible 3 dpi (OD_{600} = 0.4; Figure 21B), it can be assumed that Bs4-mediated AvrHah1$^{\Delta AD}$ recognition and cell death will take more than 2 days. Thus, testing Bs4-mediated recognition of AvrHah1 and AvrBs3

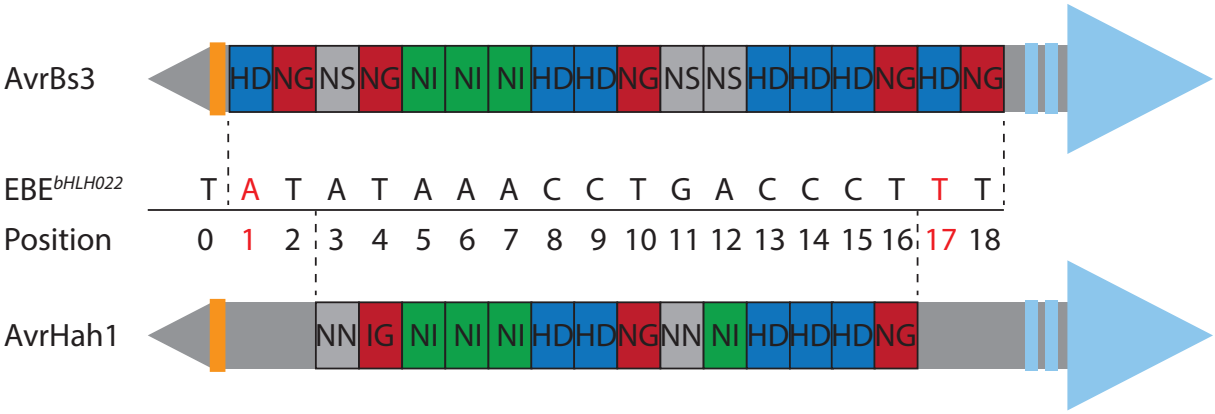

Figure 38. AvrBs3 and AvrHah1 target EBE^{bHLH022} to upregulate transcription of tomato *bHLH022*, an orthologue of pepper *UPA20*. RVD structure of both TALEs and corresponding nucleotides within the EBE^{bHLH022} are annotated. Each RVD is coloured according to its optimal binding preferences: green = "A", red = "T", grey = "N", and blue = "C".

derivatives lacking activation domains or nucleus localisation signals should be done according to the experimental conditions described in this thesis.

A competition experiment between AvrHah1 and AvrBs4 could be another alternative to determine if transcriptional upregulation of *bHLH022* has any impact on Bs4-mediated cell death. For the purposes of the proposed experiment, *Xe* 85-10 strain delivering both TALEs, AvrHah1 transcriptionally upregulating *bHLH022* and AvrBs4 triggering Bs4-mediated cell death, should be engineered and tested for an ability to suppress Bs4-mediated cell death. If AvrHah1-induced disease symptoms outcompete Bs4-mediated cell death upon recognition of AvrBs4, the proposed hypothesis might be accepted.

3.5 β-estradiol inducible system does not provide a tight control over a transgene.

Inducible systems provide the possibility to upregulate transcription of a certain gene at any stage of plant development and even in particular tissues (Borghi, 2010). Chemical inducers offer precise temporal and spatial control over transgene expression, which facilitates identification of gene functions without devastating systemic changes to plant development (Borghi, 2010). There are a few basic requirements for chemically inducible systems. However, the main parameter is the tightness of such a system, *i.e.* absence of any basal activity of the inducible promoter in the absence of the chemical inducer (Kubo *et al.*, 2013). This criterion becomes extremely important when the transcriptionally activated gene produces a toxic protein. In this case, even minimal leaky expression of such gene will have a devastating effect on the plant development.

The executor *R* genes, which were used in the experiments, cause cell death upon their expression (Römer *et al.*, 2007; Strauß *et al.*, 2012; Tian *et al.*, 2014; Chunlian Wang *et al.*, 2015). Transient expression of the executor *R* genes under the control of the EIP in *N. benthamiana*, demonstrated that the constructs had leaky expression in the absence of estradiol (Figure 16B). Thus, leaky expression of the executor *R* genes during stable transformation drastically reduced the chances of developing stable transgenic lines. However, if a transgene is integrated into an appropriate genomic context, which eliminates leakiness of the promoter (van Leeuwen *et al.*, 2001; Wilson *et al.*, 1990), it is possible to select stable lines with a tight control over expression of the executor *R* genes in the absence of the chemical inducer (Holmes *et al.*, 2020).

The second possible reason as to why the stable *EIP:Bs4C*, *EIP:Xa10*, and *EIP:Xa23* lines responsive to estradiol treatment were not identified are genetic and epigenetic effects of the insertion site (Table 1). It has been reported that the chromatin regions surrounding a transgene affect its expression (Butaye *et al.*, 2005). Thus, this creates variation in a transgene expression among different transgenic lines transformed with the same construct. Following this explanation, it is possible to hypothesise that part of the stable tomato lines, which contained corresponding *EIP:Bs4C*, *EIP:Xa10*, and *EIP:Xa23* transgenes but did not exhibit cell death upon estradiol treatment (Table 1), contained the transgenes in transcriptionally repressive chromatin areas.

The third possible reason as to why the stable *EIP:Bs4C*, *EIP:Xa10*, and *EIP:Xa23* lines were not responsive to estradiol treatment (Table 1) is a putative post-transcriptional *XVE* silencing due to its high transcriptional levels (Schubert *et al.*, 2004). In this case, *XVE* silencing would lead to the absence of the sensor protein, and thus, to non-responsiveness to liquid estradiol.

Despite numerous failures to identify estradiol-responsive tomato *EIP:Bs4C*, *EIP:Xa10*, and *EIP:Xa23* lines, a few *EIP:Bs3* lines responsive to estradiol treatment were identified (Table 1). This positive result indicates a necessity to screen for more putative T0 plants not only by phenotyping plants, but also by using qRT-PCR and immunoblot analyses for detection of the transgene-specific transcripts and translated proteins upon estradiol treatment, respectively.

3.6 Bs3-mediated pathways are preserved within solanaceous species.

This thesis describes a study of *Bs3*, an executor *R* gene from *C. annuum* encoding an FMO-like protein (Römer *et al.*, 2009), and Bs3-mediated immunity pathways in tomato. Stable tomato lines containing *Bs3* under the control of the EIP were engineered (Figures 17B and 18C). Transcriptional activation of the *Bs3* transgene with liquid estradiol in the J8 (*Bs4 Bs3*) line correlated with the cell death (Figures 17B and 17C). Alternatively, *Bs3* could be transcriptionally activated with the *Xe* 85-10 strain expressing *dTALE34* targeting the EIP and dTALE34-mediated transcriptional activation of *Bs3* in the HJ1 (*CC-Bs4 Bs3*) line was correlated with the cell death (Figures 18B, 18C, and 19). Previous experiments demonstrated that the transcriptional activation of *Bs3* by AvrBs3 causes cell death in *C. annuum* and transient overexpression of *Bs3* causes the cell death in *N. benthamiana* (Krönauer *et al.*, 2019; Römer *et al.*, 2007). The *Bs3*-mediated cell death was shown to be associated with the resistance

against *Xanthomonas* spp. in tomato and pepper (Figure 22B; Herbers *et al.*, 1992). Since all three species are highly related and share a basic set of genes (Livingstone *et al.*, 1999; Tanksley *et al.*, 1992), it is therefore non-surprising that the pathway components of Bs3-mediated cell death and immunity are preserved within solanaceous plants.

It was found that Bs3 localises to the nucleus and cytoplasm (Krönauer *et al.*, 2019). Biochemical studies revealed that *Bs3* expression does not stimulate auxin production, but coincides with elevated SA and Pip levels (Krönauer *et al.*, 2019). It was hypothesised that Bs3 catalyses an enzymatic reaction and possibly uses canonical immune signalling pathways for cell death mediation (Krönauer *et al.*, 2019). Thus, the developed pathosystem, consisting of a range of stable tomato lines expressing *Bs3* and *Xe* 85-10 strains expressing *dTALE34* for transcriptional activation of *Bs3*, can now be used to unravel elements of Bs3-mediated cell death and immunity pathways.

The J8 line (*Bs4 Bs3*) can be used for transcriptome profiling upon transcriptional activation of *Bs3* with estradiol or mock treatment. Such an experiment would unravel putative Bs3-mediated pathway components in the absence of the pathogen, thereby excluding the pathogen-associated virulence effect. In addition, a full-scale transcriptome profiling experiment should be made in the WT (*Bs4*), H11 (*CC-Bs4*), J8 (*Bs4 Bs3*), and HJ1 (*CC-Bs4 Bs3*) lines upon infiltration with *Xe* 85-10 *avrBs4*, *Xe* 85-10 *dTALE34* and *Xe* 85-10 EV strains. This experiment would enable comparison of the Bs4-mediated and Bs3-mediated immunity pathways, as well as the identification of putative shared pathway components. Subsequently, the J8 (*Bs4 Bs3*) and HJ1 (*CC-Bs4 Bs3*) lines could be used for knock-outs of putative pathway components to estimate their impact on Bs4- and Bs3-mediated signalling.

3.7 Bs3 does not mediate cell death via EDS1.

It was previously hypothesised that Bs3 may share signalling components with NLR proteins (Krönauer *et al.*, 2019). A series of preliminary experiments demonstrated that VIGS of *NbEDS1* did not abolish Bs3-mediated cell death, while silencing of *NbSGT1b* and *NbRAR1* abolished Bs3-mediated cell death (Krönauer, 2020). However, gene silencing efficiency was not quantified and assumption of silencing was based on changes in leaf morphology (Krönauer, 2020). Since VIGS may be incomplete and only knocks selected genes down by minimising but not abolishing their translation (E. Liu and Page, 2008), *EDS1*, *SGT1a*, and

SGT1b, *i.e.* master regulators of NLR-mediated immunity, were individually knocked out in tomato to test their impact on Bs3-mediated cell death.

Phenotyping of *CC-EDS1 Bs4 Bs3* with *Xe* 85-10 *dTALE34* revealed that CC-EDS1 abolishes Bs4-mediated cell death (Figure 28), indicating that this EDS1, lacking EP-domain, *i.e.* CC-EDS1, is a true null mutant and that Bs4 mediates cell death via EDS1. These results are supported by numerous studies, which demonstrate TNL signalling via EDS1/PAD4 or EDS1/SAG1 complexes, which trigger a cascade of downstream reactions resulting in SA biosynthesis and accumulation (Adlung *et al.*, 2016; G. Hu *et al.*, 2005; Lapin *et al.*, 2019; Schornack *et al.*, 2004). Nevertheless, phenotyping of *CC-EDS1 Bs4 Bs3* and *CC-EDS1 CC-Bs4 Bs3* plants with *Xe* 85-10 *dTALE34* revealed that CC-EDS1 does not abolish Bs3-mediated cell death (Figure 28). These results are in consent with the previously performed VIGS experiments (Krönauer, 2020) and led to the conclusion that Bs3 does not require EDS1 for cell death signalling.

Another point of evidence, supporting the conclusion that Bs3 does not require EDS1-mediated signalling for cell death can be found upon analysis of EDS1-mediated pathway for presence of putative substrates for Bs3. Since EDS1 and its signalling companions mediate the expression of SA biosynthesis genes *Isochorismate Synthase 1* (*ICS1*) and *AvrPphB susceptible 3* (*PBS3*; Berens *et al.*, 2019; Wildermuth *et al.*, 2001), it could be hypothesised that Bs3 plays a role in SA synthesis. However, biochemical assays suggest that Bs3 does not convert isochorismate into SA (Krönauer, 2020).

Nevertheless, the phenotypic experiments described in this thesis additionally demonstrate that Bs3-caused cell death intensity in F2 *CC-EDS1 CC-Bs4 Bs3* and *CC-EDS1 Bs4 Bs3* plants is comparatively weaker than the Bs3-caused cell death intensity in F2 *EDS1 CC-Bs4 Bs3* and *EDS1 Bs4 Bs3* plants (Figure 28). Quantification of isochorismate, SA, Pip, and N-OH-Pip content in *CC-EDS1 CC-Bs4 Bs3* and *EDS1 CC-Bs4 Bs3* tomato plants upon dTALE34-transcriptional activation of *Bs3* and in the J8 (*EDS1 Bs4 Bs3*) line upon estradiol and mock treatment should provide an explanation to this observation. Even though similar experiments were done in *N. benthamiana*, where overexpression of *Bs3* coincided with the accumulation of SA and Pip, but not N-OH-Pip (Krönauer *et al.*, 2019), until the proposed analyses in tomato are performed, it is possible to make two complimentary hypotheses: 1) EDS1-mediated signalling is a consequence of Bs3-mediated cell death; and 2) EDS1-mediated signalling may have an additive effect to Bs3-mediate cell death.

However, these hypotheses raise an additional question. Since *CC-Bs4* is a null allele and is unable to signal upstream of EDS1, what is the mechanism of the receptor TNL-independent activation of EDS1-mediated signalling in *EDS1 CC-Bs4 Bs3* line upon dTALE34-dependent transcriptional activation of *Bs3* (Figure 28)? Recent studies suggest that superoxide radical (O_2^-) can trigger EDS1-dependent SA accumulation (Mateo *et al.*, 2004; Rustérucci *et al.*, 2001; Straus *et al.*, 2010) and consecutive induction of *Pathogenesis-related Protein 1* (*PR1*) and counterbalances cell death *PR5* (Ochsenbein *et al.*, 2006). In this case, Bs3 should be able to oxidase nicotinamide adenine dinucleotide phosphate (NADPH) for production of O_2^- molecules, which trigger EDS1-mediated pathways. A recent study demonstrated that human FMO3 in addition to H_2O_2 produces O_2^- as its uncoupling products (Catucci *et al.*, 2019). Even though some Bs3 mutants are capable of elevated H_2O_2 production without causing cell death (Krönauer *et al.*, 2019), the O_2^- levels produced by those mutants and WT Bs3 had not been measured and compared (Krönauer *et al.*, 2019). This lacking information regarding Bs3-mediated O_2^- production via NADPH oxidation is important to understand the mechanism of putative activation of EDS1-dependent SA production. In addition, it would be necessary to compare levels of expression of EDS1-dependent SA biosynthesis pathway genes in the *EDS1 CC-Bs4 Bs3* tomato line in comparison to the *CC-EDS1 CC-Bs4 Bs3* line upon dTALE34-dependent transcriptional activation of *Bs3*. These gene expression data, in combination with measurement of ICS, SA, Pip, and N-OH-Pip, may suggest if the EDS1-mediated SA biosynthesis pathway has any additive effect to Bs3-triggered cell death. Additionally, bacterial growth assays in the *EDS1 CC-Bs4 Bs3* and *CC-EDS1 CC-Bs4 Bs3* tomato lines should indicate if there is any impact of *EDS1* knockout on Bs3-mediated immunity.

