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Characterisation of the cell death-inducing activity of the conserved family of *Ciborinia camelliae*-like small secreted proteins (CCL-SSPs) of *C. camelliae*, *Botrytis cinerea* and *Sclerotinia sclerotiorum*

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science in Plant Biology

At Massey University, Manawatu, New Zealand

Hannah McCarthy

2019

Abstract

Ciborinia camelliae, the causal agent of *Camellia* petal blight, is a necrotrophic fungus that sequesters nutrients from dead plant cells. Candidate effector proteins have been identified from the secretome as a highly conserved clade, termed *C. camelliae*-like small secreted proteins (CCL-SSPs). Notably, the CCL-SSPs are not unique to *C. camelliae*. Indeed, a single homolog of the CCL-SSP family has shown to be encoded by the genomes of the closely related necrotrophs, *Botrytis cinerea* and *Sclerotinia sclerotiorum*, (BcSSP and SsSSP, respectively). Previous work has identified the ability of BcSSP and SsSSP to induce cell death on *Camellia* 'Nicky Crisp' petals, whereas of the ten *C. camellia* CCL-SSPs (CcSSPs) tested, only one induced very weak cell death. The aim of this study was to determine what specific regions of the SsSSP protein confered cell death-inducing ability, and to further characterise the cell death-inducing capability of these CCL-SSPs. In this study it was shown, through generation of chimeric region-swapped proteins and infiltration into *Camellia* 'Nicky Crisp' petals and *Nicotiana benthamiana* leaves, that the region encoded by Exon 2 of SsSSP is essential for cell death-inducing activity. It was also discovered that BcSSP and SsSSP may induce cell death to different extents, as a significant different was shown in quantified cell death induced on *Camellia* 'Nicky Crisp' petals. It was also found that BcSSP can induce strong cell death on *Arabidopsis thaliana* leaves, while SsSSP does not. This research also investigated appropriate methods for characterising cell death of CCL-SSPs, and suggested addition of a C-terminus tag for future work. The results of this study have shed further light on the CCL-SSP family as candidate effector proteins and provided several avenues for future researchers to fully elucidate the function of CCL-SSPs and their role in virulence of these three necrotrophic fungi.

Acknowledgements

The first people I would like to thank for making this research possible are my supervisors, Paul Dijkwel and Carl Mesarich. It was through the guidance and support of you both that I have learnt so much during this degree. Thank you for your patience and willingness to work through problems with me, I am a better researcher because of it.

I would also like to thank the laboratory team of C5.19 for their support and friendship: Tina, Cade, Rama, Elva, Asmat, Bruce, Aakansha, Xi Xi, Meriem, and especially Nikolai. You have been a brilliant mentor Nikolai and because of you not a single day of this research has been dull. Also, many thanks to the laboratory members of C3.03 who have been very welcoming and willing to help with any problems.

Thank you to the following groups for their scholarships, who's financial support helped me achieve to the best of my abilities. Thank you to Helen E Akers, Lovell & Berys Clark, the New Zealand Plant Protection Society, J P Skipworth, and finally the Camellia Memorial Trust, special thanks for your keen interest in my research.

I would not have made it this far without the love and support of my family. To my parents, you have always been there for me and gave me the courage to follow my dreams. I would also like to thank my close friends Nicole, Jess, Steph and Jenna, who's love, and friendship have made me into the woman I am today. Finally, to my partner Sam, you never fail to make me smile and I am so grateful to have you by my side.

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Abbreviations

˚C	Degrees Celsius
%	Percentage
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
ATTA	*Agrobacteria tumefaciens* transient transformation assay
APS	Ammonium persulfate
AVR	Avirulence
Bis	Bisacrylamide
BMGY	Buffered glycerol-complex medium
BMMY	Buffered methanol-complex medium
bp	Base pair
CAZymes	Carbohydrate active enzyme
CCL-SSP	*Ciborinia camelliae*-like small secreted protein
cm	Centimetre
DAMPs	Damage-associated molecular patterns
DNA	Deoxyribonucleic acid
dpi	Days post infiltration

EDTA	Diaminoethane tetraacetic acid
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
h	Hours
H_2O	Hydrogen dioxide
HCl	Hydrochloric acid
hpi	Hours post infiltration
HR	Hypersensitive response
kDa	Kilodalton
KOAc	Potassium acetate
KOH	Potassium hydroxide
L	Litre
LB	Luria-Bertani medium
ml	Millilitre
mm	Millimetre
mM	Millimolar
M	Molar
mAmp	Milliampere
MAMP	Microbe-associated molecular pattern
MES	2-(N-morpholino)ethanesulfonic acid
mins	Minutes

$MgCl_2$	Magnesium chloride
NaOH	Sodium hydroxide
Nep1	Necrosis and ethylene-inducing peptide 1
ng	Nanogram
NLP	Nep1-like protein
NLR	Nucleotide-binding leucine-rich repeat
OD	Optical density
P	P-value
PAMP	Pathogen-associated molecular pattern
PCD	Plant cell death
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
PTI	Pathogen-associated molecular pattern triggered immunity
qRT-PCR	Quantitative real-time polymerase chain reaction
R	Resistance
ROS	Reactive oxygen species
rpm	Revolutions per minute
SA	Salicylic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	Signal peptide

SSP	Small secreted protein
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
Tricene	N-Tris(hydroxymethyl)methylglycine
Tris	Tris(hydroxymethyl) aminomethane
UV	Ultraviolet
v/v	Volume/volume
V	Volt
w/v	Weight/volume
x g	Times gravity
YNB	Yeast Nitrogen Base
YPD	Yeast peptone dextrose medium
YPDS	Yeast peptone dextrose sorbitol medium

1 Introduction

Every day, plants are subject to attack from a multitude of pests and pathogens that attempt to sequester nutrients by colonising or killing a plant's cells. Whether disease occurs or not is a matter of which organism is better adapted, the defending plant or the invading pathogen. Fortunately for gardeners and farmers, various control methods can help reduce disease incidence, through the action of biocontrol agents (Medina et al., 2017), such as antagonistic rhizobia that can control various soilborne fungal pathogens (Das, Prasanna, & Saxena, 2017), and *Paenibacillus* strains that can control pathogenic rhizogenic *Agrobacterium* (Bosmans et al., 2017). Effective control measures can also act by enhancing a plant's natural immunity, such as boosting antiviral pattern recognition receptor-mediated innate immunity (Nicaise, 2017), and using a multitiered system to stack nucleotide-binding leucine-rich repeat (NLRs) receptors and augment effector triggered immunity (M. Zhang & Coaker, 2017). Therefore, understanding the interaction between plants and their invaders is crucial to maintaining disease-free crops.

Fungi are very successful pathogens that use a variety of different mechanisms and lifestyles to cause disease. The most well studied lifestyle is biotrophy, where the pathogen acts to avoid or suppress host immunity because it can only sequester nutrients from living plant cells. Another lifestyle is necrotrophy, where the pathogen sequesters nutrients from dead host cells (Kabbage, Yarden, & Dickman, 2015). It was previously thought that necrotrophy was a less advanced lifestyle than biotrophy, and that such pathogens can only cause disease by secreting a suite of toxins and cell wall-degrading enzymes (Guyon, Balague, Roby, & Raffaele, 2014). However, it has recently been discovered that necrotrophs are just as complex as biotrophs, and that some have been suggested to use secreted proteins to activate host immunity and trick the plant into killing its own cells (Frias, Gonzalez, & Brito, 2011; Leng et al., 2018; Lorang, Hagerty, Lee, McClean, & Wolpert, 2018; Lyu et al., 2015). Necrotrophic pathogens are severe pathogens to many crops, therefore understanding how they induce disease is critical to the development of viable control methods.

1.1 Constitutive defences and pathogen-associated molecular pattern triggered immunity

While plant diseases and infections seem like frequent occurrences, when compared to the number of attempted invasions a plant faces, they are uncommon. This is because plants possess a highly effective immune system (Fig. 3.1.), and pathogens are only successful if they have adapted to overcome it. The first barriers that potential invaders face are pre-formed defences, which are constitutively active. The plant cell wall is one example. Plant cell walls have both a primary and a reinforced secondary wall which acts as a physical barrier, requiring its degradation for pathogen entry. It also acts as a chemical barrier, by storing antimicrobial compounds which are released after cell wall break down (Miedes, Vanholme, Boerjan, & Molina, 2014).

In addition to constitutive defences, plants also possess layers of immunity that activate defence responses after recognition of invasion. The first layer of the plant immune system is the innate pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). This form of immunity is quite broad and is effective in detecting and activating defence responses against a range of different invaders. PTI can be activated by detection of molecules specific to the pathogen itself, such as recognition of bacterial flagellin, the highly conserved bacterial protein EF-Tu (Hong, Zheng, Chen, Chao, & Lin, 2018), or chitin, the main component of fungi cell walls (Ray et al., 2018). PTI can also be activated by recognition of self-damage (or damage-associated molecular patterns, DAMPs), for example, detection of short oligogalacturonides released from degradation of the plant cell wall (Davidsson et al., 2017) or extracellular ATP, which is released after pathogen mediated cell rupture (Zipfel, 2014). Through recognition of PAMPs and DAMPs via pattern recognition receptors (PRRs), plants can activate immunity and protect themselves from a wide range of pathogens.

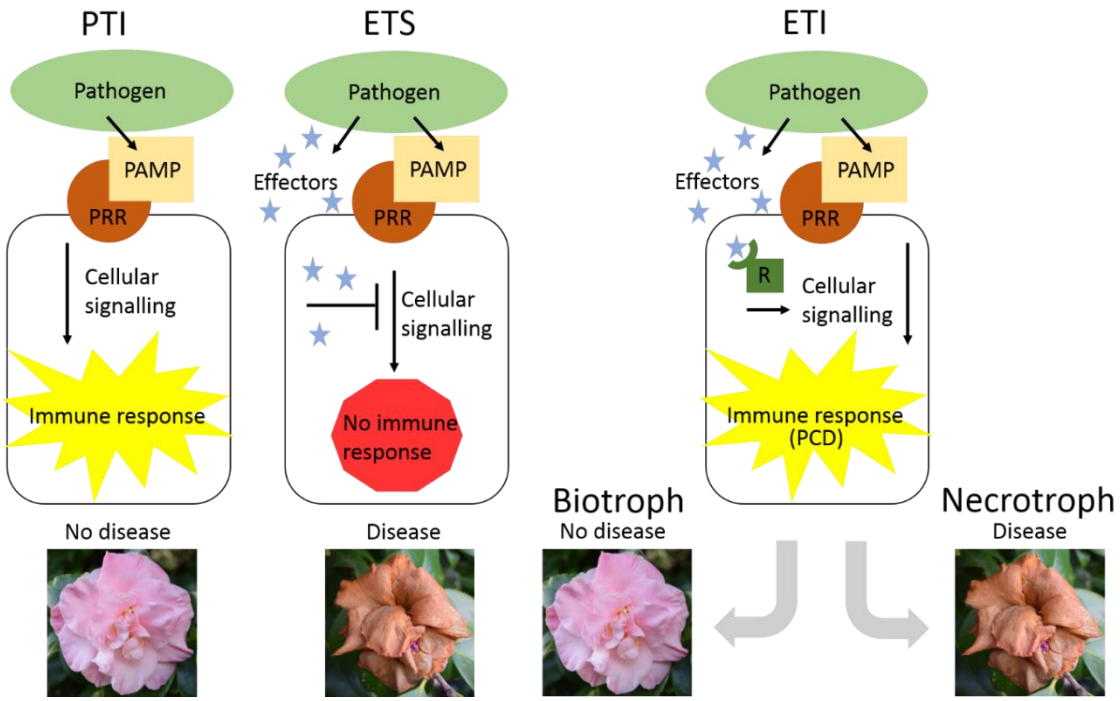


Figure 1.1. Model of plant immunity and differences in response to effector-triggered immunity (ETI) from biotrophs and necrotrophs.
Immunity can be triggered through recognition of PAMPs, (PAMP-triggered immunity, PTI), or recognition of effectors (blue stars) for ETI. PTI involves recognition of PAMPs by pattern recognition receptors (PRRs), while for ETI, effectors are recognised by resistance proteins (R). If effectors are not recognised, they act to suppress PTI and result in effector-triggered susceptibility (ETS), and disease. Generally, when effectors are recognised in the plant, activating ETI triggers an immune response resulting in plant cell death (PCD). From a biotrophic interaction this generally results in halted growth and no disease, while ETI from a necrotrophic interaction results in progression of growth, and disease.

1.2 Biotrophic suppression of PTI

Pathogens have been surviving on plants for millennia, and have developed strategies to overcome PTI, especially fungi, which are the focus of this review. The main component of fungal cell walls is chitin, and as previously mentioned, is a commonly detected PAMP. This is because one of the pre-formed defences a plant utilises is releasing chitinases into the apoplast, specifically for degrading fungal cell walls. This action releases chitin and enables its detection by specific PRRs in the plant cells (Mesarich et al., 2016). Detection of chitin activates PTI and results in a multitude of defence responses, that could include biosynthesis of antimicrobial compounds such as phytoalexins (Tiku, 2018), formation

of papillae (Troch et al., 2014), and accumulation of reactive oxygen species (ROS) (Lim, Kim, Gilroy, Cushman, & Choi, 2019). To avoid activation of these responses, the biotrophic fungus *Cladosporium fulvum* uses the small specifically binding virulent protein Avr4, an effector, to bind chitin in fungal cell walls through a chitohexaose-binding domain, preventing degradation by plant chitinases, detection as a PAMP, and subsequent activation of the plant immune response (Hurlburt, Chen, Stergiopoulos, & Fisher, 2018). This is one example of how a biotrophic fungus can suppress plant immunity to enable disease, resulting in effector-triggered susceptibility (ETS), and illustrates the adaptive potential of pathogens.

Another well studied biotrophic fungus is *Ustilago maydis*, which has been shown to use multiple effectors to manipulate host immunity and enable its own survival. *U. maydis* is the causal agent of maize smut disease and has been shown to use the four effectors; Cmu1, Pep1, Pit2, and Lip2. The chorismate mutase effector Cmu1 has been suggested to manipulate the host by inhibiting substrate levels in essential signalling pathways. Maize was shown to mount a salicylic acid (SA)-dependant defence response to Cmu1-deficient *U. maydis* transformants. Evaluation of the precursor levels in maize when infected by mutant and wildtype strains suggested that Cmu1 prevents phenylpropanoid entering the SA biosynthesis pathway and compromises the plant derived SA-dependant defence response (Djamei et al., 2011). The effector Pep1 was suggested to suppress the immune response after cerium chloride staining showed reduced ROS levels (essential for the hypersensitive response), when Pep1 was active (Hemetsberger, Herrberger, Zechmann, Hillmer, & Doehlemann, 2012). Yeast two-hybrid assays and complementation approaches suggested that Pit2 inhibits host cysteine proteases directly involved in SA-mediated defence (Mueller, Ziemann, Treitschke, Assmann, & Doehlemann, 2013). Interestingly, homologues of both Pep1 and Pit2 have been identified in other fungal species and shown to be important for virulence (Schweizer et al., 2018). Pep1 has also been found to be conserved across many dicot and monocot smut pathogens and has been described as a "fungal core effector" (Hemetsberger et al., 2015). The *U. maydis* effector Lip2 was suggested to protect against membrane damage caused by host immunity (used as an attempt to deter biotroph invasion), by maintaining lipid homeostasis (Lambie et al., 2017). The roles of these effectors from *U. maydis* illustrate the adaptive potential of biotrophic pathogens, and possible ways plant immunity can be manipulated and suppressed.

1.3 Plant adaption to pathogen effectors through ETI

While the previous examples illustrate the success of biotrophic fungi in mediating infection, plants can also evolve and adapt in response. Some plants can utilise the next layer of the immune response by recognising pathogen effector proteins. This form of immunity is effector-triggered immunity (ETI) and is more specific because it involves recognition of specific proteins by plant resistant (R) proteins (encoded by *R* genes) (Islam & Mayo, 1990). One of the first *R* genes identified was from flax (*Linum usitatissimum*), which provides resistance against the biotrophic flax rust fungus *Melampsora lini*. It has been shown that *M. lini* secretes several effectors to promote the colonization of flax (Dodds, Lawrence, Catanzariti, Ayliffe, & Ellis, 2004), with the best studied being the AvrM effector (Catanzariti, Dodds, Lawrence, Ayliffe, & Ellis, 2006). Resistance in *L. usitatissimum* to this biotrophic pathogen was found to be a result of the R protein M, which recognises the C-terminal domain of AvrM, activating ETI and mediating disease resistance (Catanzariti et al., 2010). This example illustrates how plants can adapt disease resistance by recognising pathogen effector proteins and activating immune responses.