3.8 Combination of *Bs3* and *Bs4* as a source of resistance against *Xanthomonas* spp.

Pyramiding of *R* genes, *i.e.* the accumulation of *R* genes into a single genotype or cultivar, is a method used by breeders and plant pathologists with an aim to achieve a durable and broad-scale resistance against a particular pathogen (Collinge, 2016; M.Y.A. Tan *et al.*, 2010). A combination of *Bs3* and *Bs4* might be exploited to achieve a broad-spectrum resistance against xanthomonads in numerous plant species.

Bs3 under the control of the EIP was demonstrated to provide the resistance against *Xanthomonas* spp. in tomato (Figure 22B). Moreover, *Bs3* under the control of its native promoter (*Bs3p*) is a source of resistance against *X. euvesicatoria* strains expressing *avrBs3*

and *X. gardneri* strains expressing *avrHah1* in pepper (Herbers *et al.*, 1992; Schornack *et al.*, 2008). Therefore, *Bs3* under control of *Bs3p* could be used for generation of tomato cultivars with the resistance against *X. euvesicatoria* and *X. gardneri* strains expressing *avrBs3* or *avrHah1*. Additionally, *Bs3* might be used for resistance against *Xoo* in *Oryza* spp. or against *Xcc* in *Citrus* spp. In this case, *Bs3p* should be adjusted to trap the most abundant TALEs from *Xoo* causing bacterial blight disease in rice, namely PthXo1 (Yang *et al.*, 2006), PthXo2 (Zhou *et al.*, 2015), PthXo3 (Antony *et al.*, 2010), AvrXa7 (Yang and White, 2004), TalC (Yu *et al.*, 2011), and TalF (Streubel *et al.*, 2013) as well as with PthA4 from *Xoo*, which is known to promote citrus canker disease symptoms (Y. Hu *et al.*, 2014; Z. Li *et al.*, 2014). In this case, EBEs for the afore mentioned TALEs should substitute native EBE for AvrBs3 and AvrHah1 within the *Bs3p* to form a so-called EBE-amended *Bs3p* (Hummel *et al.*, 2012).

Even though the ability of Bs4 to mediate recognition of non *X. euvesicatoria*-derived and non *Xca*-derived TALE-like proteins has not been reported so far, Bs4 might mediate recognition of TALEs, interference TALEs (iTALEs), *i.e.* TALEs lacking the AAD but retain NLSs, and truncTALEs from *Xoo* strains. It would be of a particular interest to test if Bs4 mediates recognition of iTALE, since iTALEs are known to supress activity of Xa1, a CNL from rice which mediates recognition of certain *Xoo* TALEs (C. Ji *et al.*, 2020; Z. Ji *et al.*, 2016; Read, Hutin*, et al.*, 2020; Read, Moscou*, et al.*, 2020). In case the ability of Bs4 to mediate recognition of TALEs, iTALEs, and truncTALEs from *Xoo* and cell death signalling is confirmed, it might be used to improve rice resistance against this pathogen. However, the restricted taxonomic functionality (RTF) of *Bs4* might limit its utilisation in rice (Tai *et al.*, 1999). Nevertheless, a few studies report successful transfers of NLR-encoding genes between distantly related species, *e.g.* barley Mildew resistance locus a 1 (MLA1) was demonstrated to mediate recognition of Avr_{a1} from powdery mildew fungus in *A. thaliana* (Lu *et al.*, 2016) and maize *Resistance to Xanthomonas oryzae 1* (*RXO1*) was shown to provide resistance against *Xoo* in rice (Zhao *et al.*, 2005).

Pyramiding of *Bs3* and *Bs4* within one cultivar or genotype should provide a synergetic effect in resistance to *Xanthomonas* spp., where each component is responsible for a separate task: Bs4 mediates recognition of TALE-like proteins and EBE-amended *Bs3p:Bs3* traps the most abundant TALEs.

3.9 Conclusions.

Genetic pathways that are exploited by executor proteins from *Capsicum* and *Oryzae* spp. to cause cell death and immunity against biotrophic pathogens remain to be unknown. As executor-mediated cell death and immunity represent a separate case of the ETI, it was hypothesised that NLR and executor proteins might share same pathway components. One of the main aims of this thesis was to develop a set of tools for comparison of TNL- and executor-mediated cell death and immunity pathways. The main focus was kept on TNL protein from *Solanum* spp. mediating recognition of TALEs and their derivatives, *i.e.* Bs4, and an executor protein from *Capsicum* spp., *i.e.* Bs3, which is transcriptionally activated by AvrBs3 and AvrHah1 TALEs from *Xanthomonas* species. As pepper is not amenable to transformation, the genetic dissection of Bs3-mediated pathways was done in tomato.

Tomato *Bs4* null allele (*CC-Bs4*) was engineered using CRISPR/Cas9 system. Phenotyping experiments revealed that tomato lines containing *CC-Bs4* alleles no longer showed TALE-dependent cell death. Furthermore, transgenic tomato line containing estradiol-inducible *Bs3* was generated. Upon transcriptional activation by liquid estradiol, *Bs3* led to cell death indicating that Bs3-mediated pathway components are conserved between tomato and pepper. Moreover, dTALE-mediated transcriptional activation of the *Bs3* transgene in the *CC-Bs4* background correlated with the reduced *in planta* growth of dTALE-containing *X. euvesicatoria* strain. Knocking out the ETI master regulator, *i.e. EDS1*, abolished Bs4-mediated cell death but not the Bs3-mediated cell death, indicating that Bs3 and Bs4 utilise distinct pathways to cause cell death. Nevertheless, effect of *EDS1* knockout on Bs3-mediated immunity was not quantified. Therefore, it still remains to be determined if EDS1-mediated SA biosynthesis has an additive effect to Bs3-mediated immunity. Moreover, a number of *CC-SGT1a* and *CC-SGT1b* alleles in tomato were generated using CRISPR/Cas9 system and the crosses with *CC-Bs4*- and *Bs3*-containing line were initiated. Consecutive selection of the material should be done to estimate if knockout of *SGT1a* or *SGT1b* has an effect on Bs3-mediated cell death and immunity. Additionally, the transcriptome profiling experiment upon liquid estradiol-mediated transcriptional activation of the *Bs3* transgene might reveal putative components of the Bs3-mediated cell death and immunity in absence of pathogen-associated virulence effects in the J8 (*Bs4 Bs3*) line. Consequently, knockouts of putative Bs3-mediated pathway components in the HJ1 line (*CC-Bs4 Bs3*) have to be carried

out to test impact of these knockouts on Bs3-mediated resistance to the *dTALE34*-expressing *Xe* 85-10 strain.

Moreover, transgenic lines containing other executor *R* genes, namely *Bs4C*, *Xa10*, and *Xa23*, in the *Bs4* background had been generated. The introduction of some of these transgenes to the *CC-Bs4* background by crossing was started but not finished. Selection of the homozygous lines and consecutive testing of the transcriptional activation of the transgenes using dTALE34 has to be carried out to test if they are functional in tomato. Additionally, bacterial *in planta* growth upon the transcriptional activation of these transgenes in the *CC-Bs4* background should be quantified. The resulting plant material can be used to further decipher *Bs4C*-, *Xa10*-, and *Xa23*-mediated pathways.

As for *Bs4*, it was discovered that Bs4 mediates recognition of dTALEs with different length of central repeats (34 and 35 aa-long) and that Bs4 is epistatic to TALE-induced disease symptoms. In the scope of this information, it would be worthwhile to test if Bs4 mediates recognition of TALEs and their derivatives from rice and citrus pathogenic *Xanthomonas* strains as well as TALE-like proteins from *Ralstonia solanacearum*. Moreover, the Bs4-mediated immunity to *avrBs4*-expressing *Xe* 85-10 strain was not quantified in an assay mimicking natural infection process, and therefore remains to be done.

The second part of this thesis described identification of putative tomato gene targets of AvrBs4, AvrBs3, and AvrHah1, *i.e.* TALEs from tomato and pepper pathogenic *Xanthomonas* strains. An infection assay demonstrated that AvrBs3 and AvrHah1 enhanced hypertrophy and water soaking in the *CC-Bs4* background, while AvrBs4 did not cause any visible phenotype. Transcript abundance analysis of *in silico* predicted putative TALE targets revealed that *basic Helix-Loop-Helix transcription factor 022* (*bHLH022*), a tomato orthologue of pepper *Upregulated by AvrBs3 No. 20* (*UPA20*), and a pectate lyase encoding gene, *PL*, were upregulated by AvrBs3 and AvrHah1, but not by AvrBs4. Nevertheless, the putative virulence effect upon transcriptional upregulation of *bHLH022* and *PL* by AvrBs3 and AvrHah1 haven't been quantified yet. This study will require an assay mimicking the natural infection scenario, *e.g.* dipping of leaflets into an inoculum, to enable reliable quantification of bacterial *in planta* growth.

Analysis of 5’ untranslated regions (UTRs) of *bHLH022*-like genes from numerous solanaceous species revealed presence of a conserved 63 basepair-long sequence fragment, containing the *UPA* box. These experimental outcomes indicate that AvrBs3 and AvrHah1 exploit a conserved element within 5’ UTRs of *bHLH022*-like genes to enhance similar disease symptoms in numerous solanaceous species. Moreover, the conservation of *UPA* box and its flanking sequences upstream of annotated start codons of *bHLH022*-like genes from numerous solanaceous species suggests their putative role as *cis*-regulatory elements of *bHLH022*-like genes. A further study might be initiated to reveal if *UPA* box and its flanking sequences play a role in the translational control of *bHLH022*-like genes in numerous solanaceous species.

4 MATERIALS AND METHODS

4.1 Bacterial strains, media and antibiotics.

Bacterial strains, antibiotics, and media for bacterial cultivation used in this study are listed in the Tables 5 – 7. Other solutions, expression vectors and oligonucleotides used in this study are listed in separate tables within the methods in which they were utilised.

Table 5. Bacterial strains.

Strain	Relevant Characteristics
E. coli Top 10	F- *mcr*A (*mrr-hsdRMS-mcr*Bc) 80d *lacZ*M15 *lacX*74 *deo*R *rec*A1 *ara*D139 (*ara*, *leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G
E. coli ccdB survival	F- *mcr*A (*mrr-hsdRMS-mcr*Bc) 80d *lacZ*M15 *lacX*74 *deo*R *rec*A1 *ara*D139 (*ara*, *leu*) 7697 *gal*U *l*K *rps*L (StrR) *end*A1 *nup*G *ton*A:P*trc-ccd*A
A.tumefaciens GV3101	C58 (RIF R) Ti pMP90 (pTiC58DT-DNA) (gentR/strepR) Nopaline
Xe 85-10	RifR TALE-less strain pathogenic on pepper and tomato

Table 6. Antibiotics and fungicides.

Chemical compounds	Stock solution	Dilution
Ampicillin	100 mg/ml in water	1:1000
Chloramphenicol	15 mg/ml in EtOH	1:1000
Gentamycin	15 mg/ml in water	1:1000 (*E. coli*) / 1:300 (*A. tumefaciens*; *X. euvesicatoria*)
Kanamycin	25 mg/ml in water	1:1000 (*E. coli*) / 1:250 (*A. tumefaciens*)
Rifampicin	100 mg/ml in DMF	1:1000
Spectinomycin	100 mg/ml in water	1:1000
Cycloheximide	50 mg/ml in EtOH	1:1000

Table 7. Media and buffers.

Name	Composition
Lysogeny Broth (LB)	5 g/l yeast extract, 10 g/l tryptone, 10g/l NaCl
Yeast Extract Broth (YEB)	5 g/l beef extract, 1 g/l yeast extract, 5 g/l peptone, 5 g/l sucrose, 0.5 g/l MgSO4, pH 7.2
Nutrient Yeast Glycerol (NYG)	5 g/l peptone, 3 g/l yeast extract, 20 g/l glycerol
Minimal Infiltration Buffer (MIB)	Per 100 ml: sucrose 2 g, MS basal salt mix (no vitamins) 0.5 g, MES 0.195g, 200 mM 4'-Hydroxy-3',5'-dimethoxyacetophenone dissolved in DMSO 100 ul. Adjust pH to 5.6

4.2 Plant methods.

4.2.1 Plant species and genotypes.

Plant species and genotypes used in this study are listed in the Table 8.

Table 8. List of plant species and genotypes used in this study.