The crystal structure of AvrM illustrates that a coiled-coil domain at the C-terminus mediates the interaction with protein M (Ve et al., 2013). Production of crystal structures has recently been used to investigate the function of other *M. lini* effectors such AvrP and AvrL567-A. For both proteins it was found that the domain or residues involved in the protein function, such as binding zinc for AvrP (X. Zhang et al., 2018) and interacting with cytosolic cytokinin oxidases of the host plant for AvrL567-A (Wan et al., 2018), are also important for recognition by the host R proteins. The vast number of effectors produced by *M. lini* was further established by a study of the genome of *M. lini* isolate CH5 where a total of 725 candidate avirulence (AVR) effectors were predicted with high homology to other rust fungi species (Nemri et al., 2014). Further research, which involved generating a high-density genetic linkage map, suggested that *M. lini* has a high recombination rate and a durable asexual stage that can survive chromosomal duplications and deletions. The high variability and adaption rate of *M. lini* provides an explanation as to how this fungus overcomes ETI so quickly in *L. usitatissimum* (Anderson et al., 2016). The interaction between *M. lini* and *L. usitatissimum* illustrates

the ongoing adaptive warfare that occurs between plants and pathogens, and that each organism always has the potential to outcompete the other.

1.4 Re-evaluation of plant immunity models

Until recently, the various components of plant immunity that were discussed above, such as PTI, ETI and ETS, could be used to describe the interactions between plants and pathogens (especially biotrophic pathogens). Several models were made over the years that illustrate this plant immune system, such as the well-known 'Zigzag model' (Jones & Dangl, 2006). However, recently there have been several reports of plant-pathogen interactions that do not fit with previously described plant immunity models. One such report is the finding that in plant interactions with necrotrophic pathogens, such as *Cochliobolus victoriae* (discussed in detail in section 1.5) the plant immune response does not result in a reduction of disease, but instead mediates it (Lorang et al., 2012).

The various exceptions to the original plant immunity models have been compiled and discussed in a recent review by Cook, Mesarich, and Thomma (2015). These researchers concluded that instead of restricting our understanding of the various components of plant immunity to such strict roles, plant immunity should instead be thought of as a "surveillance system" able to detect invasion in a variety of ways (Cook et al., 2015). The work presented in this review has been widely accepted and the proposed "surveillance system" model readily used (Boutrot & Zipfel, 2017; Gust, Pruitt, & Nürnberger, 2017; Luzuriaga-Loaiza et al., 2018; Phani, Shivakumara, Davies, & Rao, 2018; Y. Wang & Bouwmeester, 2017). One of the ways these reviewers suggest that we are limiting our understanding of the plant immune system, is by separating PTI and ETI, and suggesting they are distinct processes, when research suggests that there is a connection between the two. Also, the idea that PAMPs are broadly found in pathogens, while effectors are more specific, is not well supported.

The discovery of effector families, where the same type of effector has been identified in different species, was evidence to support the idea that effectors are not always highly specific molecules and may share characteristics with PAMPs. One such family are the necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs), which have been identified across three different forms of life; oomycete, bacteria and fungi (Oome et al.,

2014). Most NLPs share the conserved region II heptapeptide motif GHRHDWE, shown to be required for phytotoxic activity (Dinah et al., 2006). NLPs also generally contain the conserved region I, an 11 amino acid long region believed to have an immunogenic role as it has been found to activate ethylene (a sign of PTI) in *Arabidopsis thaliana* (Oome et al., 2014). These results further support the idea that PAMP elicitors and effectors may not be so distinct, as here NLPs have been shown to have characteristics of both PAMPs and effectors. As well as being present in vastly difference species, NLPs have also been found in fungal and bacterial species that are not pathogenic to plants (Gijzen & Nürnberger, 2006), suggesting they might play a role in processes other than virulence. Some NLPs expressed during early infection have also been shown to lack cell death-inducing activity, further supporting the concept that some NLPs might have roles outside of just activating cell death (Cabral et al., 2012). This has been further supported by the discovery that deleting a NLP from *Verticillium dahlia* compromises vegetative growth and conidiospore production as well as virulence (Santhanam et al., 2013). Therefore, the NLP family and their various roles in virulence, activators of immunity, and even vegetative growth, as well as their existence in many pathogens across all three kingdoms of life support the idea that our current models of PTI and ETI do not fully encompass the mechanisms that are involved in plant-pathogens interactions.

1.5 Necrotrophic fungi can manipulate host immunity

While the previous models discussed illustrate the interactions that occur between biotrophic fungi and plants, they cannot describe the infection strategy employed by necrotrophic pathogens. Other than the obvious difference that necrotrophs sequester nutrients from dead host cells, and therefore require killing the host plant, it was also recently suggested that necrotrophs do not act to avoid or suppress plant immunity like biotrophs. Previously, it was thought that necrotrophs were not as advanced as biotrophs as no *R-AVR* gene interaction had been found and it was believed that they induced disease through an arsenal of cell wall degrading enzymes and toxins (Guyon et al., 2014). However, it has been suggested that some necrotrophs use the plant immune system to their own advantage, by tricking the plant into killing its own cells, therefore enabling the fungus to acquire nutrients and promote disease.

Pyrenophora tritici-repentis is an example of a necrotroph that can use effector recognition through R proteins to activate immunity and mediate disease. As the causal agent of wheat tan spot, *P. tritici-repentis* is currently known to secrete three main effectors; ToxA, ToxB and ToxC, whose susceptibility is conferred in wheat by the *R* genes *Tsn1*, *Tsc2* and *Tsc1*, respectively (Faris, Liu, & Xu, 2013). ToxC has been found to be different to the other toxins as it is not a protein, and instead has been described as a "nonionic, polar, low-molecular-weight molecule" (Effertz, Meinhardt, Anderson, Jordahl, & Francl, 2002). ToxB and ToxC appear to promote virulence differently to ToxA as they both induce chlorosis rather than necrosis (Ciuffetti, Manning, Pandelova, Betts, & Martinez, 2010; Faris et al., 2013). Recently, research has further validated the role of Tsn1-ToxA and Tsc1-ToxC interactions in generating susceptibility to tan spot in wheat. Crosses to generate a wheat line carrying both *Tsn1* and *Tsc1* suggested that these two *R* genes contribute additively to susceptibility (Liu et al., 2017).

ToxA was the first effector identified and is the best characterised. Comparing the effects of ToxA in sensitive and insensitive wheat suggested that a single gene determines sensitivity and mediates the translocation of ToxA across the cell membrane (Manning & Ciuffetti, 2005). This gene was identified through gene expression profiling as *Tsn1*, and its recognition of ToxA in sensitive wheat was suggested to activate a cascade of defence signalling pathways resulting in host cell death (Adhikari et al., 2009). Further components of the *ToxA-Tsn1* interaction were investigated and it was suggested that the wheat protein ToxABP1 interacts with ToxA and is either involved in transport of ToxA into the chloroplasts, downstream events leading to cell death, or as the direct target of ToxA (Manning, Hardison, & Ciuffetti, 2007). It was later suggested through yeast two-hybrid assays that ToxABP1 does not interact with Tsn1, so it was hypothesised that Tsn1 indirectly recognises ToxA by monitoring a target which can be effected through downstream effects of ToxA (Faris et al., 2010). This is an example of indirect effector recognition, where the AVR effector targets and changes an accessory protein, which is then detected by the *R* gene product (Dodds & Rathjen, 2010). More research is required to fully understand this interaction and identify the accessory protein that ToxA interacts within wheat.

Cochliobolus victoriae is another example of a necrotroph that can manipulate the host immune response to mediate disease. This fungus causes Victoria blight on "Victoria-

type" oats and research performed on the interaction of *C. victoriae* with *A. thaliana* shows that the *R* gene *LOV1* mediates susceptibility to Victoria blight. *LOV1* is part of the nucleotide-binding site leucine-rich repeat (NBS-LRR) gene family usually associated with defence responses (Lorang, Sweat, & Wolpert, 2007). The *C. victoriae* effector victorin, has been shown to bind defence-associated thioredoxin, thereby activating *LOV1* and initiating a defence response that mediates disease (Lorang et al., 2012). Further evidence that *C. victoriae* uses the host immune response to mediate disease is the finding that silencing six genes with known roles in defence responses results in suppression of victorin-induced cell death. This silencing approach led to the discovery that defence response-associated gene *SGT1* is required for sensitivity to victorin (Gilbert & Wolpert, 2013). Recent research on the *C. victoriae-Phaseolus vulgaris* interaction showed that disease is also mediated by effector victorin, and that victorin sensitivity is a developmentally regulated, quantitative trait. Interestingly, it was also shown that the *LOV1* gene of *P. vulgaris* mediates victorin-dependent cell death in *Nicotiana benthamiana* but not *A. thaliana* (Lorang et al., 2018). These results further illustrate how necrotrophic pathogens can manipulate and hijack the host immune response to mediating disease.

1.6 *Ciborinia camelliae* as a necrotrophic pathogen

Like the necrotrophic fungi previously discussed, *Ciborinia camelliae* is believed to be a pathogen that manipulates host immunity to enable infection. As the causal agent of *Camellia* petal blight, *C. camelliae* infects the flowers of plants belonging to the *Camellia* genus, resulting in necrotic lesions and premature death of the blooms. This disease impacts several economically important industries such as the *Camellia* seed oil industry, the floriculture industry (Taylor & Long, 2000), and while of limited economic value, it also frustrates keen *Camellia* growers. While this disease is not new to growers, it is still not well understood and research on viable control methods has been limited.

So far, there has been several attempts to control the pathogen by introducing competitor microorganisms such as known sclerotial parasites (sclerotia are structures made of hardened hyphal threads that function as the dormant stage of some fungi (Loginov & Šebela, 2016)), which offered very little protection (Toor, Jaspers, & Stewart, 2005a).

Other phyllophane microorganisms were successful in preventing infection on single petals, but field trials determined that effective colonisation and protection did not occur (Toor, Pay, Jaspers, & Stewart, 2005). One method that has potential is using wood rotting fungi in mulches underneath *Camellia* plants, which was found to successfully degrade sclerotia of *C. camelliae* (Toor, Jaspers, & Stewart, 2005b). However, as *C. camelliae* is a wind-borne pathogen, the spread of spores from other infected *Camellia* petals is highly likely, especially as *Camellia* is a highly popular shrub. Genetic analysis showed that while American and New Zealand accessions of *C. camelliae* were genetically distinct, the fungi sampled within each country showed very little diversity, suggesting that any control methods devised should provide protection from *Camellia* petal blight across New Zealand (Toor, Ridgway, Butler, Jaspers, & Stewart, 2005). Therefore, it is becoming evident that to help production of viable control methods, a molecular approach needs to be undertaken to understand this disease and its interaction with the *Camellia* host.

1.7 Current research into the *Camellia* – *C. camellia* interaction

After it was discovered that some *Camellia* species have natural resistance to *Camellia* petal blight (Taylor, 2004), research was performed to determine what defence responses are utilised in these resistant species to avoid or reduce infection. It was discovered that resistant *Camellia* species tend to lignify cell walls or have cell wall modifications correlated with infection, and chitinase activity was also suggested to play a role (Taylor, 2004). An assessment of both macro- and microscopic parameters as indicators of infection severity illustrated a gradient of response in 40 different *Camellia* species. This research identified that of the species tested, the most susceptible was the interspecific hybrid *Camellia* 'Nicky Crisp', while the most resistant was *Camellia lutchuensis* (Denton-Giles, 2014).

Significant insights were made in understanding the mechanism of *Camellia* petal blight after the *C. camelliae* transcriptome was sequenced, and the secretome predicted. From this data, it was identified that *C. camelliae* is a member of the Sclerotiniaceae family and is closely related to the two necrotrophic fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum*. From the secretome data, it was shown that of the predicted protein categories, *C. camelliae* had significantly higher numbers in the small secreted protein

(SSP) group than the other two pathogens. The *C. camelliae* SSPs within this group also had high cysteine content of equal or greater than 10% (Denton-Giles, 2014). Cysteine residues are key features of effector proteins that form disulphide bonds, believed to be important for protein structure and function, and stability against proteases in the host environment (Stergiopoulos & De Wit, 2009). The importance of cysteine residues in effector proteins has been shown in a study of the *Ralstonia solanacearum* effectors RipAW and RipAR. These effectors were shown to have the ability to suppress PTI responses, but it was shown that mutation of a conserved cysteine residue completely abolished protein activity (Nakano, Oda, & Mukaihara, 2017). Similar studies have also shown that mutation or deletion of cysteine residues result in disrupted function of effector proteins, such as *S. sclerotiorum* effector SsSSVP1 (Lyu et al., 2016), *Ustilago maydis* effector Pit2 (Mueller et al., 2013), and *Cladosporium fulvum* effector Avr2 (Van't Klooster et al., 2011). The high cysteine content of *C. camelliae* SSPs suggest that these cysteine residues may participate in disulphide bonds required for protein stability. Therefore, the cysteine-rich SSPs of *C. camelliae* were shortlisted as candidate effector proteins involved in manipulating the host immune response.

Further analysis of *C. camelliae* SSPs was performed by aligning the proteins by amino acid identity. The resulting cladogram illustrated a highly supported clade of proteins within the SSPs, made up of 73 unique coding sequences. The proteins within this family were named the *Ciborinia camelliae*-like small secreted proteins (CCL-SSPs) and were predicted to have undergone gene duplication resulting in lineage-specific gene expansion. The genomes of closely related necrotrophs *B. cinerea* and *S. sclerotiorum* were also found to encode one homologue of the CCL-SSP family (BcSSP and SsSSP respectively). A search for homologues outside of the Sclerotiniaceae family identified 15 other fungi species with genomes that encoded a CCL-SSP. Interestingly, all 15 of these species have a necrotrophic life stage (were either necrotrophs or hemibiotrophs), suggesting that this protein family is specifically involved in necrotrophic processes (Denton-Giles, 2014).