Line	Species	Genotype	Publication
Nb	*Nb*	WT	
MM (WT)	*Sl*	*EDS1 Bs4*	Schornack *et al.* (2004)
C18	*Sl*	*EDS1 CC-Bs4* (mutation in TIR domain)	This thesis
C39	*Sl*	*EDS1 CC-Bs4* (mutation in TIR domain)	This thesis
D12	*Sl*	*EDS1 CC-Bs4* (mutation in NB domain)	This thesis
H11	*Sl*	*EDS1 CC-Bs4* (mutation in NB- and LRR-domains)	This thesis
J8	*Sl*	*EDS1 Bs4 EIP:Bs3-3xFLAG-T2A-mGFP5*	This thesis
HJ1	*Sl*	*EDS1 CC-Bs4 EIP:Bs3-3xFLAG-T2A-mGFP5*	This thesis
E26	*Sl*	*CC-EDS1 Bs4*	This thesis
F2 5.42	*Sl*	*EDS1 Bs4 EIP:Bs3-3xFLAG-T2A-mGFP5*	This thesis
F2 5.78	*Sl*	*EDS1 CC-Bs4 EIP:Bs3-3xFLAG-T2A-mGFP5*	This thesis
F2 5.79	*Sl*	*CC-EDS1 Bs4 EIP:Bs3-3xFLAG-T2A-mGFP5*	This thesis
F2 5.24	*Sl*	*CC-EDS1 CC-Bs4 EIP:Bs3-3xFLAG-T2A-mGFP5*	This thesis
LA2963	*Sp*	*SpBs4*	Schornack *et al.* (2004)
ECW	*Ca*	WT	Kay *et al.* (2007)
ECW-30R	*Ca*	*Bs3p:Bs3*	Römer *et al.* (2007)
79	*Cp*	*Bs4Cp:Bs4C*	Strauß *et al.* (2012)

4.2.2 Assembly of vectors for CRISPR/Cas9-mediated mutagenesis of tomato genes.

Gene-specific sgRNAs were designed using CCTop CRISPR/Cas9 target online predictor (Stemmer *et al.*, 2015) for knockout of *Bs4* (*Solyc05g007850*), while CRISPOR.ORG web-tool (Concordet and Haeussler, 2018) was used to design gene-specific sgRNAs for knockout of *EDS1* (*Solyc06g071280*), *SGT1a* (*Solyc06g036410*), and *SGT1b* (*Solyc03g007670*) in *S. lycopersicum* cv. Moneymaker (MM; Table 9). The off-target and on-target specificity of sgRNAs was predicted in *S. lycopersicum* genome v3.0 using the ITAG (International Tomato Annotation Group) 3.2 gene annotation (Jouffroy *et al.*, 2016; Shearer *et al.*, 2014; T.D. Wu and Watanabe, 2005). sgRNAs were selected based on their high on-target specificity (according to the design tool automatic annotations) and number of mismatches between on-target and off-targets. Only sgRNAs which contained minimum three mismatches in total, two of which had to be in sgRNA "core", were selected for cloning and vector assembly (Table 9). In case, the first nucleotide at the 5'-end of 20 bp-long sgRNA was not "G", it was substituted to "G" to ensure sgRNA transcription initiation by *MtU6* promoter (Bortesi and Fischer, 2015; D. Zhang *et al.*, 2017)

The CRISPR/Cas9 vector cloning system (p201N; Jacobs et al., 2015) was used for *Bs4* mutagenesis in MM tomato (Table 9). Cloning of the gRNA and assembly of the T-DNA vectors was done as previously described (Table 9; Jacobs *et al.*, 2015). On later stages, the following modifications were made to CRISPR/Cas9 vector system (p201N) to facilitate cloning process

and to integrate up to four sgRNAs (Morbitzer, unpublished). A Gateway cassette containing *ccdB* gene and recombination sites was added into p201N (Morbitzer, unpublished). Up to four *sgRNA*s and 35s promoter-driven N-terminal triple NLS-containing *GFP* were sub-cloned into pUC57 (Spectinomycin), which contained recombination sites for LR reaction (Morbitzer, unpublished). The assembled cassettes were introduced to p201N-Gateway_Cassette via LR reaction using Invitrogen LR Clonase II. This upgraded p201N vector system was used to knock outs *EDS1*, *SGT1a*, and *SGT1b* in MM tomato (Table 9).

Table 9. Selected sgRNA sequences and their target sites. If the first nucleotide at the 5'-end of 20 bp-long sgRNA was not "G", it was substituted to "G" and marked with red colour.

sgRNA	Sequence 5' – 3'	PAM	Gene	Locus	Targeted Area
1	GTTCAAAGTATTATCCTCGA	TGG	*Bs4*	*Solyc05g007850*	TIR
2	GATACCGATCTTTTATAATG	TGG	*Bs4*	*Solyc05g007850*	TIR
3	GGGGTTGGAGTCCGAAGAGC	AGG	*Bs4*	*Solyc05g007850*	NB
4	GTGATCTACGACTAAGTCGT	AGG	*Bs4*	*Solyc05g007850*	Between NB and LRR
5	GTGTTTCCTCCTGTGAATAA	CGG	*Bs4*	*Solyc05g007850*	LRR
6	GTGTTTGCAGGGCACTCGT	CGG	*EDS1*	*Solyc06g071280*	Between LP and EP
7	GTCTTCGTGCAGCAGGAGAG	TGG	*EDS1*	*Solyc06g071280*	Between LP and EP
8	GCTTTCAACTTACCGTATGA	AGG	*SGT1a*	*Solyc06g036410*	Between TPR8 and CS
9	GAAGAGTACGCAGCTTCGCC	TGG	*SGT1a*	*Solyc06g036410*	CS
10	GGGAAATGCCTCGAGTATAC	TGG	*SGT1a*	*Solyc06g036410*	Between CS and SGS
11	GGCGTCCGATCTGGAGACTA	GGG	*SGT1b*	*Solyc03g007670*	Upstream TPR8
12	GACATCGCTTGAGTATACAA	CGG	*SGT1b*	*Solyc03g007670*	Between CS and SGS
13	GGAAGGATAACTGGGCCGCG	GGG	*SGT1b*	*Solyc03g007670*	Between CS and SGS

4.2.3 Assembly of the constructs containing executor *R* genes under control of the EIP for stable tomato transformation.

Assembly of initial pER10 vector containing EIP were described in Zuo *et al*. (2000). This vector was upgraded by integration of the Gateway Cassette via *Spe*I and *Xho*I digestion for facilitation of the cloning process (Morbitzer, unpublished). Triple *FLAG*-tag and *mGFP5* reporter were fused to the 3'end of the coding sequence of the executor *R* genes and were separated by *T2A* self-cleaving peptide to produce two separate proteins (Donnelly *et al.*, 2001). This cleavage is intended for preservation of functionality of both proteins. These structural elements were assembled via GoldenGate system (Binder *et al.*, 2014) into pENTR vector containing recombination sites for LR reaction (Morbitzer, unpublished). The assembled cassettes were introduced to pER10-Gateway_Cassette vector via LR reaction using Invitrogen LR Clonase II to produce *EIP:Bs3-3xFLAG-T2A-mGFP5, EIP:Bs4C-3xFLAG-T2A-mGFP5, EIP:Xa10-3xFLAG-T2A-mGFP5,* and *EIP:Xa23-3xFLAG-T2A-mGFP5* constructs. Which

were consequently were used for transient expression in *N. benthamiana* and for stable transformation of MM tomato.

4.2.4 Cloning of *SlBs4*, *SpBs4*, and *CC-Bs4*.

Fragments of $SlBs4^{gDNA}$ and $SpBs4^{gDNA}$ were sub-cloned from isolated genomic DNA into pUC57 (spectinomycin) vectors. Mutagenesis PCR was used to introduce silent mutations for removal of *Bpi*I recognition sites within the sequence of the sub-cloned fragments. Following the Golden Gate cloning strategy, the fragments were assembled into pENTR vector via cut-ligation.

$SlBs4^{CDS}$ and $SpBs4^{CDS\text{-}\beta}$ versions were obtained upon a series of mutagenesis PCRs over sub-cloned fragments of $SlBs4^{gDNA}$ and $SpBs4^{gDNA}$ for removal of introns according to the annotated *SlBs4* gene model. The difference between $SpBs4^{CDS\text{-}\alpha}$ and $SpBs4^{CDS\text{-}\beta}$ is in SNP mutation G1873A at the end of the second exon in $SpBs4^{gDNA}$ which leads to alternative splicing resulting in inclusion of four consequent nucleotides "GTAA" (1874-1877) to the $SpBs4^{CDS\text{-}\alpha}$ and a frame-shift (Figure 8B).

Upon Sanger sequencing of *CC-Bs4* amplicons derived from H11 line, $CC\text{-}Bs4^{CDS}$ was created by introduction of the *CC-Bs4*-specific mutations into $SlBs4^{CDS}$ using a series of mutagenesis PCRs over sub-cloned $SlBs4^{CDS}$ fragments. The pENTR pre-assembled cassettes were introduced into pGWB606 vector via LR reaction using Invitrogen LR Clonase II.

4.2.5 Transient expression in *N. benthamiana*.

Electro-competent *A. tumefaciens* GV3101 strains carrying the respective binary plasmids were grown overnight (12-16 hours) at 28°C in YEB medium containing rifampicin and vector-specific antibiotics. Cultures were pelleted, re-suspended in the MIB, and adjusted to OD_{600} = 0.4. Leaves of four to five weeks-old *N. benthamiana* plants were infiltrated with a blunt end syringe.

In case of transient expression of *EIP:Bs3-3xFLAG-T2A-mGFP5, EIP:Bs4C-3xFLAG-T2A-mGFP5, EIP:Xa10-3xFLAG-T2A-mGFP5,* and *EIP:Xa23-3xFLAG-T2A-mGFP5* constructs, 24 hpi with the inocula of *A. tumefaciens* GV3101 strains carrying the respective constructs liquid estradiol or mock treatments were infiltrated with a blunt end syringe in the same leaf tissue. Leaves were

harvested two dpi with liquid estradiol or mock treatments and destained in 80% EtOH at 95°C for 60 mins. This experiment was repeated three times.

In case of transient co-expression of N-terminus *GFP*-labelled *SlBs4*, *SpBs4*, and *CC-Bs4* gDNA and CDS versions with *avrBs4* and *GFP*, the respective cultures were adjusted to OD_{600} = 0.8. The combinations of two cultures were mixed 1:1 and the respective inocula were infiltrated with a blunt end syringe in the leaves of four to five weeks-old *N. benthamiana* plants. Leaves were harvested two dpi and destained in 80% EtOH at 95°C for 60 mins. This experiment was repeated three times.

4.2.6 Stable tomato transformations and event characterisation.

The assembled constructs were introduced into *A. tumefaciens* GV3101 strain by electroporation. *A. tumefaciens* colonies containing the assembled constructs were used for stable transformation of *S. lycopersicum* cv. Moneymaker (MM) following the protocol described by Wittmann *et al*. (2016). Total genomic DNA was extracted from individual plants regenerated from calli and putative events were genotyped for a transgene (in the case of *EIP:Bs3-3xFLAG-T2A-mGFP5, EIP:Bs4C-3xFLAG-T2A-mGFP5, EIP:Xa10-3xFLAG-T2A-mGFP5,* and *EIP:Xa23-3xFLAG-T2A-mGFP5* constructs) or a mutation in a *GOI* (in the case of CRISPR/Cas9-mediated mutagenesis) using primer pairs listed in Table 10.

Table 10. Primer pairs used for genotyping of putative T0 tomato lines.

Pair	Sequence 5' – 3'	Orientation	(WT) Amplicon length, bp	*GOI*	Purpose
1	GTGGAAAAAGGGCAACGGTA	Forward	735	*Bs4*	*Bs4* Mutations
	AGCAGTTAGGGCAGTTCTCC	Reverse			
2	AAGGTGTGGGGCTGTTTA	Forward	834	*Bs4*	*Bs4* Mutations
	GTTCAAGATGAACAAGCTTTTCTGG	Reverse			
3	CATCGGAACGATGAAGCCG	Forward	574	*Bs4*	*Bs4* Mutations
	ACTTGTAAGACTCCTGCAATCTTT	Reverse			
4	AAGGTGTGGGGCTGTTTA	Forward	1892	*Bs4*	*Bs4* Mutations
	ACTTGTAAGACTCCTGCAATCTTT	Reverse			
5	ATTCTACACGCACCCGTTGA	Forward	1315	*EDS1*	*EDS1* Mutations
	ACCTGCTAGCTCAAGCCTTC	Reverse			
6	CTTGTCATGGGCTCCATTAATATCT	Forward	1100	*SGT1a*	*SGT1a* Mutations
	TGAACACAGAGCAAAGGGAAG	Reverse			
7	AAAGAGCCGAAATAATCCAAGGT	Forward	500	*SGT1b*	*SGT1b* Mutations
	ACAAACTCTTCACCATAAACCCC	Reverse			
8	TTCTGAAATTGTTCCCCGAGT	Forward	579	*SGT1b*	*SGT1b* Mutations
	CCAAGCATTCAGGGACAAAGA	Reverse			

Table 10 (Continued). Primer pairs used for genotyping of putative T0 tomato lines.

Pair	Sequence 5' – 3'	Orientation	(WT) Amplicon length, bp	*GOI*	Purpose
9	AAGGGGGCCTCTGCCCAGTC	Forward	539	*Cas9*	*Cas9* Screening
	GACAGCCGCCCCCATCCTGT	Reverse			
10	GGCGGAGCAAGCCAGGAGGAA	Forward	899	*Cas9*	*Cas9* Screening
	CTTGACAGCCGCCCCCATCCT	Reverse			
11	CCTCTTATTGTTGGAGCTGGC	Forward	607	*Bs3*	*Bs3* Transgene Genotyping
	GACCCTGTACCGAGCTTCG	Reverse			
12	TTGATCTTGATTTTGGCTAACATGC	Forward	451	*Bs4C*	*Bs4C* Transgene Genotyping
	GAGTTTCATGTTGCTGGCTCAC	Reverse			
13	GCAACTGATGCTGACGTTCTG	Forward	370	*Xa10*	*Xa10* Transgene Genotyping
	GCTGATTTCTTCGTCATCTTCACC	Reverse			
14	ATGTTGCATCATCTCAAGGAGC	Forward	338	*Xa23*	*Xa23* Transgene Genotyping
	ACAGGGAGAATAACCATCTTGTCG	Reverse			

In the case of CRISPR/Cas9-mediated *GOI* mutagenesis, amplified fragments were purified and sent for Sanger sequencing (Table 10). T0 plants with confirmed mutations in a *GOI* (mainly big sequence deletions) were kept for seed multiplication. In the next generation (T1), segregating populations were screened for the corresponding mutations in the *GOI* and for *Cas9* coding sequence by PCR amplification (Table 10). *Cas9*-free plants that are homozygous for the confirmed mutations were phenotyped minimum three times and kept for seed multiplication (Table 10). The progenies of these plants were used for phenotyping experiment and bacterial growth assays.

In case of tomato transformation with *EIP:Bs3-3xFLAG-T2A-mGFP5, EIP:Bs4C-3xFLAG-T2A-mGFP5, EIP:Xa10-3xFLAG-T2A-mGFP5,* and *EIP:Xa23-3xFLAG-T2A-mGFP5* constructs, T0 lines containing a transgene were phenotyped with liquid estradiol and mock treatments minimum three times. The T0 lines exhibiting cell death reaction upon estradiol treatment were kept for seed multiplication. gDNA of these T0 lines was used for identification of number of inserted transgene copies and their genomic locations.

4.2.7 Identification of number of inserted *Bs3* transgene copies and their genomic location in stable tomato lines.

1 µg of gDNA samples of T0 lines were separately digested with 10 U of the blunt-end cutting enzymes *Alu*I, *Bsh1236*I (*BstU*I), *Bsu*RI (*Hae*III), *Rsa*I, *Eco32*I (*EcoR*V), *Mls*I (*Msc*I), *Pvu*II, *Smi*I (*Swa*I), and *Ssp*I (*Kas*I) from Thermo Fischer Scientific in 100 µl reaction volume. The digestion was made for 16 hours at 37°C followed by heat inactivation at 80°C for 20 minutes.