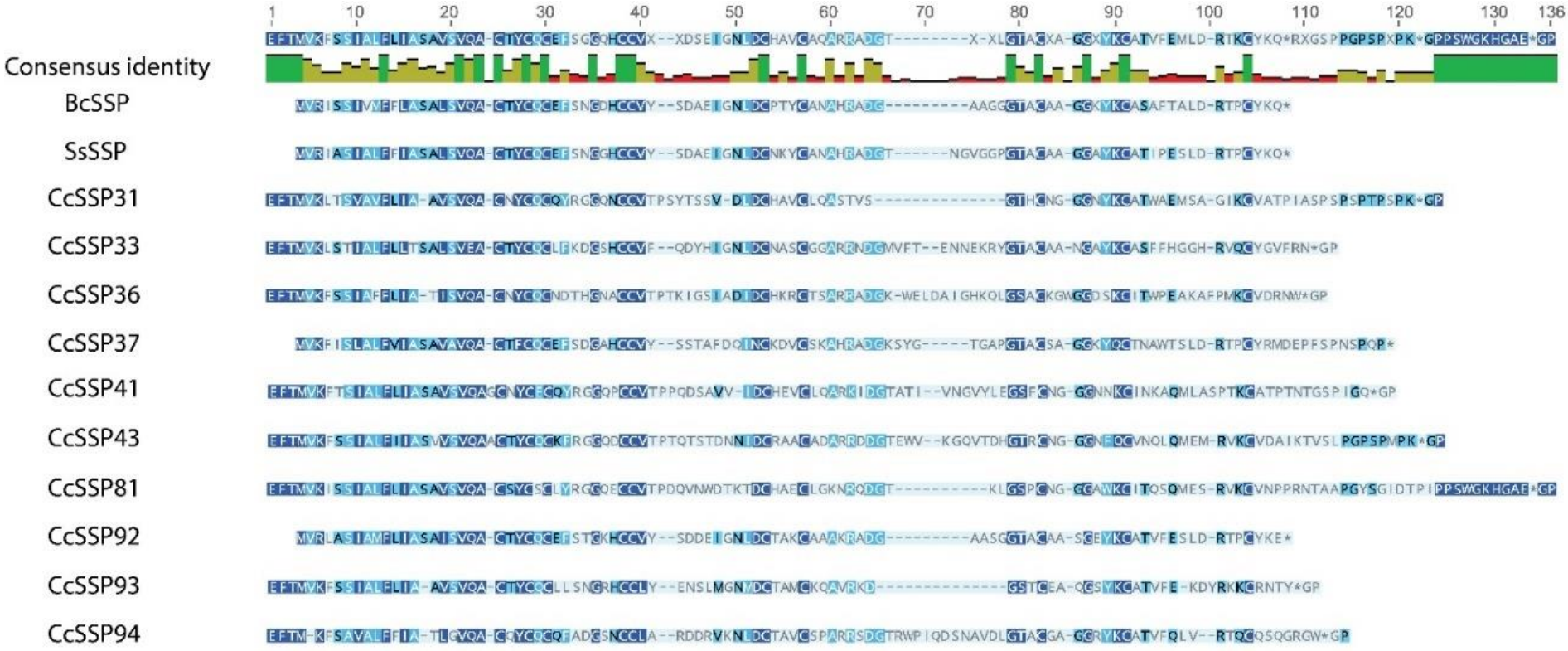
CCL-SSPs have a conserved primary structure of an N-terminal signal peptide, ten cysteine residues predicted to form the same disulphide bonds, several conserved regions, and several regions predicted to be under positive selection. Of the 73 coding sequences in this family, 72 were identified as expressed during infection, based on expressed

sequence tag detection in the transcriptome. Expression of nine CCL-SSPs of *C. camelliae* was investigated using quantitative real-time polymerase chain reaction (qRT-PCR), and it was determined that expression increased during pre-lesion development, then significantly decreased 48 hours post infiltration (hpi), coinciding with lesion maturation (Denton-Giles, 2014). These results suggest that these proteins may play a role in establishing a compatible association with the host and are therefore candidate effectors.

To determine the function of these proteins, ten *C. camelliae* CCL-SSPs (*CcSSPs*) and the homologues from *B. cinerea* (*BcSSP*) and *S. sclerotiorum* (*SsSSP*), were recombinantly expressed in *Pichia pastoris* and the cell-free culture filtrates injected into highly susceptible *Camellia* 'Nicky Crisp' petals. Interestingly, strong cell death was induced by BcSSP and SsSSP, while only one *C. camelliae* protein, CcSSP92, induced very weak cell death that covered a small area of the infiltration zone (Denton-Giles, 2014). CcSSP92 is the most similar in amino acid identity to SsSSP (75%) and BcSSP (76%) (Denton-Giles, 2014), and the amino acid identity of CcSSP92, SsSSP, BcSSP, and the other nine CcSSPs tested is shown in figure 1.2. Infiltrations into the highly resistant *Camellia* species *C. lutchuensis* also resulted in cell death from SsSSP and BcSSP but it was delayed to 24 hpi and was not as severe as was observed from the 'Nicky crisp' infiltrations. Also, BcSSP appeared to induce stronger cell death than SsSSP. Interestingly, infiltration of BcSSP and SsSSP into *Nicotiana benthamiana* leaf tissue also produced a cell death response (Denton-Giles, 2014). This suggests that SsSSP could be able to induce death in non-host plants, because while *N. benthamiana* is a host of *B. cinerea* (Fillinger & Elad, 2015), and the closely related species *N. tabacum* and *N. rustica* L. are hosts of *S. sclerotiorum*, *N. bethamiana* has not been reported as a host plant (Saharan & Mehta, 2008).

Figure 1.2. Amino acid sequence alignment of SsSSP, BcSSP, and the ten CcSSPs tested for cell death-inducing activity.

Amino acid alignment was made with Geneious v9.1.8 software using a Blosum62 cost matrix and free end gaps. Consensus identity is at the top and is illustrated by the coloured bars. Green bars illustrate sequence identity of 100%, yellow is sequence identity of at least 30 % but lower than 100%, and red is sequence identity below 30%. The sequences below the consensus are also coloured to highlight conserved amino acids. Dark blue signified that amino acids are highly similar, medium blue shows that the amino acids are similar, and light blue means the amino acids are not similar (threshold = 1).


Consensus identity
BcSSP
SsSSP
CcSSP31
CcSSP33
CcSSP36
CcSSP37
CcSSP41
CcSSP43
CcSSP81
CcSSP92
CcSSP93
CcSSP94

To determine whether the lack of cell death-inducing ability was a result of the CcSSPs not having cell death activity, or whether there was not enough protein present to elicit a response, the CCL-SSPs were tagged. A c-Myc 6xHis-tag was added to the C terminus of the CCL-SSPs tested and it was confirmed by western blot that seven of the ten tagged proteins could be detected (including SsSSP, BcSSP, and CcSSP37). Protein concentration was quantified by a chemiluminescence method and it was confirmed that all seven detected tagged CCL-SSPs were present at a concentration greater then what was observed for the 5-fold diluted tagged SsSSP. However, after infiltration of these tagged proteins it was discovered that the tag disrupted cell death activity, as tagged BcSSP failed to induce any cell death, and cell death of tagged SsSSP was delayed to 8 hpi and was reduced in strength. None of the tagged CcSSPs tested (CcSSP37, CcSSP43 and CcSSP92) induced cell death, however it could not be determined whether the lack of cell death from the CcSSPs was a result of no cell death-inducing activity, or whether addition of the tag disrupted cell death activity.

This recent discovery of the CCL-SSP family offers an exciting possibility for elucidating the interaction between *C. camelliae* and its susceptible *Camellia* hosts. Should these proteins be discovered as virulence factors, control methods could be devised to target the function of these proteins. While significant progress has been made into elucidating how this necrotrophic fungus causes disease, more work is required before a viable control method for *Camellia* petal blight can be developed.

1.8 Research objectives

The previous research on the *C. camelliae* – *Camellia* interaction, and the various literature that suggest that necrotrophic pathogens can manipulate host immunity led to the hypotheses: (1) that specific regions in the SsSSP protein can be identified which confer cell death-inducing activity, and (2) that different CCL-SSPs induce cell death in distinct plant species.

To test these hypotheses the following objectives were established:

1. Identify a specific region of the SsSSP protein that confers cell death-inducing ability. This was performed by generating chimeric region-swapped proteins containing regions from SsSSP and non-cell death-inducing protein CcSSP37 and testing the ability of these chimeric proteins to induce cell death in *Camellia* 'Nicky Crisp' petals.

2. Test the ability of SsSSP, BcSSP and CcSSP37 to induce cell death in distinct plant species. To address this, these proteins were infiltrated into multiple host and non-host plants of *C. camelliae*, *S. sclerotiorum*, and *B. cinerea* and any resulting cell death was quantified and compared between each CCL-SSP.

3. Determine whether the activity of CCL-SSPs can be modelled in different systems. This was determined by using both a *Pichia pastoris* expression system and *Agrobacterium tumefaciens* transient transformation assays to test cell death-inducing activity of CCL-SSPs.

It is my aim that the research performed to test these hypotheses will be a step forward in characterising CCL-SSPs and determining their role in virulence. It is also my aim that this research will contribute to future work on *Camellia* petal blight disease prevention and management.

2 Materials and Methods

2.1 Plant and biological materials

Plants grown in this study are as follows: *Camellia* 'Nicky Crisp', *Nicotiana benthamiana*, *Arabidopsis thaliana* Col-0, *Brassica campestris* (Pak Choi, mini toy choi), *Raphanus sativus* (Radish, pink beauty), *Ocimum basilicum* (Basil, sweet), *Phaseolus vulgaris* (Bean, climbing cobra), *Beta vulgaris* var *Cruenta* (Beetroot, action F1 hybrid), *Triticum aestivum* (Savannah wheat). Mature flowering (about 4 year old) *Camellia* 'Nicky Crisp' plants were sourced from Kilmarnock Nurseries, New Zealand, and were grown in the Massey University Plant Growth Unit greenhouse (40˚37'80.54"S, 175˚61'34.13"E) at temperatures between 1˚C and 20˚C. The greenhouse air inlets were covered with 30 mm thick polyester wadding to reduce entry of *C. camelliae* spores from the surrounding environment. All other plants were grown in a Massey University plant growth facility under short day light conditions (12 hours light, 12 hours dark), at 22˚C and at a relative humidity of about 60%. *N. benthamiana* plants were grown in pots with dimensions of 10 cm length x 10 cm wide x 11 cm tall. All plants other than *Camellia* 'Nicky Crisp' were grown in pots 6 cm length x 6 cm wide x 8 cm tall. *A. thaliana* and *N. benthamiana* seeds were lab stocks. While *B. campestris*, *R. sativus*, *O. basilicum*, *P. vulgaris*, *T. aestivum* and *B. vulgaris* seeds were sourced from Egmont Seed Company Ltd, New Zealand. All plants were grown in Premium Seed Mix^{TM} (Daltons, New Zealand)

All microorganisms used in this study are described with the appropriate antibiotics used for growth in table 2.1. These microorganisms were later transformed with various plasmids, and the selective antibiotics used after transformation are in table 2.2, and plasmid vector maps are in Appendices 7.1, 7.2, and 7.3. The various media and their recipes used to grow these microorganisms are in table 2.3. All inoculations of microorganisms from colonies were performed with fresh plates (not older than two weeks) and were done by touching the colony with a pipette tip, then pipetting up and down in the liquid media. Glycerol stocks or colonies were used to inoculate plates using a flame sterilized loop, while inoculations of plates with liquid culture were done by pipetting the appropriate volume onto the plates, then spreading with an ethanol sterilized

then flame evaporated spreader. All work with microorganisms were performed in a clean and sterile lamina flow cabinet (Clemco, Australia), treated with UV light for 10 min before use. Growth of microorganisms was often determined by measuring the OD using a spectrophotometer (U-1100, Hitachi, Japan) and 1 ml of culture in a 1 ml cuvette (unless stated otherwise).

Table 2.1. Microorganisms used in this study and antibiotics required for growth

Organism	**Strain**	**Antibiotic concentration for growth**	**Growth media and conditions**
Escherichia coli	TOP10'F (Invitrogen)	10 µg/ml tetracycline	LB media, 37˚C overnight.
	JM110 (Agilent Technologies)	100 µg/ml streptomycin	LB media, 37˚C overnight.
Agrobacterium tumefaciens	GV3101 (Laboratory stock)	50 µg/ml rifampicin	LB media, 28˚C for 2-3 days.
Pichia pastoris	X – 33 (Invitrogen)	No antibiotics	YPD media, 28˚C for 2 days

Table 2.2. Selective antibiotics used in this study

Plasmid selecting for	**Selective antibiotic**
pPICZA in *E. coli* TOP10'F	100 µg/ml zeocin
pPICZA in *P. pastoris* X-33	25 µg/ml zeocin
pTwist Amp High Copy	100 µg/ml ampicillin
pICH86988	50 µg/ml kanamycin

Plasmid maps are in Appendices 7.1, 7.2 and 7.3.

Table 2.3. Growth media used in this study

Media name	Components	Notes
YPD	1% yeast extract 2% peptone 2% dextrose	For agar, add 2% bactoagar. Add dextrose after autoclaving and media has cooled.
LB	1% peptone 0.5% yeast extract 1% NaCl	For agar, add 2% bactoagar.
Low-salt LB	1% peptone 0.5% yeast 0.5% NaCL	For agar, add 2% bactoagar.
YPDS	1% yeast extract, 2% peptone, 1 M sorbitol, 2% dextrose	For agar, add 2% bactoagar. Add dextrose after autoclaving and media has cooled.
BMGY	1% yeast extract 2% peptone 100 mM potassium phosphate (pH 6.0) 1.34% YNB* 4 x 10^{-5}% biotin 1% glycerol	Add potassium phosphate, YNB, biotin and glycerol after autoclaving and media has cooled.
BMMY	1% yeast extract 2% peptone 100 mM potassium phosphate (pH 6.0) 1.34% YNB* 4 x 10^{-5}% biotin 0.5% methanol	Add potassium phosphate, YNB, biotin and methanol after autoclaving and media has cooled.

*YNB is Yeast Nitrogen Base with Ammonium Sulfate without Amino Acids

2.2 Construct design, general polymerase chain reaction (PCR), and sequencing

CCL-SSP and chimeric region-swapped *CCL-SSP* genes were constructed and inserted into pPICZA plasmids by Genscript™ (USA). Tagged *CCL-SSP* and tagged chimeric region-swapped *CCL-SSP* genes were constructed and inserted into pTwist Amp High Copy plasmids by Twist Bioscience™ (USA). Sequence maps of all genes used in this study are in Appendix 7.4, and all primer sequences used in this study are in Appendix 7.5.

General PCR and colony PCR were routinely performed to check success of transformation. Standard PCR protocol was performed by first making a master mix in a 1.5 ml microcentrifuge tube containing 12.5 µl Taq 2x Master Mix (New England BioLabs Inc. USA), 1 µl forward primer, 1 µl reverse primer, and 10.5 µl milliQ H_2O per reaction. After preparation of the master mix, 25 µl was pipetted into 0.2 ml PCR tubes. For *E. coli* and *A. tumefaciens* the colonies were inoculated directly into the PCR tubes containing the master mix by touching the colony lightly with a pipette tip, then pipetting up and down in the master mix. For *P. pastoris* colonies an additional lysate step was required. A *P. pastoris* colony was inoculated (the same way as described above) into 10 µl of 0.02 M NaOH in a 0.2 ml PCR tube and boiled for 10 mins at 99˚C in a vapo.protect™ lightcycler (Eppendorf, Germany). After boiling, 2 µl of cell lysate was added to the master mix in each PCR tube (milliQ H_2O in master mix is reduced to 8.5 µl). All PCRs were performed using an vapo.protect™ lightcycler (Eppendorf, Germany) and the cycle; 95˚C/5 mins then 30 cycles of 95˚C/1 min, 54˚C/1 min, 72˚C/1 min, then 72˚C/7 mins. Following PCR, products were visualised on 1% or 1.5% agarose gels ran at 100 V in TAE buffer (Tris 40 mM, acetic acid 20 mM, EDTA 1 mM). Agarose gels were made by dissolving the appropriate amount of agarose powder in TAE buffer, the volume in the bottle weighed before heating in a microwave, then the volume made up with water after heating. After the gels were ran the required length of time, they were transferred into 1% ethidium bromide to stain for 15 min. The gels were then visualised under UV light in a Universal Hood II™ (Bio-Rad, USA).

Following any DNA extraction, DNA quality and concentration were measured using Nanodrop™ (Thermo Fisher Scientific, USA). DNA was considered of suitable quality if 260/230 was ≥ 1.8, and 260/280 was ≥ 1.5. Transformation of *P. pastoris* was confirmed by colony PCR with *AOX* primers, and the PCR products of two colonies per gene were

sequenced in both directions by Macrogen Ez – seq™ (Rep. of Korea). Following transformation of *A. tumefaciens* and confirmation by colony PCR with *M13* primers, the PCR products of one colony for each gene were sequenced in both directions by Massey Genome Service™ (NZ).

2.3 Preparation of electrocompetent cells

Electrocompetent *E. coli* cells of both TOP10F' and JM110 strains, and *A. tumefaciens* GV3101 were prepared by inoculating 5 ml LB broth containing the appropriate antibiotics (Table 2.1 & 2.2) with a fresh colony in a 50 ml falcon tube and grown overnight under the appropriate conditions (Table 2.1), shaking 200 rpm. The 5 ml culture was then added into 500 ml LB broth with the appropriate antibiotics (Table 2.1 & 2.2) in a 1 L flask, incubated shaking at 200 rpm until an OD_{600} of 0.5-0.7 was obtained. Cells were then chilled on ice for 20 mins and spun in prechilled 250 ml autoclavable bottles for 15 mins, 4000 x g. Supernatant was discarded, and the cell pellet resuspended in 250 ml ice-cold 10% v/v glycerol and spun again for 15 mins, 4000 x g. The process was repeated with 125 ml and then 20 ml of ice-cold 10% glycerol, with the cells kept on ice between spin cycles. Finally, the cell pellet was resuspended in 2 ml of ice-cold 10% v/v glycerol. Aliquots of 40 µl were made in 1.5 ml microcentrifuge tubes, snap frozen in liquid nitrogen and then stored at -80˚C.