1:10 dilutions of the stock solutions (100 pmol) of the adapters GWALong and GWAShort (Table 11) were mixed 1:1, denatured at 95°C for 2 minutes, and cooled down to room temperature. The digested gDNA libraries were used for ligation of adapters. The ligation was performed at 16°C for 16 hours. 1 µl of 1:10 diluted samples and the primer pairs AP1 / AMRB01, AP1 / AMRB02, AP1 / AMLB01, AP1 / AMLB02 (Table 11) were used as templates for the 1st PCR with TAKARA PrimeSTAR GXL Polymerase (40 cycles; t_a = 60°C; T_e = 3,5 mins). The 1 µl of 1:10 diluted samples and 2 pmol of the primer pairs AP1 / AMRB01, AP1 / AMRB02, AP1 / AMLB01, AP1 / AMLB02 (Table 11) were used as templates for the 1st PCR with TAKARA PrimeSTAR GXL Polymerase following the manufacturer-defined protocol (40 cycles; T_a = 60°C; t_e = 3,5 mins). 1 µl of 1:50 diluted 1st PCR reactions and 2 pmol of the primer pairs AP2 / AMRB03, AP2 / AMRB04, AP2 / AMLB03, AP2 / AMLB04 (Table 11) were used as templates for the 2nd PCR with TAKARA PrimeSTAR GXL Polymerase (40 cycles; t_a = 60°C; T_e = 3,5 mins). The 1 µl of 1:10 diluted samples and the primer pairs AP1 / AMRB01, AP1 / AMRB02, AP1 / AMLB01, AP1 / AMLB02 (Table 11) were used as templates for the 1st PCR with TAKARA PrimeSTAR GXL Polymerase following the manufacturer-defined protocol (5 cycles 98°C for 20 sec and 68°C for 3,5 mins; followed by 30 cycles of 98°C for 20 sec, 55°C for 20 sec, and 68°C for 3,5 mins).

Table 11. Primer sequences used for identification of number of inserted transgene copies and their genomic location.

Primer	Sequence 5' – 3'	Purpose
GWALong	GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT	Adapter
GWAShort	PO4 - ACCAGCCCG - Spacer C3	Adapter
AP1	GTAATACGACTCACTATAGGGC	1st PCR
AMRB01	CTTGCGAAGGATAGTGGGATTGTGCGTCATCC	1st PCR
AMRB02	CTTGAGCTTGGATCAGATTGTCGTTTCC	1st PCR
AMLB01	CCTTGCAGCACATCCCCCTTTCGCCAGCTGG	1st PCR
AMLB02	CGGCGTTAATTCAGTACATTAAAAACGTCC	1st PCR
AP2	ACTATAGGGCACGCGTGGT	2nd PCR
AMRB03	CAGTGTTTGACAGGATATATTGGCGG	2nd PCR
AMRB04	TCAGTGGAGATGGATCCTCTAGAGGCACGTGG	2nd PCR
AMLB03	CGTCCGCAATGTGTTATTAAGTTGTCTAAGC	2nd PCR
AMLB04	TAATAGCGAAGAGGCCCGCACCGATCG	2nd PCR
332	CAAATTACTCACTCTTCCAACTC	Bs3.1
333	TGTAACAGCTAGTGTATGATTCAG	Bs3.1
215	TTTATTGAGGTTCGGGGCTTT	Bs3.2
216	CGTAGATTTGTAGGGGATCAT	Bs3.2

Results of the 2nd PCR were visualised on 1% agarose gel and well amplified products were cut out of the agarose gel and purified with Thermo Scientific™ GeneJET Gel Extraction Kit. Each

amplicon was sub-cloned into *Sma*I-digested pUC57 (gentamicin) vector and sequenced. *S. lycopersicum* genome v3.0 was screened for matches with the Sanger sequencing reads. Upon successful mapping of the reads, the combinations of chromosome-specific and transgene-specific primers, namely 333 / AMRB03 and 215 / AMLB04 (Table 11), were used to confirm genomic integration of *Bs3.1* and *Bs3.2* in T0 J8 line. The combination of chromosome-specific primers, namely 332 / 333 and 215 / 216 (Table 11), were used to check if the integrated transgene copies are hemi- or homozygous in T1 generation.

4.2.8 Cloning of dTALEs and TALEs for *X. euvesicatoria*-mediated tomato infections.

dTALE34 and *dTALE35* were assembled by modular cloning into pENTR vectors (Morbitzer *et al.*, 2011). 1 µg of pSKX1 backbone containing *ahax37-FLAG* (Streubel *et al.*, 2013; Tran *et al.*, 2018), pENTR *dTALE34*, and pENTR *dTALE35* plasmids were digested with 0,5 U of Fast-Digest *BamH*I enzyme from Thermo Fisher Scientific. *BamH*I-digested pSKX1 backbone containing only transcription start site and 3'-end of *ahax37-FLAG* part, *dTALE34*, and *dTALE35* were purified from 1% agarose gel using GeneJet Gel Extraction Kit from Thermo Fischer Scientific. The purified *BamH*I-digested *dTALE34* and *dTALE35* fragments were ligated into the purified *BamH*I-digested pSKX1 backbone with T4 ligase 30 Weiss U/ µl from Thermo Fischer Scientific. The purified *BamH*I-digested pSKX1 backbone was self-ligated with T4 ligase 30 Weiss U/ µl from Thermo Fischer Scientific to produce pSKX1 Empty Vector construct. *E. coli* TOP10 competent cells were transformed with 5 µl of each ligation reaction. Colony PCR and consequent Sanger sequencing allowed to identify the clones with forward orientation of inserts.

pSKX1 avrBs4 construct was cloned in a similar fashion as previously described. However, instead of *BamH*I enzyme, the Fast-Digest *Pvu*I enzyme from Thermo Fischer Scientific was used for digestion of 1 µg of pSKX1 *ahax37-FLAG* (Streubel *et al.*, 2013; Tran *et al.*, 2018) and pBinar *avrBs4-FLAG-Avi* (Lutz, unpublished). *Xe* 85-10 electrocompetent cells were transformed with the assembled pSKX1 constructs. Colony PCR was performed to select the colonies containing the whole dTALE/TALE sequence. 10-20 positive clones were tested for an ability to transcriptionally activate their respective targets and to cause associated cell death phenotype in numerous tomato and pepper genotypes: *Bs3* in tomato HJ11 line and MM (lacking *Bs3*) for dTALE34 / dTALE35 as well as *Bs4C* in *Cp* "79" and *C. annuum* Early Calwonder (ECW; lacking *Bs4C*; Strauß *et al.*, 2012). Per each strain, one clone continuously causing

expected phenotypes in three independent phenotyping pre-tests were selected for further bacterial growth assays and phenotyping experiments. pDSK602 *avrBs4*, pDSK602 *avrBs4Δ227*, and pDSK602 *EV* (Schornack *et al.*, 2004) as well as pDSK602 *avrBs3* and pDSK602 *avrHah1* (Strauss, unpublished) were generated in separate studies.

4.2.9 Phenotyping of plants with the liquid estradiol and mock treatments.

20 mM of β-Estradiol ≥ 98% (CAS # 50-28-2) from Sigma-Aldrich was dissolved in dimethyl sulfoxide (DMSO) and stored as a stock solution for maximum of two weeks at -20°C. 1:1000 dilution of the stock solution in Milli-Q water was used for preparation of liquid estradiol-containing inoculum (20 µM). Mock treatment was prepared by 1:1000 dilution of the DMSO in Milli-Q water. Both inocula were syringe-infiltrated into the leaflets of four to five weeks-old tomato plants. Phenotypes were observed two dpi. Phenotyping experiments were independently repeated three times with similar results. Samples for the transcripts abundance measurement via qRT-PCR were collected 12 and 24 hpi. And the transcripts abundance analyses were made only once per experimental design.

4.2.10 Phenotyping of tomato and pepper plants using *X. euvesicatoria*.

Xe 85-10 strains were grown on solid NYG medium containing rifampicin and a vector-specific antibiotic (gentamycin for pSKX1 or spectinomycin for pDSK602) for two days at 28°C. Bacterial cells were re-suspended in sterile water, inocula were adjusted to OD_{600} = 0.4, and were infiltrated into the abaxial side of the leaflets of the four- to five-weeks-old tomato or pepper plants. Upon infiltration with the inocula, the plants were kept for two days at 22 ± 1°C with relative air humidity of 40-50% a 16h/8h day/night cycle. Representative leaflets exhibiting cell death phenotypes were collected 2 dpi (or 3 dpi in case of phenotyping with *Xe* 85-10 expressing *dTALE35*). Harvested leaflets were destained in 80% EtOH at 95°C for 60 mins. Phenotyping experiments, where the impact of CC-EDS1 on Bs3-mediated signalling was tested, were repeated twice with similar results. While the remaining phenotyping experiments were independently repeated three times with similar results.

4.2.11 Bacterial *in planta* growth assay.

Four- to five-weeks-old tomato plants were transferred to Outsunny PVC Transparent Greenhouse (200 x 100 x 80 cm) two days prior to infiltration to create high humidity

conditions for bacterial growth. *Xe* 85-10 strains containing *dTALE34* and EV pSKX1 were grown on solid NYG medium containing rifampicin and gentamycin, while *Xe* 85-10 strains containing *avrBs4* and EV pDSK602 were grown on solid NYG medium containing rifampicin and spectinomycin for two days at 28°C. Bacterial strains were re-suspended in sterile water, inocula were adjusted to OD_{600} = 0.00004 (10^5 CFU/ml), and were infiltrated into the abaxial side of the leaflets of the prepared plants. Upon infiltration with the inocula, the plants were kept in extra humid conditions at 22 ± 1°C with a 16h/8h day/night cycle. Infiltrated leaf tissue samples (0.64 cm^2) were harvested at 0, 4, and 7 dpi for *Xe* 85-10 pSKX1 *dTALE34* and *Xe* 85-10 pSKX1 EV. In case of *Xe* 85-10 pDSK602 *avrBs4* and *Xe* 85-10 pDSK602 EV strains, the samples were harvested at 0, 4, 7, and 9 dpi. The harvested samples were grinded in 100 µl 1mM $MgCl_2$ solution in a Retsch Mixer Mill 200 (20 f / 40 seconds). Series of sample dilutions (10^0 to 10^{-6}) were plated on solid NYG medium containing cycloheximide and corresponding antibiotics and incubated at 28°C for 36 to 48 hours. Log_{10} values representing number of CFUs per cm^2 of infiltrated leaflet surface were used for statistical analyses. These *in planta* bacterial growth experiments were independently repeated minimum three times with similar results.

4.2.12 *In silico* analysis of putative AvrBs4, AvrBs3, and AvrHah1 EBEs in tomato genome.

TALgetter (Grau *et al.*, 2013) were used for prediction of putative EBEs within the 1 kb promoter regions (upstream of translation start codon, both strands) of annotated tomato genes (*S. lycopersicum* genome v 3.0 and ITAG3.2). The RVD composition, *i.e.* TALE-code, of each effector were used for prediction of putative EBEs. In order to shortlist potential target genes of AvrBs3-like proteins, strict selection criteria were introduced to EBE location in the promoter regions of their putative targets. First, an EBE of a putative target should start with "T" at the position "0". Second, an EBE should be in the forward orientation towards a predicted transcription start site of a putative target. Third, an EBE should be located 50 – 300 bp upstream a predicted start codon of a putative target. Top candidates satisfying the selection criteria and having high likelihood of the predicted TALE-EBE interaction were selected for further analysis.

4.2.13 TALE and *Bs3p-EBE^{GeneID}:GUS* reporter assay.

avrBs4, *avrBs3*, and *avrHah1* were assembled into pGWB605 using Invitrogen LR Clonase II. Empty pGWB605 was used as a negative control. pGWB3* vector containing the native pepper

Bs3 promoter (343 bp upstream of ATG) introduced via Golden Gate assembly (Binder *et al.*, 2014; D. Wu *et al.*, 2019) was used for integration of EBE^{GeneID} by PCR-based mutagenesis. Primers used in this study were listed in Table 12. *Bs3p-EBE^{Bs3}:GUS* and *Bs3p-EBE^{Bs4C}:GUS* constructs in pGWB3* vector used as positive controls very assembled in the previous study (Jordan, unpublished). All constructs were transformed into *A. tumefaciens* GV3101 strain by electroporation. *A. tumefaciens* were grown in YEB medium containing rifampicin and spectinomycin (for pGWB605-containing strains), or rifampicin and kanamycin (for pGWB3*-containing strains) at 28°C for 16 h. Bacterial cells were harvested by centrifugation, resuspended in the MIB and adjusted to an optical density at 600 nm (OD_{600}) of "1.2". Combinations of pGWB605-containing and pGWB3*-containing strains were created by mixing inocula with the adjusted OD_{600} in 1:1 ratio. Inocula were infiltrated into 4-week-old *N. benthamiana* leaves with blunt-end syringe. 36 hpi minimum four leaf discs from infiltrated area were harvested and stained in GUS staining solution (pH = 7.0, 0.1 M sodium phosphate, 5 mM EDTA, 1 mM K3[Fe(CN)6], 1 mM K4[Fe(CN)6], 0.1% Triton X-100 and 0.05% X-Gluc) for 24 h, followed by washing in 70% ethanol for two days. Four representative stained leaf discs were scanned.

Due to the big number of samples and controls within this assay, all combinations of infiltrations were divided into two separate groups. *i.e.* expected positive interactions and expected negative interactions. These two groups were tested separately. However, the standard controls, *i.e.* *Bs3pEBE^{Bs4C}:GUS*/*avrBs4-GFP*, *Bs3pEBE^{Bs4C}:GUS*/*GFP*, *Bs3pEBE^{Bs3}:GUS*/*avrBs3-GFP*, *Bs3pEBE^{Bs3}:GUS*/*avrHah1-GFP*, *Bs3pEBE^{Bs3}:GUS*/*GFP*, 35s:GUS, were included into each set. The whole assay was made once.

Table 12. Primer sequences used for integration of EBE^{GeneID} sequences into pGWB3* vector containing the native pepper *Bs3* promoter.

Primer	Sequence 5' – 3'	*GeneID*
145	PHO-ATAAAATTGGTCAGGCAAACGTGTTCATTG	*Bs3p*
146	TATGTACACCTCCCCCTCTTCACAACTTCAAGTTATCATCCCCTTTCTC	*PE*
147	TCTGTAAACCTAACCCAATTCACAACTTCAAGTTATCATCCCCTTTCTC	*AP*
148	TATATAAACCTGACCCTTTTCACAACTTCAAGTTATCATCCCCTTTCTC	*bHLH022*
149	TTTAATTATTAATCCACTTTCACAACTTCAAGTTATCATCCCCTTTCTC	*bHLH073*
150	TACAACTACTAATCCCCTTTCACAACTTCAAGTTATCATCCCCTTTCTC	*UP*
151	TATATTTAGTACTCCTCTTTCACAACTTCAAGTTATCATCCCCTTTCTC	*RING*
152	TATAATTATTAATTCACTTTCACAACTTCAAGTTATCATCCCCTTTCTC	*BCP*

4.2.14 TALE-mediated tomato target gene upregulation assays in tomato.

A. tumefaciens GV3101 strains expressing *avrBs4*, *avrBs3*, *avrHah1* and *GFP* (pGWB605 EV) or *Xe* 85-10 strains containing *avrBs4*, *avrBs3*, *avrHah1*, and EV pDSK602 were infiltrated at OD_{600} = 0.4 with the blunt-end syringe into leaflets of four- to five-weeks-old tomato plants (Moneymaker and its corresponding mutants). In case of *A. tumefaciens*-mediated transient expression of TALEs, the infiltrated tissue samples were harvested 48 hpi, while for *Xe* 85-10 inocula, the infiltrated tissue samples were harvested 24 hpi. Each of these experiments was made once.

4.2.15 RT-PCR and qRT-PCR.

Total RNA was isolated from infiltrated tissue samples with the Roboklon Universal RNA Kit following to the protocol provided by the manufacturer. The quality of total RNA isolated from the samples was checked by nanodrop. Additional off-column treatment with *DNase*I was made following the protocol from RevertAid First Strand complementary DNA (cDNA) Synthesis Kit (Thermo Scientific). Equal amounts of *DNase*I-treated total RNA (400 ng) for all samples within one experiment were prepared for cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the protocol provided by the manufacturer.

Table 13. Primer pairs used for RT-PCR.

Pair	Sequence 5' – 3'	Orientation	Amplicon length, bp	*GeneID*
15	TGGACCGCGAAAGACGATAATT	Forward	317	*PE*
	AGTTTTGGAGGATAGCTGACG	Reverse		
16	TATGAGTGGGGGAGGCCAA	Forward	315	*AP*
	GTAGTTGTCGTCCTTTCATGT	Reverse		
17	AGGCCCTTATGTTGGATGAG	Forward	340	*bHLH022*
	CCCCATAAGAGCTGTCCAATAC	Reverse		
18	AAGAAGAAATGGAACTTGGACC	Forward	339	*bHLH073*
	TCCCTCCATATCCAGCATTTCT	Reverse		
19	CATACAAACCATCAATAGTGCC	Forward	303	*UP*
	TCAGTATTTCCATCGTCATCAG	Reverse		
20	TTTCTCTACTTGTACCATCCCT	Forward	209	*RING*
	TGTCTTCATTTCTCTTGCATCG	Reverse		
21	GGTGCATCATGTGGTCGGAGAA	Forward	351	*BCP*
	GCTACAGCCATTAATCCAACAT	Reverse		
22	AGTCAACTACCACTGGTCAC	Forward	205	*EF1α*
	GTGCAGTAGTACTTAGTGGTC	Reverse		

RT-PCR was performed in 20 µl reactions, each containing 1 pmol of forward and 1 pmol of reverse primers (Table 13), 2 µl of 1:20 diluted cDNA, 4 µl of 5x Phusion High Fidelity Buffer from New England Biolabs Inc., 0,2 µl of Phusion High Fidelity Polymerase, and Mili-Q water following the PCR protocol (35 cycles of 98°C for 10 sec, 53°C for 15 sec, and 72°C for 30 sec). *EF1α* (*Solyc06g009970*) from *S. lycopersicum* (Table 13) was used as reference gene for comparison with expression levels of putative target genes (Szczesny *et al.*, 2010). RT-PCRs were repeated twice on one set of template samples.

qRT-PCR was performed in 8 µl reaction (384-well Thermo Scientific™ PCR Plates, CFX384 Touch qRT-PCR Detection System BioRad), each containing 1 pmol of forward and 1 pmol of reverse primers (Table 14), 2 µl of 1:4 diluted cDNA, and 4 µl of MESA BLUE qRT-PCR MasterMix Plus for SYBR Assay No ROX (Eurogentec). Standard program was used for all qRT-PCR runs (95°C for 5 min; 40 cycles of 95°C for 15 sec, 58°C for 45 sec). The primer specificity was checked by running a melting curve after all qRT-PCR runs (0.5°C elevation every 5 sec from 58 to 95°C). The primer efficiency was checked by running a trial qRT-PCR with minimum five different cDNA samples of various dilutions (1:4, 1:16, 1:64, and 1:256 or 1:5, 1:25, 1:125, and 1:625). Each sample had three technical replicates. The mean of Ct values for five biological replicates were used to determine qRT-PCR primer efficiencies. *TIP41-Like* (*Solyc10g049850*) from *S. lycopersicum* (Table14) was used as a reference gene for internal normalisation (Lacerda *et al.*, 2015). Relative expression was calculated with $2^{-\Delta Ct}$ method and this raw data was used for statistical analyses. qRT-PCRs were made once for each set of template samples.

Table 14. Primer pairs used for qRT-PCR.

Pair	Sequence 5' – 3'	Orientation	Amplicon length, bp	Efficiency, %	*GeneID*
23	CGGTTGGCTGTGGCAATTC	Forward	98	97,21	*Bs3*
	ACGACCCTGTACCGAGCTT	Reverse			
24	GCTTAGGGTTGATGGAGTGCT	Forward	104	95,57	*TIP41-Like*
	CTCTCCAGCAGCTTTCACGA	Reverse			
25	TACTACATCACAGGCAGCTGAAG	Forward	147	96,57	*bHLH022*
	TCATCTGCACCCCATAAGAGC	Reverse			
26	GCTTGTGCAAAGGATGCCAA	Forward	91	95,64	*PL*
	TCCTCCAATTGCATACATCTCCC	Reverse			
27	CAGGCCTTGTCTTCGAAAGGA	Forward	128	97,66	*TIP41-Like*
	TTTTTACAGGACACTCCAACATGG	Reverse			

4.2.16 Mining of plant genomes for tomato *bHLH022* orthologues and alignment of sequences.

Genomic sequence of tomato *bHLH022* (*Solyc03g097820*) was used as a query for mining of *bHLH022*-like gene sequences within the genomes of *Solanum*, *Capsicum*, *Nicotiana*, *Petunia*, *Coffea*, and *Vitis* species using the genomic browsing platforms available at https://www.solgenomics.net and https://blast.ncbi.nlm.nih.gov. Sequences of the *bHLH022*-like genes and the sequences upstream of their predicted (annotated) start codons were extracted for further analysis (Supplementary Information 5.2). 303 bp-long sequences (300 bp upstream and 3 bp representing the start codon) were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004). The most conserved nucleotides were annotated with the BoxShade algorithm at (https://embnet.vital-it.ch/software/BOX_form.html). The complete alignment of these sequences is available as Figure S2.

4.3 Quantification and statistical analyses.

Statistical analyses of raw data were performed in R v4.0.3. Where applicable, unpaired two-samples Wilcoxon test and pairwise Wilcoxon rank sum test followed by FDR *p*-value adjustment method for multiple comparisons were used to compare significance levels between different groups. Significance levels are represented by number of asterisks, where *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; and ****, $p \leq 0.0001$. All details regarding applied statistical tests are described in the figure legends. The box plots were deployed to represent the distribution of the numerical data points and skewness. *n* represents numbers of independent biological replicates.

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SUPPLEMENTARY FIGURES

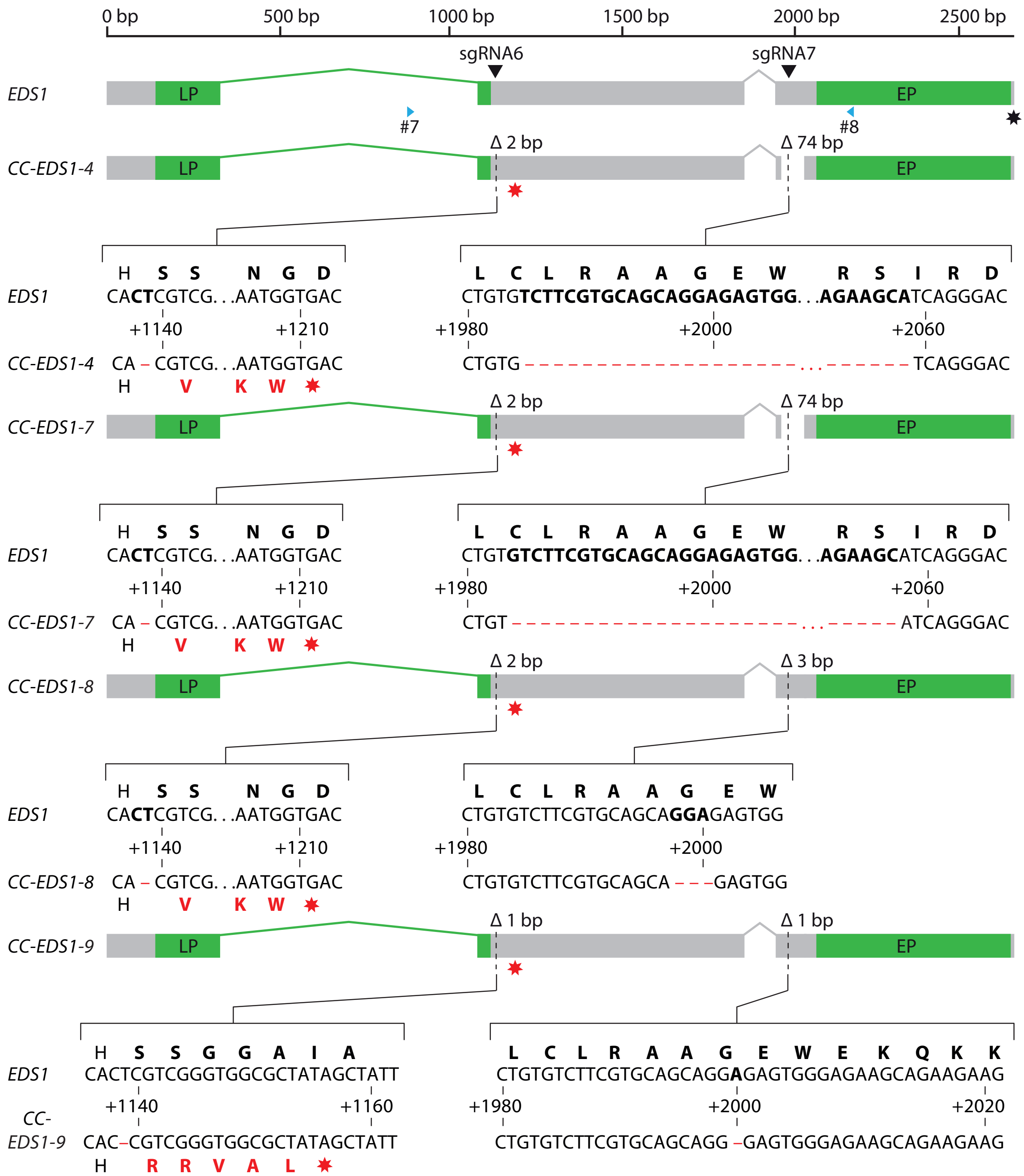


Figure S1. Gene models and CRISPR/Cas9-generated mutations within 13 *CC-EDS1* alleles identified in *Cas9*^{EDS1}-free plants from F2 generation. Green blocks represent sequence regions encoding functional domains. Black triangles and vertical dashed lines indicate the sgRNA6 and sgRNA7 target sites. Blue triangles represent primers used for genotyping. WT *EDS1* genomic and protein sequences impacted by mutations are highlighted with bold black font. *CC-EDS1* mutations within genomic and protein sequences are marked with bold red font. Black and red stars represent the locations of native and premature stop codons, respectively.