Electrocompetent *P. pastoris* X-33 cells were prepared by inoculating a 5 ml YPD broth (Table 2.3) in a 50 ml falcon tube with a fresh colony and growing under the appropriate conditions (Table 2.1), shaking at 200 rpm. 0.1 ml of this culture was then added to 500 ml YPD broth in a 2 L flask and incubated under the appropriate conditions (Table 2.1) shaking at 200 rpm until an OD_{600} of 1.3-1.5 was obtained. The culture was then centrifuged at 1,500 x g for 5 min at 4˚C, and the pellet resuspended in 250 ml ice-cold sterile water. The process was repeated with 125 ml ice-cold water and then 20 ml of ice-cold 1 M sorbitol, with the cells kept on ice between spin cycles. Finally, the pellet was resuspended in 1 ml ice-cold 1 M sorbitol, 40 µl aliquoted into 1.5 ml centrifuge tubes and kept on ice to be used the same day. These cells cannot be stored.

2.4 Electroporation transformation

Transformation of *E. coli* strains TOP10F' and JM110 and *A. tumefaciens* GV3101 was performed by electroporation. Electrocompetent cells were thawed on ice, while a 0.2 cm electroporation cuvette (Bio-Rad, USA) was placed on ice (one per sample). In an ice-cold microcentrifuge tube, 2 µl of DNA suspended in TE buffer (10 mM Tris pH 7.5 with HCL, 1 mM EDTA pH 8 with NaOH) was mixed with 40 µl of the electrocompetent cells and incubated on ice for about 1 min. A Micropulser™ (Bio-Rad, USA) was set to "Ec2" for the *E. coli* cells and to "Ec3" for the *A. tumefaciens* cells. The DNA and cell mixture were transferred into an ice-cold cuvette, tapped to the bottom and placed in the machine to electroporate with one pulse. Immediately after electroporation, 1 ml of low-salt LB liquid media was added (Table 2.3), and the cuvette mixed by inversion to resuspend the cells (timing is essential for transformation efficiency). The samples were then transferred to 15 ml falcon tubes and incubated under the appropriate conditions (Table 2.1) not shaking for 1 hour. After incubation 25 ml – 200 ml of samples were plated on low-salt LB agar (Table 2.3) containing appropriate selective antibiotics (Table 2.2).

Transformation of *P. pastoris* was also performed by electroporation and followed a similar procedure. For *P. pastoris* transformation, 10 µg of linearized DNA (in sterile water) was used. The Micropulser™ (Bio-Rad, USA) was set to "Pic" and following electroporation, 1 ml of ice-cold 1 M sorbitol was added immediately and mixed by inversion. Cells were then incubated in a 15 mL falcon tube under the appropriate conditions (Table 2.1) for 1.5 hours. Following recovery, the cells were plated on YPDS media (Table 2.3) containing zeocin (Table 2.2). Various volumes between 10 µl and 200 µl were plated, and the plates were incubated at 28˚C for 3-10 days, wrapped in tinfoil (zeocin is light sensitive).

2.5 Plasmid purification

Following several transformations in this study, isolation of plasmid was required. For plasmid extraction from *E. coli* TOP10F', an alkaline lysis method was used, where 5 ml LB with the appropriate antibiotics (Table 2.1 & 2.2) was inoculated with a fresh colony and grown overnight under the appropriate conditions (Table 2.1). The 5 ml culture was then pelleted at 6,000 x g for 5 mins, the supernatant discarded, and the pellet resuspended

in 200 µl ice-cold H_2O in a 1.5 ml microcentrifuge tube. Then 200 µl of room temperature 0.2 M NaOH/1% SDS was added and the tube gently inverted to mix. Next, 200 µl ice-cold solution III (2 M KOAc, pH 6.0) was added and gently inverted to mix. A thick white precipitate was observed. The tubes were then spun at 15,500 x g for 7 mins, and the supernatant recovered and transferred to a new 1.5 ml microcentrifuge tube. An equal volume of ice-cold 100% isopropanol was added to precipitate the DNA, and the tubes spun again. The DNA was washed by adding 400 µl of ice-cold 70% ethanol and spinning at 15,000 x g for 3 mins. The supernatant was discarded and the tubes air dried in a fume hood until all ethanol had evaporated. The DNA was then resuspended in 10 µl of sterile milliQ water and stored at -20˚C.

For isolation of plasmid from *E. coli* JM110 cells, the High Pure Plasmid Isolation Kit™ (Roche, Switzerland) was used, and the optional wash step to eliminate high nuclease activity was performed. Following Golden Gate ligation, plasmid from *E. coli* JM110 cells was isolated again, using the alkaline lysis method described above.

Plasmid to be used for transformation into *P. pastoris* is required to be linear. Therefore, restriction digestion with SacI was performed. 1 µg DNA, 1 µl SacI-HF™ (New England BioLabs Inc., USA), and 5 µl Cut Smart™ buffer (New England BioLabs Inc., USA) were added into a 1.5 µl tube and made up to a 50 µl reaction volume with milliQ water. The tube was incubated at 37˚C for 15 min, then 65˚C for 20 min in a heating block. Results of restriction digests were visualised on 1.5% agarose gels (the same process as described for visualising PCR products in section 2.2). The digested plasmid was then cleaned up prior to transformation by a phenol/chloroform (1:1) method. Equal volume of phenol/chloroform (1:1) was added to the samples, and spun for 5 mins at 12,000 x g. The top layer was transferred into a new 1.5 ml microcentrifuge tube and 1/10 volume of 3 M sodium acetate, and 2.5 volume of 95% ethanol was added to the tubes and mixed by pipetting up and down before spinning for 5 mins at 12,000 x g again. After this DNA precipitation step, the supernatant was discarded, and the DNA was washed by adding 200 µl of 75% ethanol and spinning for 5 mins at 12,000 x g. The ethanol was removed and the tubes air dried before the DNA was resuspended in 10 µl sterile milliQ water, and stored at -20˚C.

2.6 Golden Gate ligation

The tagged *CCL-SSP* genes to be used for *Agrobacterium tumefaciens* transient transformation assays (ATTAs) were ordered in pTwist Amp High Copy entry plasmids and were ligated into the destination vector PICH86988 using Golden Gate cloning before transformation into *A. tumerfaciens*. Following plasmid extraction from *E. coli* JM110, Golden Gate cloning (Engler, Kandzia, & Marillonnet, 2008) was performed. In a 0.2 ml PCR tube; 1 µl T4 DNA ligase (New England BioLabs Inc., USA), 2 µl 10 x T4 DNA ligase buffer (New England BioLabs Inc., USA), 1 µl BsaI-HF™ (New England BioLabs Inc., USA), 2 µl CutSmart™ buffer (New England BioLabs Inc., USA), 1.2 µl of 50 ng/µl PICH86988 plasmid, and 0.4 µl of 50 ng/µl insert (region swapped DNA) were added, and made up to a final reaction volume of 50 µl with sterile milliQ water. The mixture was placed in the vapo.protect™ lightcycler (Eppendorf, Germany) where the following run protocol was performed; 10 cycles of 37˚C/5 min and 16˚C/10 min, followed by 50˚C/5 min, 80˚C/5 min, and 10˚C/hold. Following completion of the Golden Gate ligation reaction, the reaction mixture was treated with Sepharose™ 4B with bead diameter 45-165 µm (Sigma-Aldrich, Germany). A small hole was made in a 0.6 ml tube with a razor blade. The tube was then placed in a 1.5 ml tube and 150 µl of Sepharose™ was added. The tubes were spun twice with the lids open at 1,000 x g for 2 min. The collection tube was then replaced with a new tube, and the entire Golden Gate reaction was added into the tube containing Sepharose™ and spun again. The reaction mixture that collected in the 1.5 ml tube was then stored at -20˚C to be all used for transformation. The efficiency of Golden Gate ligation is low, therefore after electroporation and the cells have recovered, the cell culture was spun down, 950 µl of supernatant removed, and the cells resuspended in the remaining 50 µl. The 50 µl of cells were then plated on a single plate containing selective antibiotics (Table 2.2).

2.7 *P. pastoris* protein expression and analysis of cell death-inducing ability

Following transformation of *P. pastoris*, a single colony was selected for each chimeric region-swapped gene to express the protein and test ability to induce cell death. 5 ml BMGY broths (Table 2.3) in 50 ml falcon tubes were inoculated with fresh colonies and grown overnight at 28˚C until an OD_{600} of 2-6 was obtained (0.5 ml of culture was diluted

in 0.5 ml of BMGY medium for OD measurements). Cells were then harvested by centrifuging 2 ml of culture at 3,000 x g for 5 min. The pellet was resuspended in BMMY liquid media (Table 2.3) until the OD_{600} was approximately 1.0. Cultures were grown in 50 ml falcon tubes covered with two layers of sterile Miracloth^{TM} (EMD Millipore Corp., USA) for 48 hours at 28˚C, adding 100% methanol to a final concentration of 0.5% after 24 hours to maintain induction of protein expression. Cells were then pelleted by spinning at 3,000 x g for 5 mins and as the proteins for this study are secreted, the supernatant was collected, and the pellet discarded. Culture filtrates were sterilized through a 0.2 µm filter to remove all cells, 1 ml aliquoted into 1.5 ml microcentrifuge tubes, snap frozen in liquid nitrogen, and then stored at -20˚C.

To test whether CCL-SSP and chimeric region-swapped CCL-SSPs could induce cell death, infiltration of the culture filtrates into plant material was performed. *Camellia* 'Nicky Crisp' petals were infiltrated using a syringe with a needle (0.5 x 16 mm, Terumo Corp. Japan) at the thick central base of the petal and infiltrated until the entire half lobe was visibly infiltrated (although keeping each infiltrated lobe separate). Detached petals were kept in trays on paper towels moistened by MilliQ water and covered in plastic wrap. *N. benthamiana* attached leaves were infiltrated by pressing the base of a needless syringe to the underside of a leaf and pushing down the plunger to force the liquid into the leaf. The *S. sclerotiorum* host plants (*A. thaliana, B. campestris, R. sativus*, *O. basilicum*, *P. vulgaris*, *B. vulgaris*, and *T. aestivum*) were also infiltrated by pressure infiltration with needless syringes as described above.

2.8 Protein analysis by Tricine-SDS-PAGE

Visualisation of protein produced by recombinant *P. pastoris* expressing CCL-SSPs and chimeric region-swapped CCL-SSPs was performed using Tricine-SDS-PAGE, because it separates proteins smaller than 30 kDa especially well. Tricine gels were made following the methods described by Schägger (2006). A 16% separating gel was made first with 4 ml 40% acrylamide/Bis solution (37.5:1), 3.3 ml 3 x gel buffer (3 M Tris, 1 M HCl, 0.3% SDS, pH 8.45), 2.4 ml glycerol, 1.7 mL water, then to polymerise; 33.3 µl APS (10%) and 3.3 µl TEMED were added last). A few drops of acetone were added on top to remove any air bubbles. Once the gel polymerised, a 4 % stacking gel was made

on top with 0.4 ml 40% acrylamide/Bis solution (37.5:1), 1 ml 3x gel buffer, 2.6 mL water, then to polymerise; 30 µl APS (10%) and 3 µl TEMED were added last. The combs were added, and the gel left to polymerise. These quantities are adequate for two 7 cm x 8 cm gels.

Protein was concentrated using a TCA precipitation method (Koontz, 2014). 1 volume of TCA stock (100% w/v Trichloroacetic acid) was mixed into 4 volumes of culture filtrate (200 µl volume was used) and incubated for 10 min at 4 ˚C. Tubes were then spun at 14,000 rpm for 5 min, and the supernatant discarded. The protein pellet was then washed by adding 200 µl of cold acetone and spinning again at 14,000 rpm for 5 min. This washing step was repeated and then the pellet was dried by placing the open tubes in a 95 ˚C heating block until all acetone had evaporated. The pellet was then resuspended in 21 µl of sterile water, and 5 µl of reducing sample buffer A (30% glycerol, 1 M Tris/HCL pH 6.8, 12% SDS, 0.05% coomassie blue, and 6% mercaptoethanol) was added. Tubes were boiled at 95˚C for 10 min before loading 10 µl into the gel.

Tricine gels were run with a 1 x cathode buffer (10 x stock made with 1 M Tris, 1 M Tricine, 1 % SDS, pH 8.25) and a 1 x anode buffer (10 x stock made with 1 M Tris, 0.225 M HCl, pH 8.9). The gels were run at 15 mAmp initially to let the protein stack at the top of the separating gel, then they were run at 30 mAmp until completed. Finished gels were then immediately fixed in 30 % ethanol, 10 % acetic acid. Tricene gels were then stained using the Pierce^{R} Silver Stain Kit^{TM} (Thermo Fisher Scientific, USA), following the described procedure.

2.9 *Agrobacterium tumefaciens* transient transformation assays

A. tumefaciens transformed with tagged CCL-SSPs and chimeric region-swapped *CCL-SSPs* in the PICH86988 plasmid were used for ATTAs. 3 ml broths of LB (Table 2.3) containing the appropriate antibiotics (Table 2.1 & 2.2) in 50 ml falcon tubes were inoculated with a fresh colony and grown overnight at 28 ˚C, shaking at 200 rpm. The cell cultures were then spun down at 2500 x g for 5 min, the supernatant discarded, and the pellet resuspended in 1 ml of infiltration buffer (10 mM $MgCl_2$, 10 mM MES-KOH, 100 µM acetosyringone, and H_2O to make up to the desired volume). The OD_{600} of the culture was then checked by mixing 50 µl of the culture with 950 µl of infiltration buffer

to make a dilution factor of 20. The desired volume of infiltration culture was made to an OD_{600} of 0.4 by diluting the culture with infiltration buffer. The cultures were then left at room temperature for 3 hours before infiltration into leaves of 4-6-week-old *N. bethamiana* plants. Infiltrated leaves were photographed 7 dpi, then detached and photographed under UV light in a Universal Hood II™ (Bio-Rad, USA) commonly used for exposing electrophoresis gels. The intensity of the photo was adjusted to the settings of high = 3330 and low = 830.

3 Results

3.1 Cell death triggered by SsSSP in susceptible *Camellia* 'Nicky Crisp' petal tissue can be successfully replicated

It has previously been shown that the CCL-SSP homologues from *S. sclerotiorum* (SsSSP) and *B. cinerea* (BcSSP) trigger rapid and strong cell death when infiltrated into highly susceptible *Camellia* 'Nicky Crisp' petals, while ten of the 73 *C. camelliae* CCL-SSPs (selected to spread across and represent the *C. camelliae* CCL-SSP clade), do not (Denton-Giles, 2014). In an attempt to replicate these results, recombinant *P. pastoris* expressing BcSSP, SsSSP and CcSSP92 (one of the *C. camelliae* CCL-SSPs previously tested (Denton-Giles, 2014)), and the empty vector (negative control), were grown. The culture filtrate was then harvested, spun to down and filtrate sterilized to remove all cell, and then the cell-free culture filtrate was infiltrated into highly susceptible *Camellia* hybrid 'Nicky Crisp' petals. As expected, the empty vector negative control did not trigger cell death (Fig. 3.1). BcSSP and SsSSP triggered cell death as early as 2 h post-infiltration (hpi), and this cell death was limited to the site immediately adjacent to the infiltration zone at the base of the petal (Fig. 3.1). Notably, at 20 hpi, the cell death triggered by SsSSP was present throughout the entire infiltration zone, while the cell death triggered by BsSSP was present throughout only half of the infiltration zone (Fig. 3.1). Unlike BcSSP and SsSSP, the CcSSP92 protein did not trigger any visible cell death, although some wounding from mechanical damage was observed (Fig. 3.1). These results are consistent with those that have been found from other studies (Denton-Giles, 2014), illustrating that the results were replicated and that this is a robust assay for CCL-SSP cell death-inducing activity.

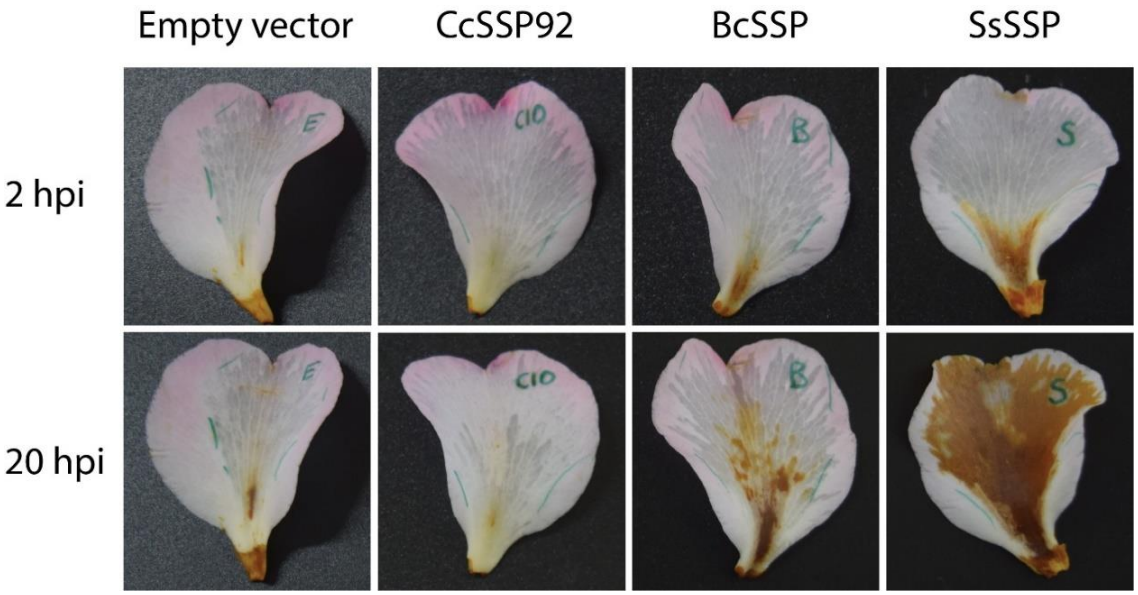


Figure 3.1. 'Nicky Crisp' *Camellia* petals infiltrated with cell-free culture filtrate of recombinant *Pichia pastoris* expressing CCL-SSPs.
Camellia 'Nicky Crisp' petals were infiltrated with cell-free *P. pastoris* culture filtrate and the infiltration area marked with green pen. Photographs are taken 2 and 20 h post-infiltration (hpi). Images are representative (n = 3).

3.2 Alignment of SsSSP and CcSSP37 identified variable sequence that can be swapped to generate chimeric proteins

The above results show that BcSSP and SsSSP can activate cell death, while no such response has been shown for the ten of 73 *C. camelliae* CCL-SSPs tested (Denton-Giles, 2014). Therefore, the protein sequences of SsSSP and CcSSP37 were aligned to identify variable sequence regions that may be responsible for cell death activity. Upon identification, I suggest that variable regions could then be swapped between SsSSP and CcSSP37 to generate chimeric proteins that, when infiltrated into highly susceptible *Camellia* 'Nicky Crisp' petals, could be used to confirm the region or regions responsible for cell death activity. The alignment showed that the protein sequence encoded by the second exon of *SsSSP* and *CcSSP37*, when compared to the first exon, contained the greatest level of amino acid variability (Fig. 3.2A). Based on this finding, the region encoded by the second exon was selected as a region swap (Figs. 3.2A and B). Other swaps were designed to refine the region or regions within Exon 2 that may be responsible for the cell death-triggering ability of SsSSP. More specifically, three other swaps were

designed to include areas were the amino acids between SsSSP and CcSSP37 were different, and were labelled “Mid 1”, “Mid 2”, and “C term” (Fig. 3.2B). The region swaps “Mid 2” and ‘C term” were also designed to not disrupt the Y/F/WxC motif, which is three amino acids long and has a variable second amino acid (Fig. 3.2A). These results show that by aligning SsSSP and CcSSP37, variable amino acid sequence can be identified for the generation of chimeric domain-swapped proteins that may assist in the identification of regions important for the cell death activity of SsSSP.

3.3 Transformation of chimeric region-swapped CCL-SSP-encoding genes into *P. pastoris* cells can be performed successfully

It has previously been shown that the yeast *P. pastoris* can be used to recombinantly express fungal proteins that contain cysteine residues and produce disulphide bonds (Frias, Brito, Gonzalez, & Gonzalez, 2014). I suggest that transformation of *P. pastoris* with the chimeric region-swapped *CCL-SSP* genes will generate successfully recombinant colonies, and that *P. pastoris* will be a successful expression system for these proteins. The chimeric region-swapped genes were obtained in the expression vector pPICZA from Genscript^{TM}, and then transformed by electroporation into *E. coli* TOP10F’ for propagation. The DNA was then extracted and used for transformation of *P. pastoris* X-33 cells by electroporation. Colony PCR was performed to determine the success of *P. pastoris* transformation, the PCR products were then sequenced, and Tricene–SDS–PAGE of the cell-free culture filtrates was performed to determine expression and secretion of the proteins. Transformation of *E. coli* TOP10F’ was successful after the first attempt, and the transformation of *P. pastoris* was successful after six attempts (Table 3.1). This was confirmed by colony PCR (Fig. 3.3A), where all colonies tested amplified a PCR product of the correct size. The PCR products were then sequenced and found to contain the correct chimeric gene sequences (Fig. 3.3B). The Tricene–SDS–PAGE was successful in showing that protein was secreted by recombinant *P. pastoris* into culture medium, as protein bands were identified at 10 kDa (Fig. 3.3C). However, the empty vector control also showed similar bands. These experiments show that the chimeric region-swapped *CCL-SSPs* were successfully transformed into *P. pastoris.*

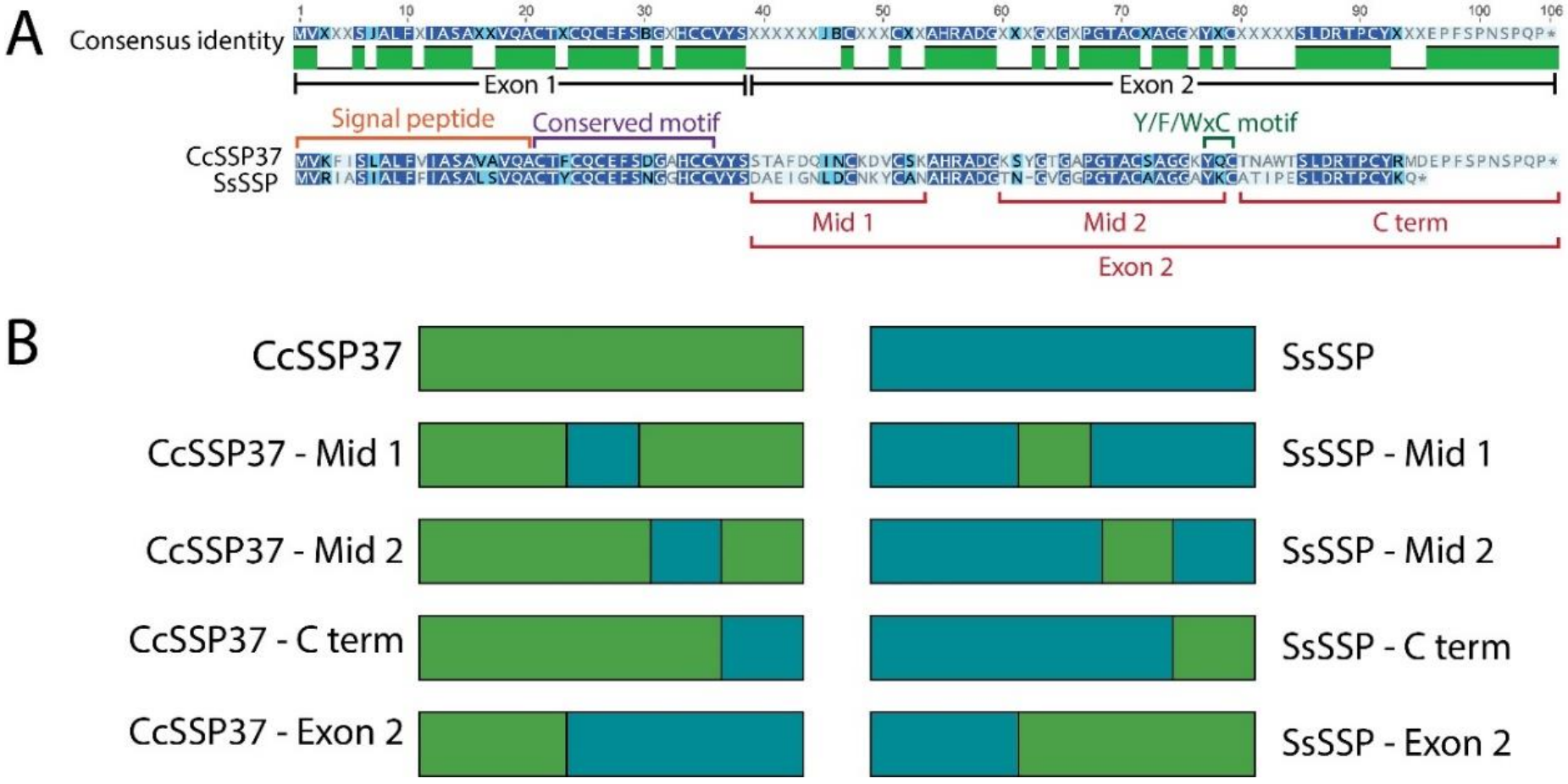

A
Consensus identity
Exon 1
Exon 2
Signal peptide
Conserved motif
Y/F/WxC motif
CcSSP37
SsSSP
Mid 1
Mid 2
C term
Exon 2
B
CcSSP37
SsSSP
CcSSP37 - Mid 1
SsSSP - Mid 1
CcSSP37 - Mid 2
SsSSP - Mid 2
CcSSP37 - C term
SsSSP - C term
CcSSP37 - Exon 2
SsSSP - Exon 2

Figure 3.2. Region swap designs between CcSSP37 and SsSSP.
A – Alignment of SsSSP and CcSSP37 protein sequences using a Blosum62 cost matrix and free end gaps (made with Geneious v9.1.8 software). The consensus identity at the top shows the conserved amino acid residues with green bars representing 100% sequence identity. The amino acid sequence similarity between SsSSP and CcSSP37 is shown through the shades of blue; dark blue means the amino acids are identical, medium blue means the amino acids are similar, and light blue means the amino acids are not similar (threshold = 1). The orange line illustrates the *Ciborinia camelliae* signal peptide, the purple line shows the conserved motif "CTYCQCLFPDGSHCC" (Denton-Giles, 2014), the green line illustrates the three amino acid Y/F/WxC motif (Denton-Giles, 2014), the red lines show which amino acid regions will be swapped between SsSSP and CcSSP37 to generate chimeric region-swapped genes, the names of each region swap are also in red, the black lines just below the consensus identity highlights exon 1 and 2 of SsSSP and CcSSP37. **B** – A basic view of the chimeric region-swapped protein design. Regions from CcSSP37 are coloured green, while regions from SsSSP are coloured blue.

However, the success of *P. pastoris* as an expression system was not confirmed by this method as presence of the chimeric region-swapped proteins in the culture filtrate was not confirmed.

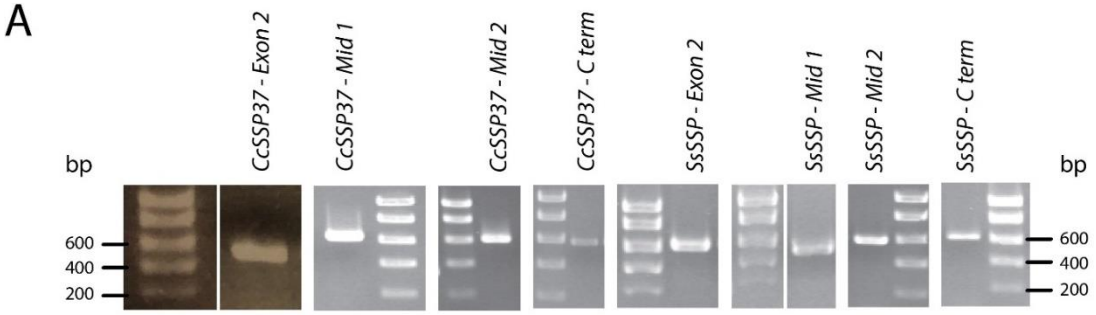
3.4 Infiltration of chimeric region-swapped CCL-SSPs into susceptible *Camellia* 'Nicky Crisp' petal tissue identified the protein region encoded by Exon 2 of SsSSP as essential for cell death activity

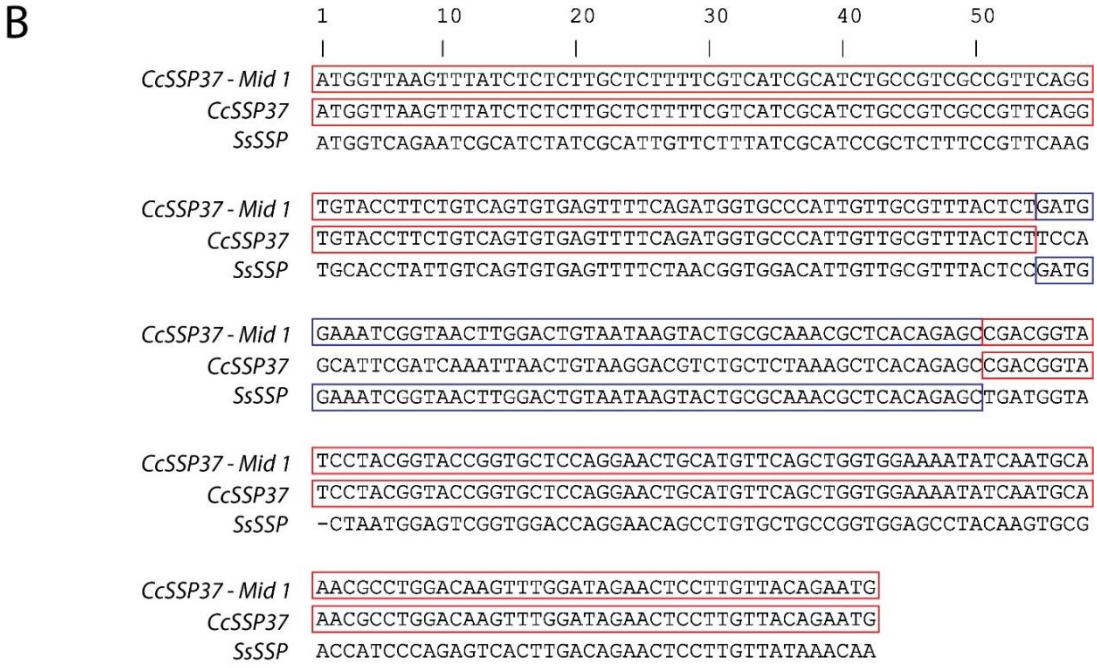
It was shown above that the chimeric region-swapped *CCL-SSP* genes were successfully transformed into *P. pastoris.* I hypothesise that infiltration of these chimeric proteins into highly susceptible *Camellia* 'Nicky Crisp' petals will provide information on which region of SsSSP is required for its cell death-inducing activity. Recombinant *P. pastoris* cells expressing BcSSP, SsSSP, CcSSP37, the chimeric region-swapped CCL-SSPs, and empty vector, were grown, the culture filtrate collected, all cells removed by centrifugation and filtration, and infiltrated into petals of the highly susceptible *Camellia* hybrid 'Nicky Crisp'. As expected, the negative control of infiltrated water, and CcSSP37 did not induce any cell death, although some browning from wounding damage was visible from CcSSP37 (Fig. 3.4A). SsSSP and BcSSP also performed as predicted. SsSSP induced cell death strongly throughout the entire infiltration area, and BcSSP induced strong cell death throughout most of the infiltration zone (Fig. 3.4A). Of the chimeric region-swapped proteins, only CcSSP37 – Exon 2, which is a chimeric protein containing

Table 3.1. Number of transformations performed in *Escherichia coli* and *Pichia pastoris* and their various success rates

Organism transformed	**Attempt No.**	**DNA transformed**	**No. colonies tested by PCR**	**Transformation successful / not**
E. coli TOP10F'	1	Empty vector	2	Successful
		CcSSP37 – Exon 2	6	Successful
		CcSSP37 – Mid 1	6	Successful
		CcSSP37 – Mid 2	6	Successful
		CcSSP37 – C term	6	Successful
		SsSSP – Exon 2	6	Successful
		SsSSP – Mid 1	6	Successful
		SsSSP – Mid 2	6	Successful
		SsSSP – C term	6	Successful
P. pastoris X - 33	1	All constructs*	0	Not successful
	2	*CcSSP37 – Mid 2*	0	Not successful
		CcSSP37 – C term	0	Not successful
	3	All constructs*	0	Not successful
	4	*CcSSP37 – Exon 2*	5	Successful
	5	All constructs*	0	Not successful
	6	Empty vector	3	Successful
		CcSSP37 – Mid 1	9	Successful
		CcSSP37 – Mid 2	2	Successful
		CcSSP37 – C term	2	Successful
		SsSSP – Exon 2	3	Successful
		SsSSP – Mid 1	4	Successful
		SsSSP – Mid 2	3	Successful
		SsSSP – C term	3	Successful

***** "All constructs" refers to the following chimeric region swaps which were performed in separate cells but collected into one column to save space; *CcSSP37 – Mid 1*, *CcSSP37 – Mid 2*, *CcSSP37 – C term*, *SsSSP – Exon 2*, *SsSSP – Mid 1*, *SsSSP – Mid 2*, *SsSSP – C term*, and empty vector.