Figure S1. Continued

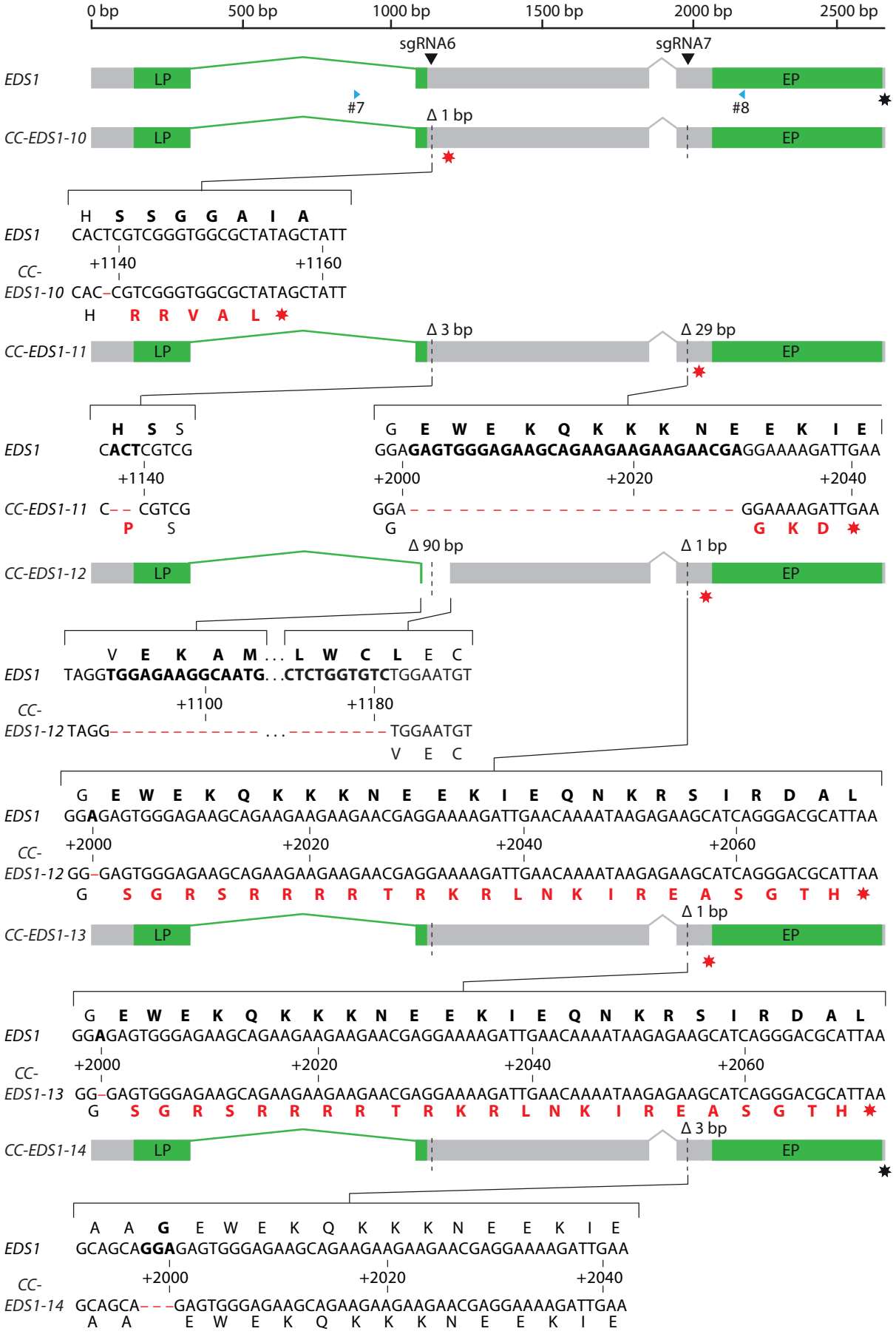
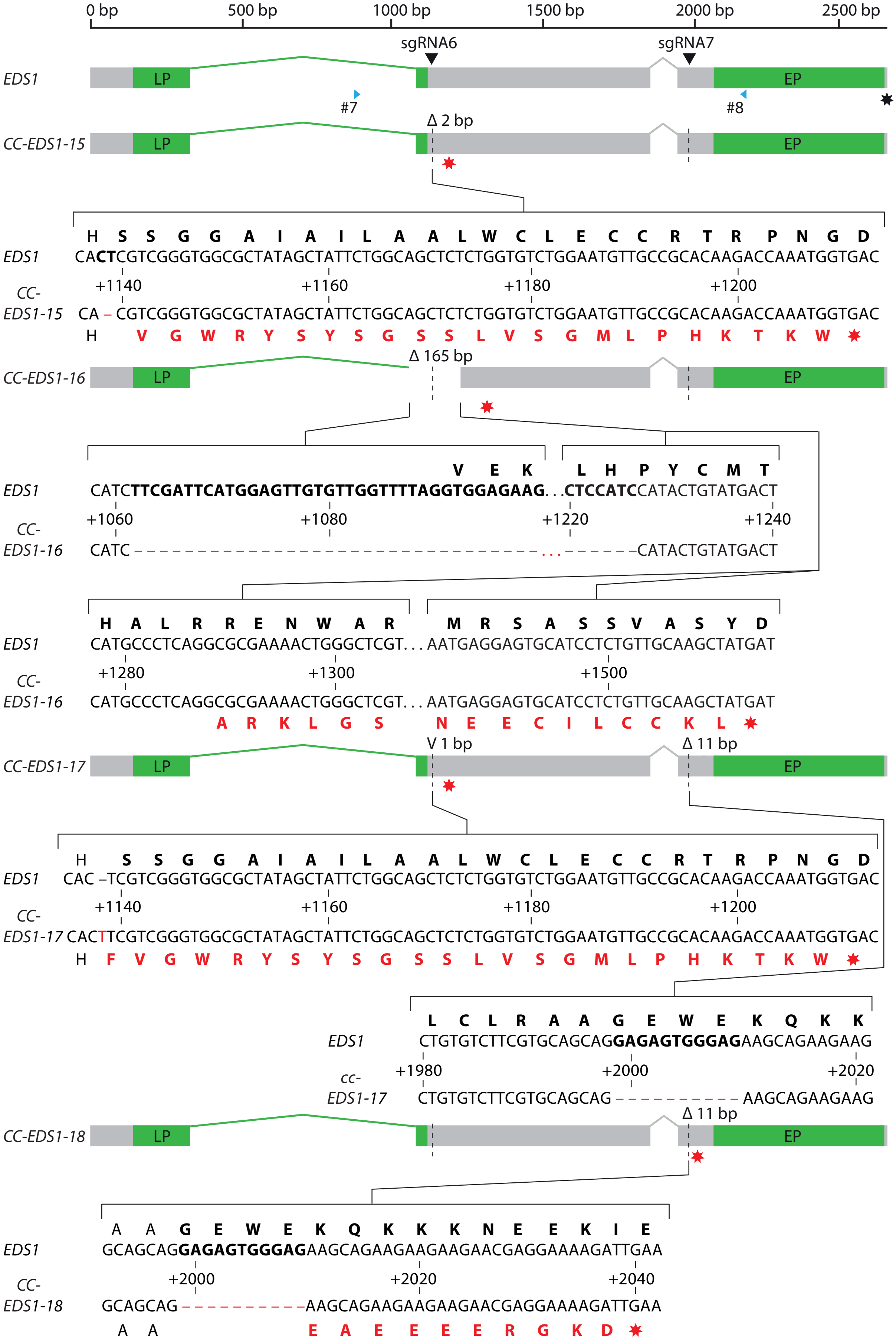


Figure S1. Continued



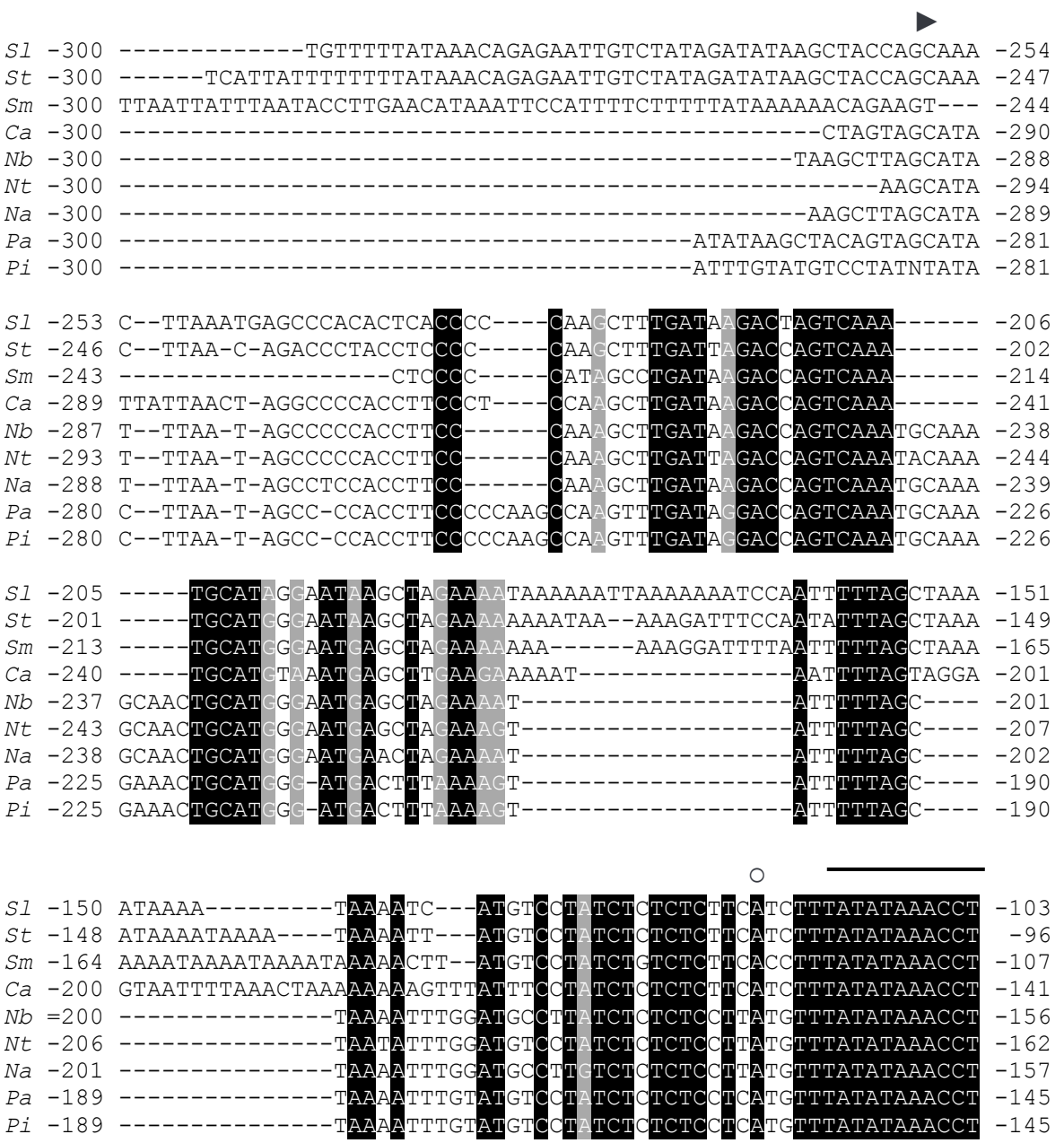
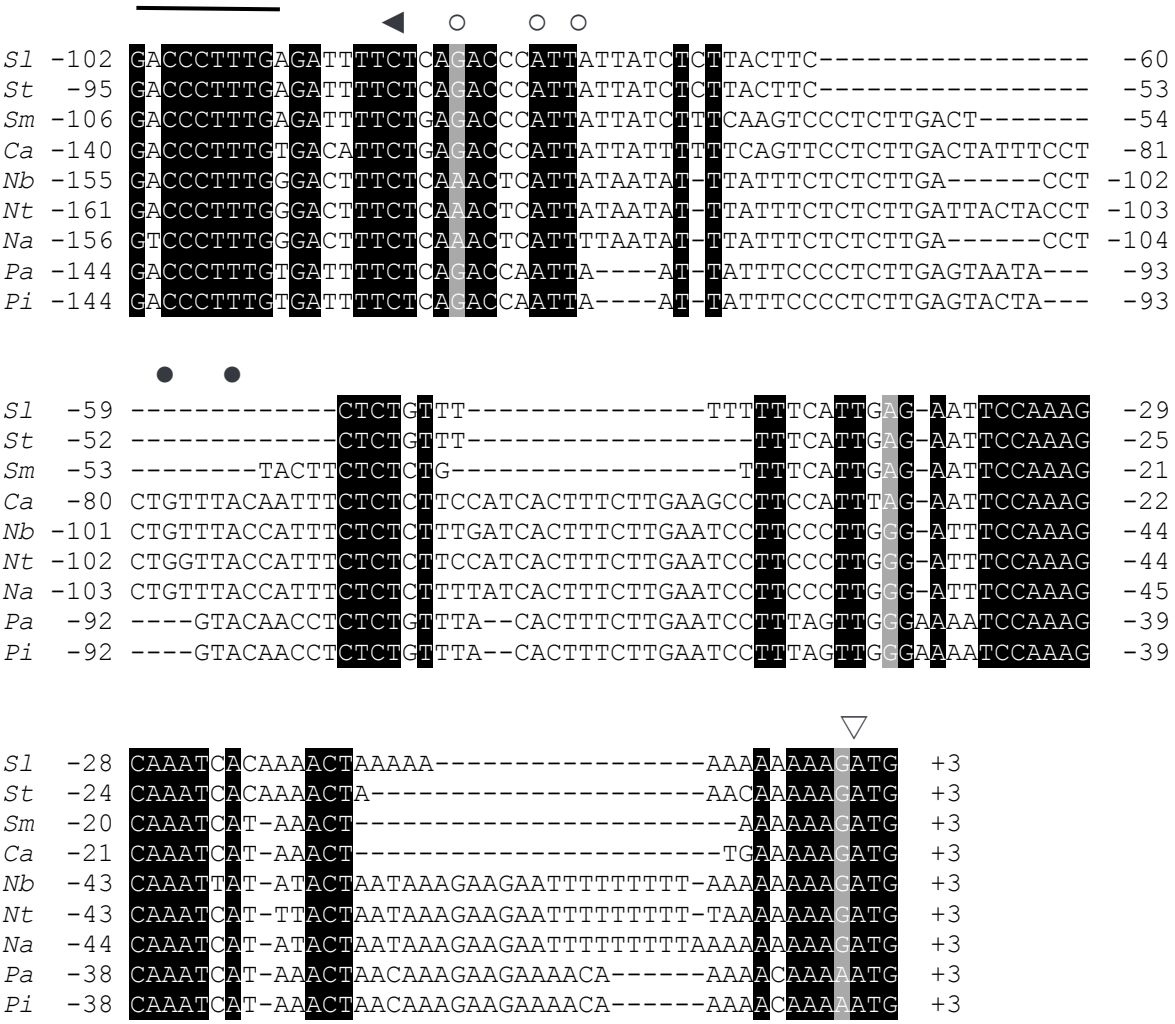


Figure S2. Alignment of sequence fragments upstream of start codons of *bHLH022*-like genes from genomes of solanaceous species, namely *Solanum lycopersicum* (*Sl*), *Solanum tuberosum* (*St*), *Solanum melongena* (*Sm*), *Capsicum annuum* (*Ca*), *Nicotiana benthamiana* (*Nb*), *Nicotiana tabacum* (*Nt*), *Nicotiana attenuata* (*Nt*), *Petunia axillaris* (*Pa*), *Petunia inflata* (*Pi*). 303 bp-long sequences (300 bp upstream and 3 bp representing the start codon) were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004). The most conserved nucleotides were annotated with the BoxShade algorithm. Shaded (black and grey) nucleotides represent the most conserved sequence fragments. Black horizontal line represents $EBE^{bHLH022}$ and EBE^{UPA20}, *i.e.* target sites of AvrBs3 and AvrHah1, respectively. White triangle (▽) represents annotated CDS start site of *bHLH022*-like genes. White circles (○) represent identified TSSs of *UPA20* from non-infected *C. annuum* plants (Kay *et al.*, 2007). Black circles (●) represent identified TSSs of *UPA20* from *C. annuum* upon transcriptional activation with AvrBs3 (Kay *et al.*, 2007). Black triangles represent the start (▶) and the end (◀) of the duplicated sequence region upstream of start codons of the *bHLH022*-like gene from the *Pi* genome (Supplementary Information 5.2).

Figure S2. Continued



SUPPLEMENTARY INFORMATION

5.1 Genomic locations of the integrated *EIP:Bs3-3xFLAG-T2A-GFP* transgene copies within the chromosome 7 of the J8 (*Bs4 Bs3*) line.

Identification of genomic integration sites of the *Bs3* transgene copies in *S. lycopersicum* T0 J8 line (*Bs4 Bs3*) was done using Genome Walking method (Materials and methods, chapter 4.2.7; Cottage *et al.*, 2001; Shapter and Waters, 2014). The shaded nucleotides (grey) represent the identified *Bs3* T-DNA parts (right or left border). The non-shaded nucleotides (white) represent identified genomic fragments of chromosome 7 before or after which the *Bs3* T-DNA copies were integrated.