A
bp
CcSSP37 - Exon 2
CcSSP37 - Mid 1
CcSSP37 - Mid 2
CcSSP37 - C term
SsSSP - Exon 2
SsSSP - Mid 1
SsSSP - Mid 2
SsSSP - C term
bp
600
400
200
600
400
200


B
1 10 20 30 40 50
CcSSP37 - Mid 1 ATGGTTAAGTTTATCTCTCTTGCTCTTTTCGTCATCGCATCTGCCGTCGCCGTTCAGG
CcSSP37 ATGGTTAAGTTTATCTCTCTTGCTCTTTTCGTCATCGCATCTGCCGTCGCCGTTCAGG
SsSSP ATGGTCAGAATCGCATCTATCGCATTGTTCTTTATCGCATCCGCTCTTTCCGTTCAAG
CcSSP37 - Mid 1 TGTACCTTCTGTCAGTGTGAGTTTTCAGATGGTGCCCATTGTTGCGTTTACTCTGATG
CcSSP37 TGTACCTTCTGTCAGTGTGAGTTTTCAGATGGTGCCCATTGTTGCGTTTACTCTTCCA
SsSSP TGCACCTATTGTCAGTGTGAGTTTTCTAACGGTGGACATTGTTGCGTTTACTCCGATG
CcSSP37 - Mid 1 GAAATCGGTAACTTGGACTGTAATAAGTACTGCGCAAACGCTCACAGAGCCGACGGTA
CcSSP37 GCATTCGATCAAATTAACTGTAAGGACGTCTGCTCTAAAGCTCACAGAGCCGACGGTA
SsSSP GAAATCGGTAACTTGGACTGTAATAAGTACTGCGCAAACGCTCACAGAGCTGATGGTA
CcSSP37 - Mid 1 TCCTACGGTACCGGTGCTCCAGGAACTGCATGTTCAGCTGGTGGAAAATATCAATGCA
CcSSP37 TCCTACGGTACCGGTGCTCCAGGAACTGCATGTTCAGCTGGTGGAAAATATCAATGCA
SsSSP -CTAATGGAGTCGGTGGACCAGGAACAGCCTGTGCTGCCGGTGGAGCCTACAAGTGCG
CcSSP37 - Mid 1 AACGCCTGGACAAGTTTGGATAGAACTCCTTGTTACAGAATG
CcSSP37 AACGCCTGGACAAGTTTGGATAGAACTCCTTGTTACAGAATG
SsSSP ACCATCCCAGAGTCACTTGACAGAACTCCTTGTTATAAACAA

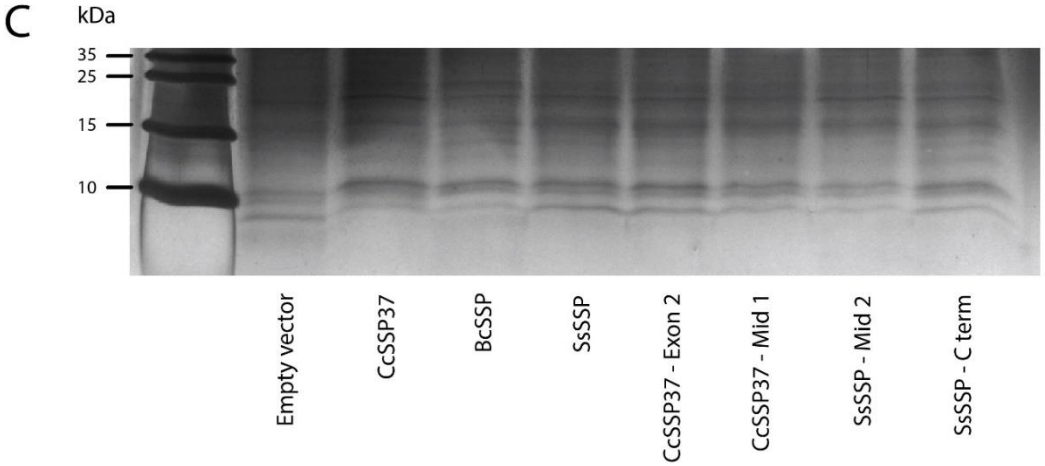

C
kDa
35
25
15
10
Empty vector
CcSSP37
BcSSP
SsSSP
CcSSP37 - Exon 2
CcSSP37 - Mid 1
SsSSP - Mid 2
SsSSP - C term

Figure 3.3. Quality control checks of transformed *Pichia pastoris* colonies by colony PCR, sequencing, and Tricene-SDS-PAGE gels of *P. pastoris* culture filtrates.
A – Colony PCR of *P. pastoris* colonies transformed with chimeric region-swapped genes. *AOX* forward and reverse primers were used (primer sequences are in Appendix 7.5), the expected product sizes for each gene are as follows; CcSSP37 - Exon 2 (576 bp), CcSSP37 - Mid 1 (612 bp), CcSSP37 - Mid 2 (609 bp), CcSSP37 - C term (579 bp), SsSSP - Exon 2 (612 bp), SsSSP - Mid 1 (576 bp), SsSSP - Mid 2 (579 bp), SsSSP - C term (609 bp). **B** – Comparison of sequencing results of chimeric region-swapped *CcSSP37 – Mid 1* with known sequences of *CcSSP37* and *SsSSP*. The top sequence is the sequencing data received from Macrogen Ez – seq™ (Rep. of Korea) of a *P. pastoris* colony transformed with *CcSSP37 – Mid 1*. The middle and bottom sequences are the known nucleotide sequences of *CcSSP37* and *SsSSP*. The red boxes illustrate the nucleotides shared with CcSSP37, and the blue boxes illustrate the nucleotides shared with SsSSP. This image is representative of all chimeric region-swapped genes; as all were sequenced and found carry the correct region-swapped DNA. The forward and reverse sequences were checked for two colonies per gene. **C** – Silver-stained Tricene–SDS–PAGE gel of culture filtrate from recombinant *P. pastoris* expressing CCL-SSPs and chimeric region-swapped CCL-SSPs. The expected molecular weight of the proteins irrespective of any post-translational modifications are: CcSSP37 (11.2 KDa), BcSSP (9.43 KDa), SsSSP (9.67 KDa), CcSSP37 – Exon 2 (9.64 KDa), CcSSP37 – Mid 1 (11.18 KDa), SsSSP – Mid 1 (9.69 KDa), SsSSP – C term (10.96 KDa).

Exon 1 of CcSSP37 and Exon 2 of SsSSP, triggered cell death (Fig. 3.4A). A time course of cell death induction, based on SsSSP, BcSSP and CcSSP37 – Exon 2 at 0, 2, 6 and 12 hpi, revealed that all three proteins triggered cell death as early as 2 hpi, with SsSSP triggering the strongest cell death covering almost the entire infiltration area, at this time point (Fig. 3.4B). The general trend from the petals tested suggested that SsSSP induced cell death more rapidly than BcSSP or CcSSP37 – Exon 2 (Fig. 3.4B). Quantification of the observed cell death showed that after 24 h, the percentage of necrosis induced by SsSSP and CcSSP37 – Exon 2 was not significantly different (Fig. 3.4C). In contrast, a significant difference was found for BcSSP, water, CcSSP37, and all other chimeric region-swapped proteins (although they were not significantly different to each other). These results show that infiltration of chimeric region-swapped CCL-SSPs identified that Exon 2 of SsSSP is essential for cell death activity.

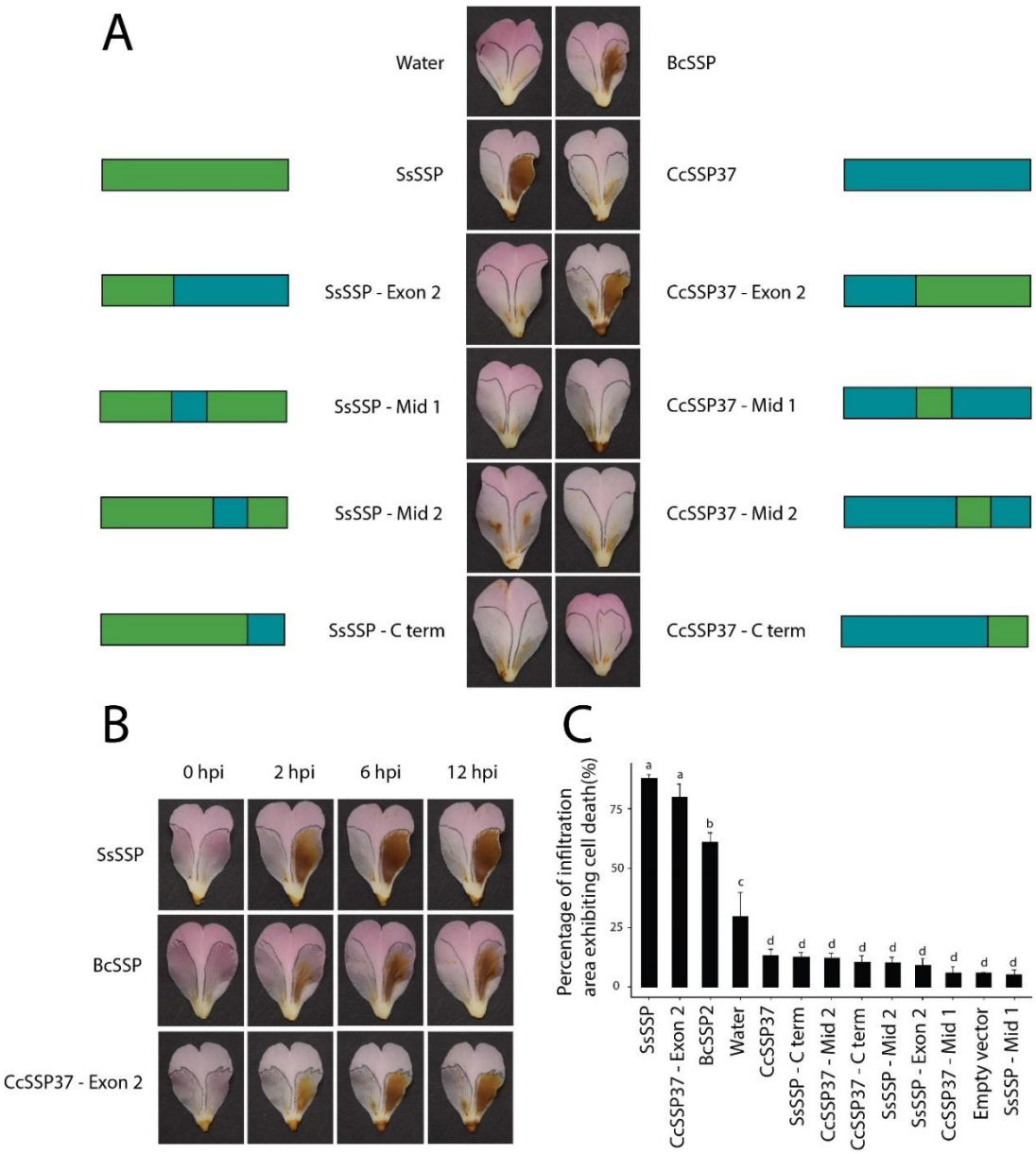


Figure 3.4. *Camellia* 'Nicky Crisp' petals infiltrated with culture filtrate of recombinant *Pichia pastoris* expressing CCL-SSPs and chimeric region-swapped CCL-SSPs.

A – *Camellia* 'Nicky Crisp' petals were infiltrated with cell-free culture filtrate of recombinant *P. pastoris* expressing CCL-SSPs or chimeric region-swapped CCL-SSPs and photographed 24 h post-infiltration (hpi). The left side of the petals were infiltrated with empty vector, and the right side with CCL-SSPs or chimeric region-swapped CCL-SSPs. The infiltration area was marked with black pen, and the basic designs of the chimeric region-swapped proteins are illustrated on the far left and right of the figure. **B** – Petals infiltrated with SsSSP, BcSSP, and CcSSP37 – Exon 2 from 0 hpi to 12 hpi (0 hpi to 48 hpi photos of petals infiltrated with all CCL-SSPs and chimeric region-swapped CCL-SSPs are in Appendix 7.6). **C** – Quantification of the percentage of infiltration area that has undergone cell death after 24 h, using ImageJ 1.52a software and analysed with Ri386 v3.5.1 software. The letters above the bars represent significance of P = 0.05 produced

by a Tukey test. Bars with the same letter are not significantly different to each other. Standard error is represented by lines above each bar. Images are representative (n = 9).

3.5 CCL-SSPs can induce cell death on *Nicotiana benthamiana* leaves

It has been shown above that the protein region encoded by Exon 2 of SsSSP is required to trigger cell death upon infiltration into susceptible 'Nicky Crisp' petals. It has also been shown that upon infiltration of *N. benthamiana* leaves with SsSSP and BcSSP, strong cell death is observed, while infiltration of CcSSP93 (one of the ten of 73 *C. camelliae* CCL-SSPs shown to not induce cell death) did not show an effect (Denton-Giles, 2014). I suggest that infiltration of *N. benthamiana* leaves with chimeric region-swapped CCL-SSPs will induce results similar to what was found from infiltrated *Camellia* 'Nicky Crisp' petals and will show that *N. benthamiana* can be used as a model species for testing activity of CCL-SSPs. Recombinant *P. pastoris* cells expressing chimeric region-swapped CCL-SSPs or empty vector were grown, the culture filtrate recovered, and all cells removed. The cell-free culture filtrate was then infiltrated into *N. benthamiana* leaf tissue and observed 24 hpi for cell death. As expected, infiltrated water, empty vector, and CcSSP37 did not induce any cell death (Fig. 3.5A). SsSSP infiltration induced strong necrosis as predicted, which covered most of the infiltration area. As was expected from the *Camellia* 'Nicky Crisp' infiltrations above, the only chimeric region-swapped protein that induced cell death was CcSSP37 – Exon 2 (Fig. 3.5A). All other chimeric region-swapped proteins were found to only induce wounding, evident by the small circle of browning, consistent with the size of the syringe which was used for infiltration (Fig. 3.5A). Quantification of induced cell death through visual lesion scoring illustrated a significant difference between SsSSP and CcSSP37 – Exon 2 (Fig. 3.5C). The other chimeric region-swapped proteins had an average lesion score of just over 1, which according to the lesion key (Fig. 3.5B), signifies wounding from mechanical damage, not induced cell death. There were also several significant differences between the other chimeric region-swapped proteins. These results illustrate that CCL-SSPs can induce cell death on *N. benthamiana* leaves and confirm that the protein region encoded by Exon 2 of SsSSP is essential for inducing cell death.

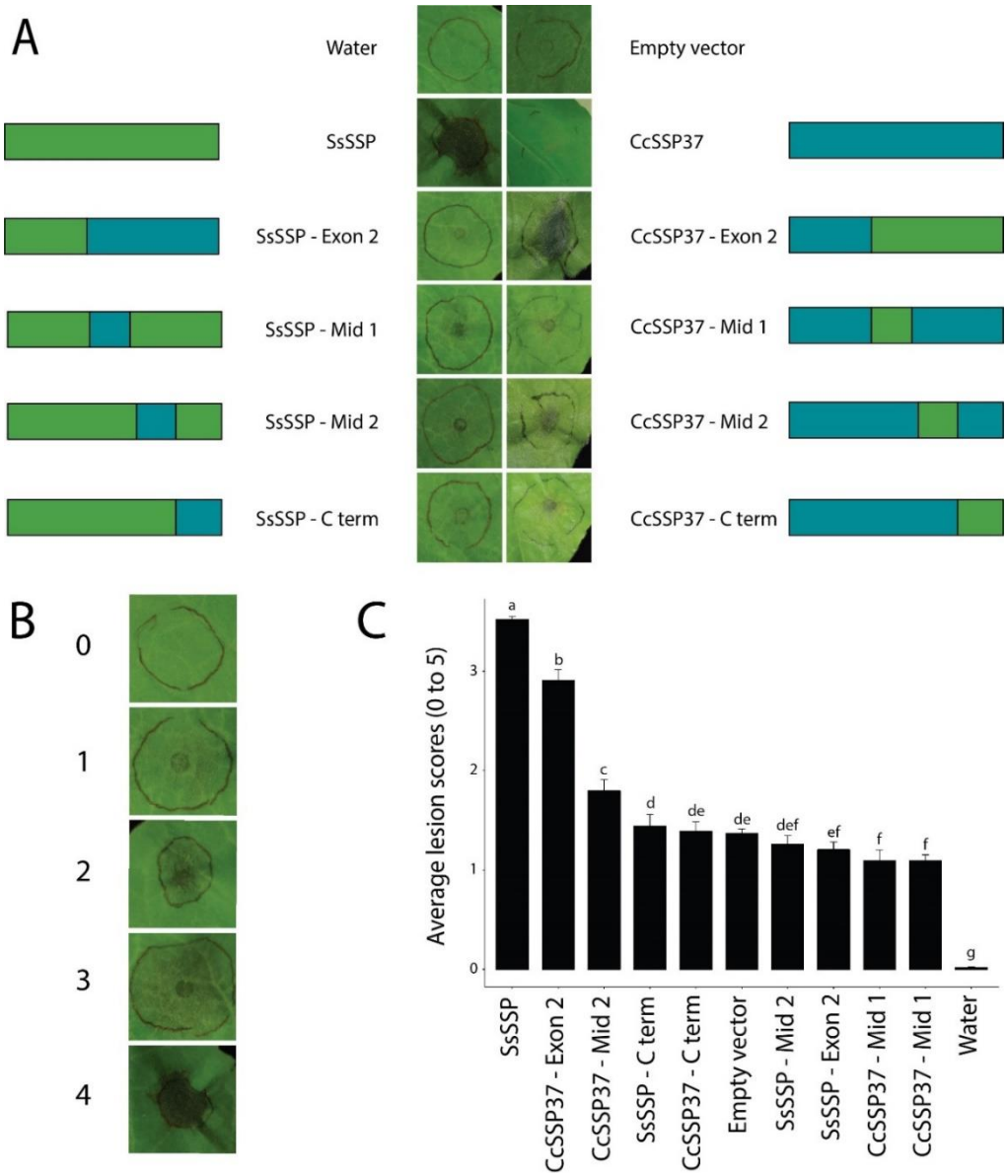


Figure 3.5. ***Nicotiana benthamiana*** **leaves infiltrated with cell-free culture filtrates of recombinant** ***Pichia pastoris*** **expressing CCL-SSPs and chimeric region-swapped CCL-SSPs.**

A – Infiltration area is circled with black marker and images were taken 24 hpi. The basic designs of the chimeric region-swapped proteins are illustrated on the far left and right of the figure. **B** – Key used to grade cell death induced by infiltrated CCL-SSPs, with 0 = no cell death, 1 = mechanical damage, 2 = weak cell death, 3 = moderate cell death, and 4 = strong cell death. This key was made after examination of all infiltrations and represents the range of cell death observed. **C** – Average lesion scores of infiltrated leaves. Scoring was performed through visual inspection by three different people to minimise bias and was analysed with Ri386 v3.5.1 software. The letters above the bars illustrate significant differences between groups to a level of P = 0.05, produced by a Tukey test. Groups with the same letters are not significantly different to each other. Standard error is represented by lines above each bar. Images are representative (n = 18).

3.6 Quality control checks of *Agrobacteria tumefaciens* show successful transformation with new chimeric *CCL-SSP* region-swapped genes

It has been shown above that chimeric genes can be generated and successfully transformed into *P. pastoris*. Therefore, I suggest that new chimeric region-swapped genes can be generated and successfully transformed into *A. tumefaciens*. A cloning strategy was devised, transformations were performed as described in section 2.4, and the resulting transformants were tested by colony PCR and sequencing. Basic design of the new chimeric region-swapped genes includes the PR1a signal peptide for expression in *A. tumefaciens*, a N-terminal 3 x FLAG tag, and previously designed region swaps "Mid 1" and 'C term" (Fig. 3.6A). The cloning strategy (Fig. 3.6B) consists of multiple steps to obtain these new chimeric region-swapped genes in the destination vector PICH86988. The genes were first transformed into *E. coli* TOP10F' to amplify the plasmid. Plasmid DNA was then extracted and used for Golden Gate cloning however, a DNA methylation site was discovered over the region where pICH86988 joins the inserted *CCL-SSP* and prevented digestion of the *Bsa*I restriction site. Therefore, use of a non-methylating *E. coli* (JM110) was required for plasmid amplification (Fig. 3.6B). Golden Gate cloning was then performed to insert the chimeric genes into the PICH86988 plasmid, which was then used for transformation of *A. tumefaciens*. Transformation of *A. tumefaciens* was confirmed as successful by colony PCR, where the correct PCR products were obtained (Fig. 3.7A). Sequencing the *A. tumefaciens* colonies also confirmed that the correct chimeric gene sequences had been successfully transformed (Fig. 3.7B). These results show that *CCL-SSPs* and new chimeric region-swapped *CCL-SSPs* can be successfully transformed into *A. tumefaciens.*

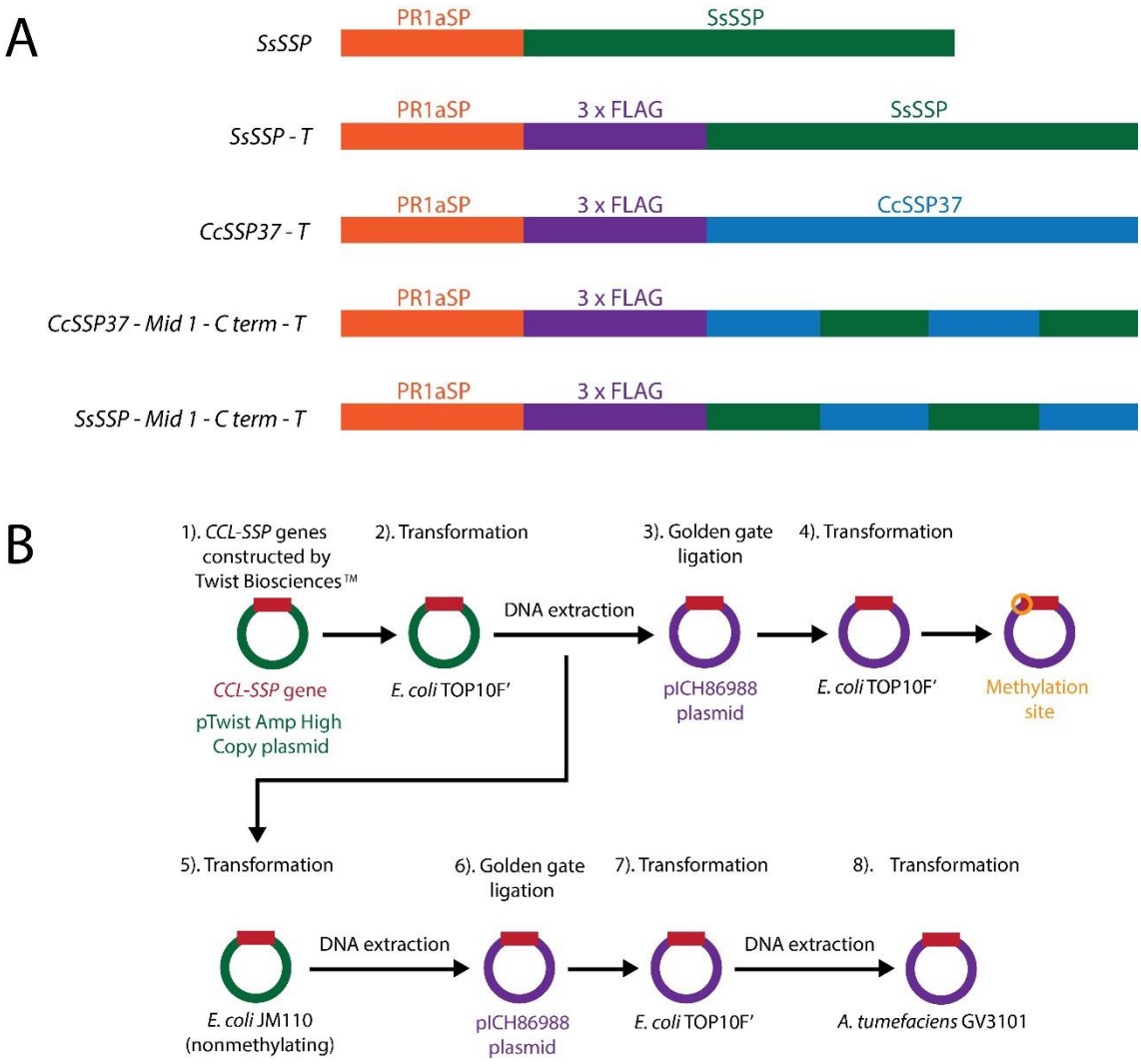


Figure 3.6. New tagged chimeric region-swapped *CCL-SSPs* and the cloning strategy for their transformation into *Agrobacterium tumefaciens*.

A – Basic design of new tagged chimeric region-swapped *CCL-SSP* and *CCL-SSP* genes containing a N terminus 3 x FLAG tag (labelled by addition of "T" at the end of the gene name). Gene regions are colour coded; the PR1a signal peptide (PR1aSP) is orange, the 3 x FLAG tag is purple, *SsSSP* is green, *CcSSP37* is blue, and the new chimeric region-swapped genes (*CcSSP37 – Mid 1 – C term – T* and *SsSSP – Mid 1 – C term – T*) containing regions from *SsSSP* and *CcSSP37*, are coloured accordingly. **B** – Cloning strategy implemented for transformation of *A. tumefaciens* with the new chimeric region-swapped *CCL-SSPs* and tagged *CCL-SSPs*. Images and labels are colour coded; the *CCL-SSP* or chimeric region-swapped gene is red, the pTwist Amp High Copy plasmid is green, the pICH86988 plasmid is purple, and the methylation site is orange.

Table 3.2. Outcomes of the multiple transformation steps that were performed prior to transformation of *Agrobacterium tumefaciens*

Organism transformed	**Plasmid**	**DNA transformed**	**Number of colonies tested by PCR**	**Transfor-mation success**
E. coli TOP10F'	pTwist Amp High Copy	*SsSSP – Mid 1 – C term - T*	4	Successful
		CcSSP37 – Mid 1 – C term - T	4	Successful
		SsSSP	4	Successful
		SsSSP - T	4	Successful
		CcSSP37 - T	4	Successful
E. coli JM110	pTwist Amp High Copy	*SsSSP – Mid 1 – C term - T*	3	Successful
		CcSSP37 – Mid 1 – C term - T	3	Successful
		SsSSP	3	Successful
		SsSSP - T	3	Successful
		CcSSP37 - T	3	Successful
E. coli TOP10F'	pICH869 88	*SsSSP – Mid 1 – C term - T*	3	Successful
		CcSSP37 – Mid 1 – C term - T	3	Successful
		SsSSP	3	Successful
		SsSSP - T	3	Successful
		CcSSP37 - T	3	Successful
A. tumefaciens GV3101	pICH869 88	*SsSSP – Mid 1 – C term - T*	3	Successful
		CcSSP37 – Mid 1 – C term - T	3	Successful
		SsSSP	3	Successful
		SsSSP - T	3	Successful
		CcSSP37 - T	3	Successful

"T" represents that these genes contain the 3 x FLAG tag at the N-terminus

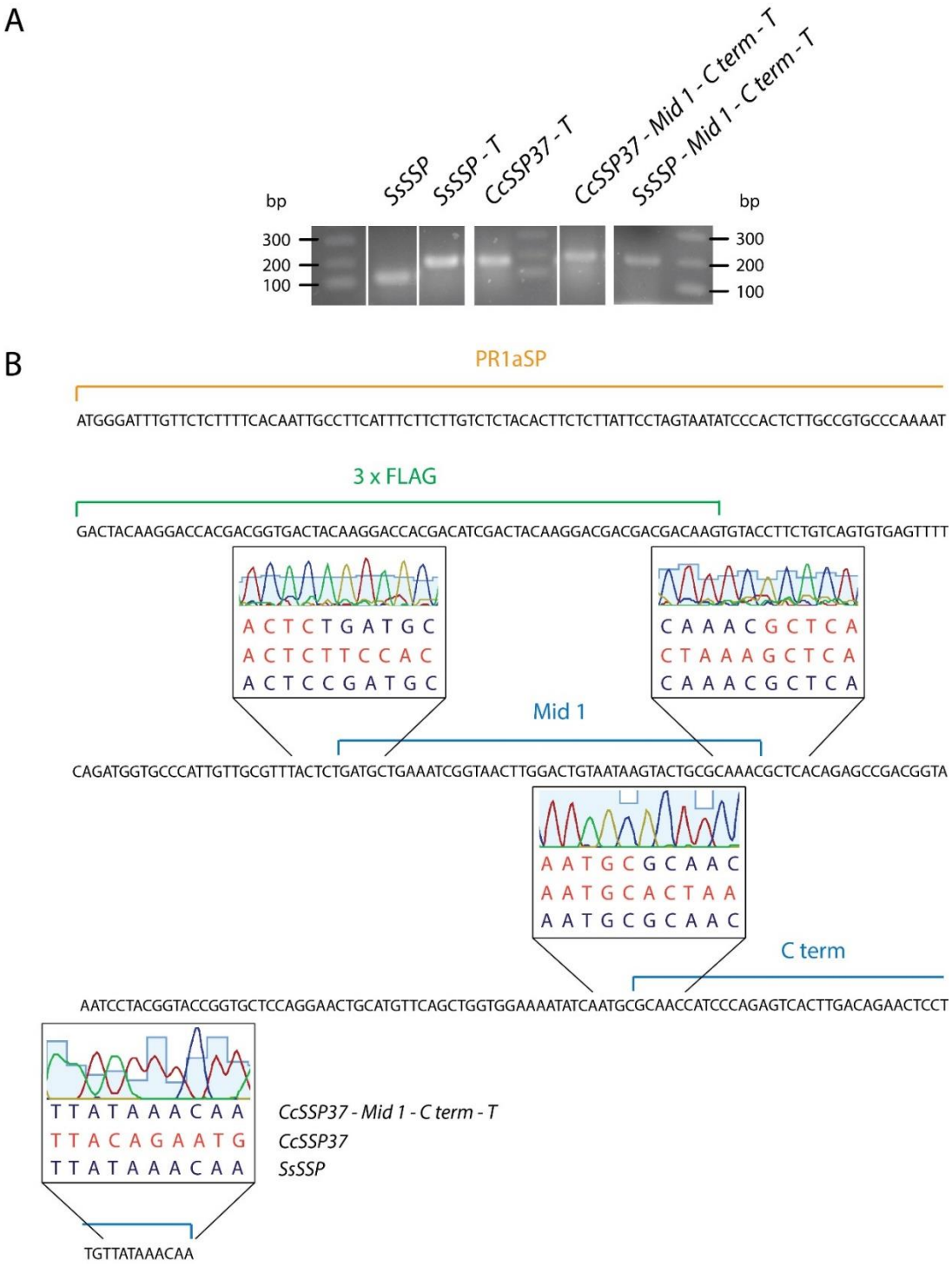


Figure 3.7. Quality control checks of *Agrobacterium tumefaciens* transformed with new tagged *CCL-SSPs* and chimeric region-swapped *CCL-SSPs*, by colony PCR and sequencing. **A** – PCR was performed using forward primer PR1aSP - 5' and reverse primers specific to the gene. Expected PCR products are as follows: SsSSP (102 bp), SsSSP – T (168 bp), CcSSP37 – T (156 bp), CcSSP37 – Mid 1 – C term – T (171 bp), SsSSP – Mid 1 – C term – T (189). Primer sequences are found in the Appendix 7.5. Images are representative (n = 3). **B** – PCR products from a single colony were sequenced in both direction by Massey Genome Service™ (NZ) for

each gene. Illustration of the success of transformation by quality of sequencing data for *CcSSP37 – Mid 1 – T*. The main nucleotide sequence (in black) is the expected sequence for *CcSSP37 – Mid 1 – C term – T*. The orange band illustrates the sequence encoding the PR1a signal peptide, the green band shows the sequence encoding the 3 x FLAG tag, and the blue bands highlights the chimeric sequence from *SsSSP*. The boxes cover the region-swap boundaries, and show the chromatogram received from the sequencing data. Underneath the chromatogram is the nucleotide sequence of *CcSSP37 – Mid 1 – C term – T* obtained from the sequencing results, underneath that is the expected *CcSSP37* sequence, and beneath that is the expected *SsSSP* sequence. These sequences are colour coded; red is DNA from *CcSSP37* and blue is DNA from *SsSSP*. These results are representative of the other genes (*SsSSP – Mid 1 – C term – T, SsSSP, SsSSP – T, CcSSP37 – T*) which were also sequenced and found to match the expected sequence.