>*Solanum lycopersicum* T0 J8 line (*Bs4 Bs3*) *Bs3* t-DNA copy 1 Right Border (grey) is located at chromosome 7 (non-shaded; positions 1587709-1588013)
gDNA digestion enzyme: *HaeIII*
First PCR primers: AMRB03 & AP1 (Table 11)
Second PCR primers: AMRB02 & AP2 (Table 11)
Sequencing primer: AMRB02 (Table 11)

CNNNNNNNNNNNNNNCCGTGGCGGCACGTGGCAAGCTTGGATCCACGATATCCTGCAGGCATGCAAGCTTAGCTT
GAGCTTGGATCAGATTGTCGTTTCCCGCCTTCAGTTTAAACTATCAGTGTTTGATTTAGTGNGAAAAATTAAAAA
AATATAAAAATATTTTGGTTTGTGAATTTTGATGGGAAAATTGTTATATATATAATTATTATTATTATTTTG
AATTTTAAAGATATANTGAGGNNAAANNANGTGANTCTGGAGGCTTTTTGGGATGATGANTTGAATGNTTATGCA
ANGGGCNNGTGATTNNTCCATTTTAATTAAGAAATGCTGAATCATACACTAGCTGTTACACATTAATATTCCTNA
AAAAATTGGTCAAAAAATNNCANTTTNCTCATTCTTTTTATTTTTGNANTCCCCCAAGANCAAN

>*Solanum lycopersicum* T0 J8 line (*Bs4 Bs3*) *Bs3* t-DNA copy 1 Right Border (grey) is located at chromosome 7 (non-shaded; positions 1587709-1587939)
Confirmatory PCR primers: 333 & AMRB03 (Table 11)
Sequencing primer: AMRB03 (Table 11)

NNNNTTNNNNNNANNNNNNNNNNNNNNGGATCCTCTANAGGCACGTGGCGGCACGTGGCGGCACGTGGCGGCACG
TGGCAAGCNAGGATCCACGATATCCTGCAGGCATGCAAGCTTAGCTTGAGCTTGGATCAGATTGTCGTTTCCCGC
CTTCAGTTTAAACTATCAGTGTTTGATTTAGTGTGAAAAATTAAAAAAATATAAAAATATTTTGGTTTGTGAATT
TTGATGGGAAAATTGTTATATATATAATTATTATTATTATTATTTTGAATTTTAAAGATATAATGAGGAGAAAAA
ACGTGATTCTTGATACTTTTTGGAATGATGAATTGAATAATTATCCAAGTGGCTAGATATTAATCCATTTTAATT
AAGAAATGCTGAATCATACACTAGCTGTTACANNN

>*Solanum lycopersicum* T0 J8 line (*Bs4 Bs3*) *Bs3* t-DNA copy 2 Left Border (grey) is located at chromosome 7 (non-shaded; positions 2668199-2668868)
gDNA digestion enzyme: *HaeIII*
First PCR primers: AMLB04 & AP1 (Table 11)
Second PCR primers: AMLB01 & AP2 (Table 11)
Sequencing primer: AMLB01 (Table 11)

NNAATTTGTTTTTAGTTGTCTAAAAATATATATACAACCAATACGATAAAGTTAATTCTAATTATTAATATTTGC
ATGTTAGATAAATTTCGGAGTGAAGCTAGAATATATTAAATATTAGTATTGATTGGTTTCTAATCRAAGCTGAGG
GTTGAGGACCTAAAGCCCCRAACTTTAATAAATGATAAGGTAGGAACAGACCTAAGCAAAGCGTGGCAGTGGGAT
GGGGGTGGAGCCTTTATAAAAATCATGAATCCGCCAATAGAAAATGAATCTGACAAAATATGTAGTACTGAGTTA
GGATTGATCTCAGATTTCAAATCRAAGTTAGAATTGGAGACCTAAAGCCCCGAACCTCAATAAATGATAAGGGAG
GAATGGACCGAAGCAAAGGGTGAGAGTGGGATGGGGGTTGAACCAAGTGAATTGATTTTATTTTTGGTGATAATG
TAATTACTTATTTTTTGCTCGTCAAGTTATTGTTTAAATAAAATTCTACATCTTATCTTAAAAAGTAAGTATTAT
AATTGTCATCTTAAAAYCCTGAATTCGCTTTTGTCAATAGTCAGTAATTTGAAAAGTCTAAAATTTTGCTATGGG
TCATNNNNNAATGTAATTAGTATACCTGTTATTTAAAAATTCTGAANTCGTCTCTSNCAGTTANCATGATMAANN
TTTTGCNATGAATCATNNAACAACNNNNCAACTTTGCNNTNNNNGNTGACNNGNNNNTCNTCATGMANNNGGACC
CTNAANNNCCTTAACGCTGTNNANTTGNNNNNNNTTCNNTGAAANGACAAANGNNTNNNNNNNNNNNNCTTNNNT
GGNNNNNCNNNAAANNNNNNNNTNNNNNTNNNNNNNNNNNNNNNNNNNNNTTNNNNNNNNNCNNANNANNNANNN
NNNNNNTNNNNNNNN

>*Solanum lycopersicum* T0 J8 line (*Bs4 Bs3*) *Bs3* t-DNA copy 2 Left Border (grey) is located at chromosome 7 (non-shaded; positions 2668537-2668868)
Confirmatory PCR primers: 215 & AMLB04 (Table 11)
Sequencing primer: 215 (Table 11)

NNNWNGGGNTTAGGTCTCANTTCTAACTTCGATTTGAAATCTGAGATCAATCCTAACTCAGTACTACATATTTTG
TCAGATTCATTTTCTATTGGCGGATTCATGATTTTTATAAAGGCTCCACCCCCATCCCACTGCCACGCTTTGCTT
AGGTCTGTTCCTACCTTATCATTTATTAAAGTTCGGGGCTTTAGGTCCTCAACCCTCAGCTTCGATTAGAAACCA
ATCAATACTAATATTTAATATATTCTAGCTTCACTCCGAAATTTATCTAACATGCAAATATTAATAATTAGAATT
AACTTTATCGTATTGGTTGTATATATATTTTTTAGACAACTTAAAAACAAATTGACGCTTAGACAACTTAATAAC
ACATTGCGGACGTTTTTAATGTACTGAATTAACGCCGAG

5.2 Genomic sequences of *bHLH022*-like genes and their upstream genomic sequences identified within the genomes of solanaceous species.

Genomic sequences of *bHLH022*-like genes (and their corresponding 3000 bp upstream fragments) from genomes of *Solanum*, *Capsicum*, *Nicotiana*, *Petunia*, *Coffea*, and *Vitis* species. Sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm (Edgar, 2004). The most conserved nucleotides were annotated with the BoxShade algorithm (Materials and Methods, chapter 4.2.16). Black shaded nucleotides represent EBE^{bHLH022} and EBE^{UPA20}, *i.e.* target sites of AvrBs3 and AvrHah1, respectively. Grey and black shaded nucleotides represent the most conserved sequence fragments within the genomes of the examined species. Bold script represents the duplicated sequence region upstream of the start codon of the *bHLH022*-like gene from the *Pi* genome. The most homologous sequence to EBE^{bHLH022} in the genome of *Coffea arabica* contains four bp insertion (TGAC) between positions 14 and 15. The most homologous sequence to EBE^{bHLH022} in the genome of *Vitis vinifera* contains two bp with switched positions ("T" at the position 15 and "C" at the position 16).

>*Solanum lycopersicum* v3.0 ch3 *bHLH022* (*Solyc03g097820*)

AAATCCTTGCAAGGACAAAACATTCATCTGGCATATAGAGACAGAAAAAAAAGGGAGGTATTGACCAAACTATTA
ACGGAGCAAAGATATTTTTGGACCAAAATATTGATAGCAAGAATATATTTATTCGTTTCACATAGTTGAAGTTCT
TTTTTAATTATTCTCCATAGTTTCTATGATTAAAAAGTTGTTTCCTTATATAGTACTATTTTAACAATTTTTACT
CACAAACTAGGGTATAGAATTATAAGTTACATTTTATGTACAAAGACATGCGGATCGAAATGACCAAAACATGTG
GTTTATATCTAATTACACTTGCTTAATCGATATGTAAAAGAGATAACAGATATAGAATGAGCCTTAGGTCTAGTG
TTAATAGCTGGCAGCATCACCAGGCAAAGCTTGATTTTGTAAATATAAATTAGGGAGTAACATTGTTAAATCTCC
TTTAACTATTTTTTTATATTTTGAAAAATCATGCTTTCGCCACCATTTCAATCACGTTTTGAGGATAATTATAGT
ATTTGATGTTGTCCCTCCTAATTATTCACTTTCAACCTATTTTTGTTGCCACATATCACTTCTTGATTCGTCCAT
ATGTCATGCTCGATTTTAATCATAAAGATTATTAATTTAATTTTTTTTTTAAGGATAGAATCATTCATACCTGTAT
TATTAGAGTACTAATTATGGTATAATAATGCAAAATTCCACTTTTGCTTTAATCAAGAATATAGTAAGCTATTTT
AGAATGGGCCATGACAAAACTAATGCTAGAAATATTAGACTCAACTATAGGGGTTTGTGATGAGTACAATAATAA
TTAAAAGATGAGAAACACAAGTAACAATTAATGGCAGCAATGAATAGATGTTTTTATTTACGTTTCAACTCTTGT
GAATTGTCATATTTTTAGATACATAAAAACAACTGACTTGAAAGTTGAATCATCATATCTCAGTGTTTGCATCTC
TGATTCACTGATGAGAAAGAGTCATTCAACGGAAGACCAAAAAAAGCAACTGACATTTTCAATATAATTGGGGTG
TGTCATGCATGTATTACGAAATTTTAACACAAACAAGTAACAACAACAATAATACACTCATCTTCATCACCAACA
AGCAAGGTCAACTTGACAAATTAATACTATTTCAATATCGTTATATCTACGAATTAAATTTAGTGTAACACTTTT
TTTTTATATTCATTATGTCATCTTTTTAAATTCTTGAGTTTGATTTTATTATTTTAACTATATACTATACATTTT
GATTGAAAATTTATATTCTTTGTTCTTATTTCATAAGGTTTGCATATATTTTGTCTTCCATAAATATCACTCCTA
GAATTATACTGAGTATATGATGGTCGTCAATTTATATATGTAATAAGAAGGAAAAAAAGTAAGACCTCTTTATGA
AGCTGGCTTTAGGTCCCAATCTTGTTGAATTTTTAACACTTGAGAAGCACAGGATGGATGCAACTTATACCTATT
GTGTCGGGTAGGAATATATATTATTTTTATTAGGCTTTCGGATTTAAAAGAAAAGTTTTCGACACACAAAAGAAG
ACATGATATAAAGCAAATAAAACAACAAATAGTAATACCTAATGAAAATAGAAGAATGTGTCACTACTAAGGGGT
CTTCGACTTAAAGACAAGTTATGTTGAGATTAATTATGATAGAATGAGTTATATTGAGATAAATTATGATCAAAT
TAGTTATGTTAATATTATTTTTTATTAATTAATTGGTTTATAAAACATGTATGACATACATAATAAGTTGTTGTT
TACAAAAATATCCTCTAAGAATAAGTTGTTTGTTTATAAAAGAGGATTTAAGGGGGATATTAACAAATTTGTTAT
TTTACATTTTTATATTGGAATAAGTTATTCGAGGATTAATATTTCACCCAAAAATAGAGATTATATATCCCAGCC
ATAATTATTAATCACGGAATAAGTTGTCTCACGATTAGTATTTCACCCATAAGTAAGGATAATAAATCCTAGTCA
TAATTATTAATCTTTAAATACATTATCTTACACTTTGTAACCAGACAAAAGAATTGAGTAAAGCTTTTATACATA
TCTCAATATTATTTTTCTTTATTCATCACACCAAACAACCCCTCCTACAAAATATGCAACAACACTAGATTATCT
ACTACAATCTTTATTCTAATCTTAAACGTTCATAGCTTTCTTTTTAGGATCATGTTACCAGTAAGCTGAAATTAT
ATCATATTTTATCTAATTACCTTCATTCATTTTTTTTACACCATTTTCATCTCTCGAACTGTTGCTATAGTCCCG
TTTATAAATCTCTGCTACTATTATTCTAATATCATTATCATTTTTAGTTTTCTATTTATATACTCGATACTCATA
TTAAAATCTAATCAAATTTTGATCATATAGAAATATATCATATTGGCGTAAAAATCAACGAAATTGATCTAAAAA
ATACCGATATAACCCCAGGATAATTCTTGGAGTTTTCTCCATGCCCGATGGAGAAGTAAAAATATTTAATAAAAT
TTAATTTATTTTAATAATAAACAAAACTAATAGGATTGATTATGTATCAGAACTAAAACACTCAAATTCAAAAAT
CTCTTCTCTATAGTGGCTTATTCATACTCATTATCTACTTAATTATTTAATATCTTAAACATAAATTTCATTATT
TGTTTTTATAAACAGAGAATTGTCTATAGATATAAGCTACCAGCAAACTTAAATGAGCCCACACTCACCCCCAAG
CTTTGATAAGACTAGTCAAATGCATAGGAATAAGCTAGAAAATAAAAAATTAAAAAAATCCAATTTTTAGCTAAA
ATAAAATAAAATCATGTCCTATCTCTCTCTTCATCTTTATATAAACCTGACCCTTTGAGATTTTCTCAGACCCAT

TATTATCTCTTACTTCCTCTGTTTTTTTTTCATTGAGAATTCCAAAGCAAATCACAAAACTAAAAAAAAAAAAAG
ATGGCTGCTTTTTCATCACACCAATTACAACACAATAACCCATTTCTTCTTGATTCAGTATTTTTGCCAACTTCT
CCTATTAAAATGTCTGGCTTTTTTGAGGAACCAAACAATTCTTGTATAGTACAACAGTTTTACCAACAAGAATTC
CCTTCCAATTTAATTTCTCATGAAAATAGCTTTTGCCTTGACCCTAAAAGTAGCAGCAGTATAAGCTTAGATATG
GATGCTTCCTCTGTTACTGATAAAATTGAAAGTGGAATTAATAATAATAAGGCTAATGTTAGTCCTTTGGATAAG
AAAAGAAAATCTAGTGAAGGGTCTTCTTCTATGACTTCTGCTCATTCTAAGGTAATTTGGAATTTTCCATTTCTG
TTCTGTAAATTATTCATTTTCTTGGAATTGGGATTAATGATTACTCTGTTTTTTTGTTTTTTGTTTTTATTATTA
GAATGAGAAACAGGGTGATAATGGGAAAAAGAAGAAAATTATCAGCAAGTTAGTAGCCAAAGATGAAAAGAAAGC
TAATGAAGAAGCACCAACAGGGTACATTCATGTTAGAGCAAGAAGGGGCCAGGCAACTGATAGCCATAGTCTTGC
TGAAAGGGTATAATTCTACTTTTTGCCCCTTTTAACTATTGGTCCCTCGGTTCGATTTGATACGGAGTTTTAAGA
AAAAAATATTATTTGTATGATTATATATTATTTCATTAATAATAAAAAAATAAAGTTAAATTATTATTTAATATA
GTTATATGTCATTTCAAAAACACTAACTAAAAAGAAAAATAAGTTATAAATAGAAATGGAACGGATACTAAAATT
TATAATGTGGTGTGAAAATTGAAACAACTAAATTTAAATTCATGTTGAAAAATAACACATACGAACTAAATACTT
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GATGAAGATATTGCAATCTCTTGTTCCTGGTTGTGACAAGGTAAATGACAAAGTTAATTCAGTAAAAAAATTTTG
TCTTTTATTTGTCTTTCCATGTAGTACAATTGAAAGGCACTTTTCAAGAAATTAGTATTTAAAAATCTTTTAAAT
TATAATTCTTCTGACAATTTTCATGTGACTTGAATGAGATCAGGTAACTGGAAAGGCCCTTATGTTGGATGAGAT
AATTAATTATGTCCAATCTTTGCAAAACCAAGTTGAGGTATTAAACAAGAGAACAATCAATTAATAACTTCCTTT
ATATTTTAACTTGCATAATTATAAAATATTAATACTAATTATATTATGTGTTTCTCATGTTTCAGTTTCTCTCAA
TGAAACTTACTTCTTTGAATCCAATGTACTATGACTTTGGAATGGACTTAGATGCACTCATGGTCAGACCTGATG
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>*Solanum tuberosum* PGSC DM v3 ch03 PGSC0003DMS000003852
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>*Solanum melongena* v4.0ch03: 13395157-13401156

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>*Capsicum annuum* CM334 v1.55 ch3 *UPA20* (*Ca03g22700*)
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>*Petunia axillaris* v1.6.2 Peaxi162Scf00171g01120

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>*Petunia inflata* v1.0.1 Peinf101Scf03000g00020

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>*Coffea arabica* ch04 CM011113.1

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TTATTGTTCTTGAAAATAAAAACCCTTAAATGCTTACTTTTCTCTGACATAAACAATTAAGGCAATAACTATATT
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AAGGATGAAAATATTGCAAGCACTTGTTCCAGGTTGTGACAAGGTGAAGTTTTTTCATCAGCATCTTGTCTTTAT
TTTGTTTGTCTCGCTAAAGTTTTTTTTTTCTGACAATTTGAAGTGAATTCGACTGATAAACAGGTAACCGGAAAG
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TTGTTATTTGGTAAAGTTTTTGAAGCTTGCTGTTATATAATCCATGCAAAATATTTCAAATTCTTGCAGTTTCTA
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GAACCGGTACATGTTGCACAATGTTCTAACACAATTGTGTACCAAGTTGTTATATTTCAGAGAATGTTTCACCAT
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GGTCTTCTATCTCTCTGATCACATGAATAAAAAGAAAAGCAAAGTAAAAGGGCATGAAGAGGATAGAAATCAA

>*Vitis vinifera* Contig VV78X035637.14 AM473672.2

TGGATGAGTTCAAATCTCGAGGCTCTTTCTCTCTGCTTCACATGCAGGGGCACAAAAGGCTCTTTATGGGCAGCG
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AGACATGGAATTGTTTTGTTCTTAGGATGAGTTCCAATGGAGGGGGCTTCTTCTTCTCAAAAGCTATGATTCCAT
CATTTCCAAGTCCAGTTGTTTAAAGGAGAAGGGTATCTGAACAATGAATAAAGGGGCAGCAAAATCAATGGAGGA
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TCTAAACAAATATTTATATATATGTAATGTATGTAAAAAAAATAATCCCATGTATGTCCTTCCATGCATTATAAA
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CAACAGGATGATCCACCTCTCATCCCCTCTAGAAACACCCACTTCATTTCAGTTCTCCCCCTTTATCTTCCACCA
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GCTACTGCCAACTAGAAACAAGCATGCAGATCAGTAAATGCTGGATCATACATGTAGCTCACCAACTCATCAAAT
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GTTGGCAGCAGCATCATCTATCAGCAACTGTCATATCTTCGCGTGGCGTGTTTGATCCTTTCCCGGAAGATTTAT
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GGCTAGGGTGCTAGCTAGAAGGAGCCAATTTCATGAACAAAAATAACCATGTCTATACTCTAATAATGTTTATGC
AAAATGGCATGTCATATGACTGGTATCTTAATTATGTAATTAATAATTTGGTGTAAAATTGAGAATATTTATATT
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GGGATATGTGAGTTTGAGTTTTTGCGTCTTCACATGCAAGTATGAACCTGTTCTATGATAACATCATATAGATAC
TGTCTACTTTCATCCTGTAAATTTTCGTGACTTTAAAACAAATTTATATATAATAGATGAGTCATTCTATACAAG
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AGTTGGATTGACCTATTGCTCGAGACCTAATAAATATTATATATGGTTTGTAAATTGTGAAACTATATATATTTA
TGGTGGGGGACTAATTTTGATAGTGACGACCCATGTAAATATTAATATCCTCAAACTCTCTGGTATGCTCTTTCC
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AATGCACATATGTGCACATTGACCAATAGTGACGTGCCACATGGGCATACCTGAAGGGCTTGCGTCCACATTTAT
GATGATGATGATAGAAATGGATGACCCATCAAAATCTAGATGGGGCATGATGGAGGGTCTGGATGGATGTGAATG
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GGTGCCCAAACCCCATACGGAATAGGATGTCGACATCATTTGGAAAGGGAGACATGTGTCATGTCATTTGATGGG
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GGCTCCGCCTCCTTCGTGACTTTGATATCCCACATGCGAGTTGACTCAAAATGCTAATCATCCCTTTCATTTTTT
CCCCTTTTTTTTTCCATCTTTTAAAGGTGAAAATTTTGGGAAATGGAATATTCCCTCTTTATTTTCCATATTCAG
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GAAGATAATAGAAAAAGGCATCTTTGGAAAGAAGACTTTCACTTTCACAGTTTCCAAACACAACTTTTTCAAAAA
TAACTTTATTTATTTTTCCCATAACCTTGTTCTACTTTCTTCCTCCTGTCTTTTCCCTTTAAGAAGCACCAAGTG
TCCTTATCGCCAATGCGTAACTAGCCCACTTTCTCCTCCAAGTGATTTTCAAAACCATCTCTTTCACCTCTTTTT
ATCTTCTTCATTTCATAATAAGCCAAAAAAAAAATAAAAAAATAAAAATAAATA

5.3 *UPA* box containing transcribed RNA sequences identified in solanaceous species.

Previously identified 63 bp-long sequence fragments containing the *UPA* box and conserved within genomes of solanaceous species was identified as part of the transcribed RNA sequence in *Nicotiana*, *Petunia*, and *Capsicum* spp. (Figure 36; Figure S2; Kay *et al.*, 2007). These results suggest that the identified sequence fragment is a part of 5' UTR, and therefore is important for regulation of translation. Black shaded nucleotides represent the *UPA* box, *i.e.* EBE^{bHLH022} and EBE^{UPA20}, *i.e.* target sites of AvrBs3 and AvrHah1, respectively. Grey shaded nucleotides represent the most conserved sequence fragments within the genomes of the examined species.

>Transcriptome Shotgun Assembly: *Nicotiana attenuata Na*_454_85814 Transcribed RNA Sequence Genebank Accession GBGF01081108 (Singh *et al.*, 2015)

TGTTTTTTTCTTCTTCTTTTTTTGTACTGTGTATGTTTCTAATTATAGTACTGTTCTGACATCATTATCTGCTAG
CTTAAATTATTTATTGTGTTAAAGGAAAATTATTTTTTAGAAAAAAAAAGAGTTGTATTTGGATATAAGCTTAGC
ATATTTAATAGCCTCCACCTTCCCAAAGCTTGATAAGACCAGTCAAATGCAAAGCAACTGCATGGGAATGAACTA
GAAAATATTTTTAGCTAAAAATTTGGATGCCTTGTCTCTCTCCTTATGTTTATATAAACCTGTCCCTTTGGGACT
TTCTCAAACTCATTTTAATATTTATTTCTCTCTTGACCTCTGTTTACCATTTC

>Transcriptome Shotgun Assembly: *Nicotiana tabacum* Locus 15016 Transcript 2/5 Confidence 0.692 Length 1902 Transcribed RNA Sequence (Reverse Compliment) Genebank Accession GDGU01095412

CTTATCTCTCTCCTTATGTTTATATAAACCTGACCCTTTAGGACTTTCTCAAACTCATTATAATATTTACCTCTC
TCTTGACTACTGCCTCTGTTACCATTTCTCTCTTTCATCACTTTCTTGAATCCTTCCCTTGGGATTTTCAAAGAA
AATCATATACTAATAAAGAAGAATTATTTGTAAAAAAAAGATGGCTGCTTTTTCAGACCAATTACAGCACACAAA
CCCTTTCCTTCTTGACTCAGTTTTTTTGCCAAGTTCTCCTATTAAGATGTCTGGTTTTTTAGAGGAACAAAACAA
TTCTATAGTGCAGAATTGTTTTACTCAATTTTACCAACCAGAATCTTTTCAGCAGCTCCCAACTGCCAATGTGAT
TGTTCATGAAAGTAGCTATTGCCTTGACCAAAGCACAAATGTTACACTTGGCCAAAATGAGCCTAATTCTGTGAC
CAACAACCGTAGCAGCAGTGTTAGCTTGGATATGGATTCTTCCTCTGTCACTGATAAAATAGAAAGTAGGAATAA
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GCAAACTAGCCCTACTAACATTACATCACAGGCAGCTGAAGTTATTCCTAACATTAATAATAGTGGCTATCCTTT
CTTGGATAATTCAGCTTCACTCATGTTTCAACAAGTCCATTTTCCTAATTCCATTTCTCAGGGTAATGGACAGCT
CTTATGGGGTGCAGATGACCAAAGACAAAAATTTATTAATCAGTCAGGACTCAGCAACAACTTTTGTTCTTTCCA
TTAATGTAACACCAAAGCCCTGCCCTACTTAACATCTGCACTTTGGAAAGGAGGTTTTATCCAGCAAAAGAAGTG
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AAGCTAAATCACCCTATCAAGGCTCGGATTCTTCTTAAATATTCACTAGATTGAAATGATAGGCCTTTTGGATGG
ATGTTATGAATCAAGAAATCAGTGGAAGCCAAAAGAGTACAAAGAGTCAACTGGCGGAATAAGAAGGCAGAGATC
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CTAGAGCCATGTGATGGTGGTGGGTGGGTGTTAAACCCTAGCTTAGCTAAAAGAAGGGGAAAAAATGTAATTCGT
TTTTTTGCATCTTCTTCTAGTAATTGATATTATTATTGTTGTTTCTTTGTTATTACTGTTAATTTTTACGGTTTT
AGTAGGGTTTGATGTTATTTTGTGAATGCCACATGATTGGTTTGCATAAAAGATGGAGGATTGTTGCTTTCATAT
CTATGTTTGTACGATGGAATCTTTCAG

Transcriptome Shotgun Assembly: *Petunia axillaris* Locus 28275 Transcript 1/2 Confidence 0.667 Length 1831 Transcribed RNA Sequence Genebank Accession GBRU01063012 (Guo *et al.*, 2015)

AAGTATTTTTAGCTAAAATTTGTATGTCCTATCTCTCTCCTCATGTTTATATAAACCTGACCCTTTGTGATTTTC
TCAGACCAATTAATTATTTCCCCTCTTGAGTACTAGTACAACCTCTCTGTTTACACTTTCTTGAATCCTTTAGTT
GGGAAAATCCAAAGCAAATCATAAACTAACAAAGAAGAAAACAAAAACAAAAATGGCTGCTTATTCATACCAATT
ACAGCACACAAATCCTTTTCTTGTTGACACAGTTTTTTTGCCAAGTTCTCCTATTAAGATGTCTGGTTATTTGGA
GGAACCAAACAACCAAAACTATTTTTCTCAATTTTACCAATTACCAGAATCTTGTCAGCAGCTCCCTACAAATAT
GATTGTTCATGAAAGTAGCTATTGCCTTGACCAAAGCACAAATGTTACTCTTAGCCAAAATGAGCCTTCTGAGAC
TAACAAAAGTAGCAGCAGCAATATCTTGGATATAGAATCTTCTTCAGTTACTGATAAAATTGAAACTGGGAATAA

GCCTATGAACAAGAAGAGAAAAGCCAGAGAAGGGTCTAATTCTTCTATGAGTTCTGCTAATTCTAAGAATGTAAG
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TGACAACAAGAAAGAGGAGAACAAATCTCATGAAGAGGCACCTACTGGCTACATTCATGTTAGAGCAAGAAGGGG
CCAAGCAACTGACAGCCATAGTCTTGCTGAAAGGGTGAGGAGAGAGAAAATAAGTGAAAGGATGAAGATACTGCA
ATCTCTTGTTCCTGGTTGTGACAAGGTAACTGGGAAGGCCCTCATGTTGGATGAAATAATTAATTATGTCCAATC
TTTGCAAAACCAAGTTGAGTTTCTCTCCATGAAACTTGCTTCTTCAAATCCAATGTACTATGACTTTGGCATGGA
CTTAGATGCACTCATGGTCAGACCTAACCAGAATTTGGGTGGTTTGGAGACACCACTGCCAAACATTCAGCAAAC
CAGCATTACTGATACTACACCACAGGCTGCTGCAGTCATTCCTAACATTAATAATGGCTATCCTTTCTTGGATAA
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CAACACCAAGCGCTGCCCTACTCAACATCTGCTAGCACTATGGAAGGTAGTTTTATCGAGCAAAAGAGGTGGGGC
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ATCCTTTTGATTGTTGCTGTCCAATATTTGACTTAGTTCTCCTGAATCTATTGAGACACAAGTTCTTAGAGCCAT
GTGATGGTGGTGGGTGTGATTAAGCCTTAGCTAGCTTAGCTAGAAAGAGGAAGCATCTAAATGTAGAGAAAAAAT
GCACTAGTTTTTGTTTCATTTTCTTTTTGTGTATCATCTTCTTTTATCACTAGTACTGAATATTGTAATGATCTG
TTGTTTTAATGTATGGCGCATTCTTTCTGTT

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