3.7 *A. tumefaciens* transient transformation assay of CCL-SSPs induces irregular cell death and cell death from the empty vector control

It has been shown in *Camellia* 'Nicky Crisp' and *N. benthamiana* that the protein sequence encoded by Exon 2 of SsSSP is essential for cell death activity. However, these results were identified from infiltration with *P. pastoris* culture filtrate, which also contains many yeast proteins and likely fragments of yeast cell walls that could act as PAMPs that activate host immunity. I suggest that using an *A. tumefaciens* transient transformation assay (ATTA) of the new chimeric region-swapped genes and *CCL-SSPs* in *N. benthamiana* will not generate immunity triggering particles and will provide clear results of induced cell death. *A. tumefaciens* GV3101 was transformed with the new tagged chimeric region-swapped genes and tagged *CCL-SSPs* by electroporation, the cells grown to the appropriate OD_{600}, mixed with infiltration buffer, infiltrated into *N. benthamiana* leaf tissue, and then assessed for cell death and photographed 7 dpi. It was found that visible responses in the leaves were very weak, and therefore the leaves were also photographed under UV light to better visualise any cell death that is occurring through fluorescence of phenolic compounds that are released during cell death (Koga, Zeyen, Bushnell, & Ahlstrand, 1988).

The controls in this experiment were the negative controls of empty vector and 14801 - T, a protein identified from *Venturia inaequalis* culture (Win, Greenwood, & Plummer, 2003). As was expected, 14801 - T did not induce any cell death that could be observed under white light or under UV light (Fig. 3.8A). In contrast, the empty the vector behaved irregularly. It was observed that of the leaves tested by ATTA with empty vector, half induced cell death and half did not. In the cases where the empty vector did not induce

cell death, no visible difference is noticeable from the leaves under white light, and no fluorescence was observed under UV light (Fig. 3.8B). However, when the empty vector did induce a response, the leaves visibly exhibited discolouring, and under UV light, the infiltrated leaf area fluoresced at a level visibly equal in intensity to the positive control of INF1 - T (Fig. 3.8B). The other controls used were INF1 - T (an apoplastic protein from *Phytophthora agathidicida* (unpublished)), and SsSSP, acting as a control for the 3 x FLAG tag which was added to all other proteins. INF1 - T induced the strongest response, visible on the leaves as discolouration and small spots of cell death, and under UV as intense levels of fluorescence (Fig. 3.8A). SsSSP did not appear to induce any visible response in the leaves, while a small level of fluorescence was observed under UV (Fig. 3.8A).

Of the other CCL-SSPs tested, SsSSP – T (the tagged version of SsSSP) induced the strongest response, visible as discolouration of the leaf and medium levels of fluorescence (Fig. 3.8A). SsSSP – Mid 1 – C term – T induced very weak responses; slight discolouration and low levels of fluorescence, while CcSSP37 – Mid 1 – C term – T did not appear to induce any response (Fig. 3.8A). Quantification of the level of cell death was performed by measurement of fluorescence intensity, normalized to the empty vector of each leaf. These results show that the only proteins that induced a response significantly different to the empty vector, were INF1 - T and SsSSP – T (Fig. 3.8C). These results show that the ATTA performed with *N. benthamiqnq* produced irregular cell death and cell death from the empty vector control. Therefore, the ATTA system did not provide clear results of induced cell death.

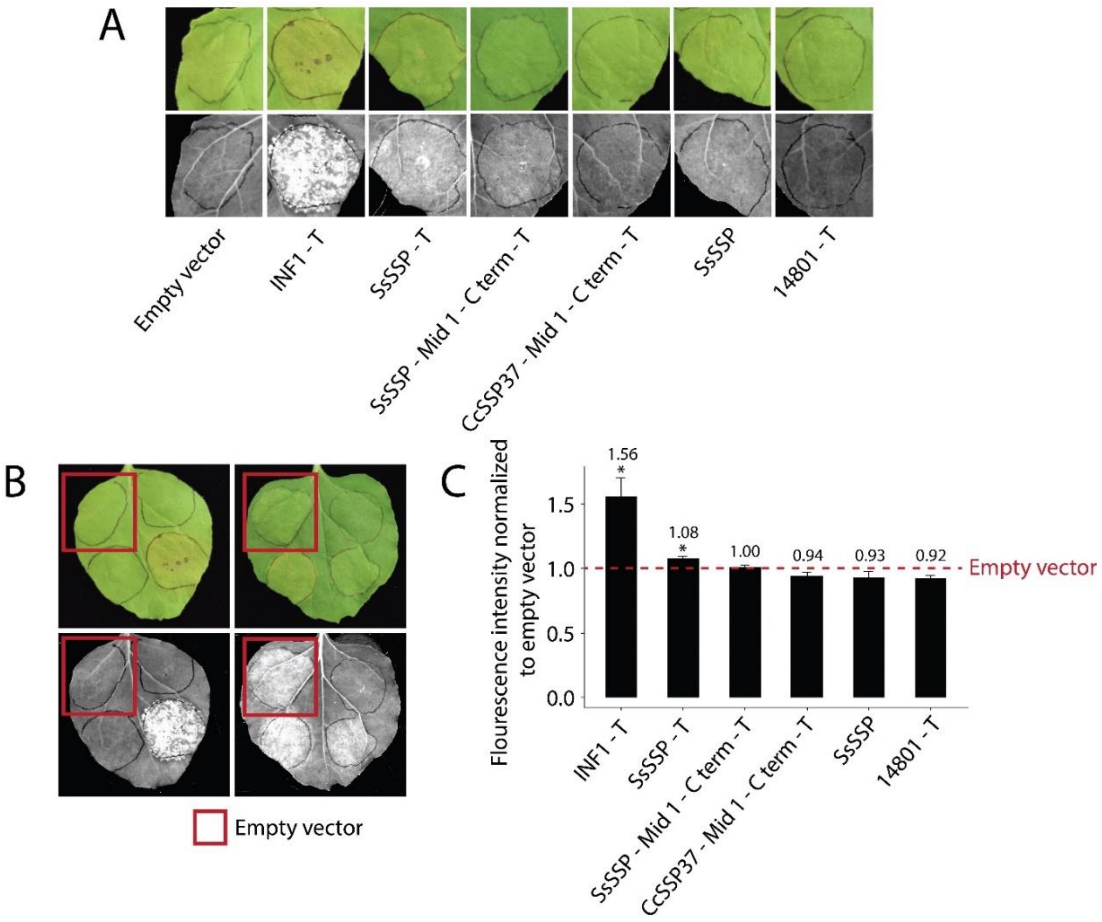


Figure 3.8. Plant cell death induced by an *Agrobacterium tumefaciens* transient transformation assay of *Nicotiana benthamiana* leaves with CCL-SSPs.
A – *N. benthamiana* leaves 7 days post infiltration (dpi) with recombinant *A. tumefaciens*. The top row of images was taken under normal white light, while the bottom row was taken under UV light. INF1 is an apoplastic protein from *Phytophthora agathidicida* (unpublished) and is being used here as a positive control for cell death. 14801 is a protein that was discovered from *Venturia inaequalis* culture (Win et al., 2003). It was used as a negative control as it has been shown to have no effect when recombinantly expressed in *N. benthamiana* leaves using an ATTA (unpublished). Infiltration area is marked with black pen. Representative images are shown. **B** – Whole *N. benthamiana* infiltrated leaves illustrating the variable fluorescence observed from the empty vector. The top row of images was photographed under normal white light, while the bottom row was photographed under UV light. In all images, the red box illustrates the area that was infiltrated with the empty vector. For the leaf on the left, the other infiltrations were made with; infiltration buffer (top right), 14801 - T (bottom left), and INF1 - T (bottom right). For the leaf on the right, the other infiltrations were made with; infiltration buffer (top right), SsSSP (bottom left), SsSSP – Mid 1 – C term – T (bottom right). Of the 16 leaves that were infiltrated with empty vector, 8 showed fluorescence and 8 did not. Photographs were taken 7 dpi. **C** – Intensity of the fluorescence emitted from the infiltrated leaves was quantified using ImageJ 1.52a software and normalized to the empty vector control that was present on each leaf (therefore empty vector is equal to 1 and is illustrated by the dotted red line). Bars with an asterisk (*) above them are significantly different to the empty vector, with a significance level of P = 0.05.

Significance was tested using the Wilcoxon signed rank test and Ri386 v3.5.1 software, and the lines above the bars represent standard error. The number of samples used in this experiment are as follows; INF1 (n = 8), 14801 (n = 6), SsSSP - T (n = 8), SsSSP (n = 4), CcSSP37 - Mid 1 - C term - T (n = 2), SsSSP – Mid 1 – C term - T (n = 6). All genes with 'T' at the end of the name contain a N terminus 3 x FLAG tag.

3.8 CCL-SSPs tagged with a N terminal 3 x FLAG tag induce cell death to the same extent as untagged CCL-SSPs

It has been previously shown that adding a tag to the C terminus of CCL-SSPs reduces cell death-inducing ability (Denton-Giles, 2014). It was also shown in this study that running Tricene–SDS–PAGE gels with untagged proteins is not successful in identifying presence of the chimeric region-swapped proteins in *P. pastoris* culture filtrate. Here, I suggest that addition of a 3 x FLAG tag to the N terminus of CCL-SSPs will not disrupt cell death inducing activity. *CCL-SSPs* containing the 3 x FLAG tag were constructed by Twist Biosciences^{TM} (USA) and transformed into *A. tumefaciens* by electroporation. The cells were then grown to the appropriate OD_{600}, mixed with infiltration buffer, infiltrated into *N. benthamiana* leaf tissue, then assessed for cell death and photographed 7 dpi. The positive control for the tagged proteins was SsSSP, which was not tagged. SsSSP induced a very weak response; slight discolouration was visible on the leaf and low levels of fluorescence was observed (Fig. 3.8A). The tagged version of SsSSP (SsSSP – T) induced slighter stronger responses of discolouration and fluorescence (Fig. 3.8A). The difference in response between the tagged and untagged SsSSP is shown from quantification of the fluorescence intensities; SsSSP – T is significantly different from the empty vector while SsSSP is not (Fig. 3.8C). Therefore, these results suggest that tagged CCL-SSPs can induce cell death to the same or greater extent than untagged CCL-SSPs.

3.9 *A. tumefaciens* transient transformation assay of *N. benthamiana* leaves with new chimeric region swapped CCL-SSPs induces weak cell death

It has been shown above that the protein region encoded by Exon 2 of SsSSP is essential for cell death induction in *Camellia* 'Nicky Crisp' and *N. benthamiana* tissues. I suggest that use of an ATTA with the new chimeric region-swapped genes SsSSP – Mid 1 – C term – T and CcSSP37 – Mid 1 – C term – T will provide more information about which specific region within Exon 2 of SsSSP is required for cell death activity. The new

chimeric region-swapped genes were constructed by Twist Biosciences™ (USA) and transformed into *A. tumefaciens* by electroporation for ATTAs as described in section 2.9. The cells were grown to the appropriate OD_{600}, mixed with infiltration buffer, infiltrated into *N. benthamiana* leaf tissue, then photographed 7 dpi. As discussed above, the controls 14801 – T and INF1 – T behaved as expected, but the empty vector induced strong responses in half of the samples. Of the two chimeric region-swaps, SsSSP – Mid 1 – C term – T appeared to induce the strongest response, which was still relatively weak compared to the positive control INF1 – T. SsSSP – Mid 1 – C term – T induced slight discolouration in the leaf and low levels of fluorescence (Fig. 3.8A). CcSSP37 – Mid 1 – C term – T in comparison did not appear to induce any discolouration or fluorescence (Fig. 3.8A). Quantification of the intensity of fluorescence across all samples illustrated little difference between the two chimeric proteins. SsSSP – Mid 1 – C term – T and CcSSP37 – Mid 1 – C term – T had average fluorescence intensities of 0.94 and 0.93 respectively (Fig. 3.8C). Both proteins were also shown to not induce fluorescence at a level higher than the empty vector (Fig. 3.8C). Therefore, these results show that weak cell death is induced from ATTAs of the new chimeric region swapped CCL-SSPs, however, some cell death was also induced by the empty vector control.

3.10 SsSSP and the chimeric region-swapped protein CcSSP37 – Exon 2 do not induce cell death when infiltrated into *Arabidopsis thaliana* leaves

It has been shown that SsSSP and the chimeric region-swapped protein CcSSP37 – Exon 2 can induce cell death in *N. benthamiana* leaf tissues. I suggest that these two proteins will induce cell death in *S. sclerotiorum* host plant *A. thaliana* (Saharan & Mehta, 2008) as well. Cell-free culture filtrate from recombinant *P. pastoris* expressing CCL-SSPs and CcSSP37 – Exon 2 was infiltrated into *A. thaliana* leaves of 8-week-old plants. As expected, the negative controls of water and empty vector did not induce cell death (Fig. 3.9A), although small amounts of browning were observed from mechanical damage (Fig. 3.9B). CcSSP37 also did not induce cell death (Fig. 3.9A). Unexpectedly, the protein which induced the greatest level of cell death was BcSSP (Fig. 3.9A). Quantification of the results show that BcSSP induced cell death in about 40% of the infiltration area (Fig. 3.9B). While SsSSP and CcSSP37 – Exon 2 appeared to not induce any cell death (Fig. 3.9A), and their levels of response were not significantly different to the negative controls

(Fig. 3.9B). These results illustrate that my hypothesis was not correct, SsSSP and CcSSP37 – Exon 2 do not induce cell death in *A. thaliana*, while BcSSP does.

3.11 SsSSP induces cell death when infiltrated into *S. sclerotiorum* host plant *Ocimum basilicum*

SsSSP has been shown to induce strong cell death in *Camellia* 'Nicky Crisp' petals and *N. benthamiana* leaves; however, the same result was not found on *A. thaliana* leaves. I suggest that SsSSP will induce cell death in *S. sclerotiorum* host plant *O. basilicum* (basil) (Saharan & Mehta, 2008). The cell-free culture filtrate of recombinant *P. pastoris* cells expressing SsSSP and empty vector were infiltrated into leaves of 5-week-old basil plants. Empty vector was a negative control and as expected, did not induce cell death (Fig. 3.10A). However, empty vector did induce quite a strong wounding response, suggesting that basil leaves are sensitive to infiltration. SsSSP was the only protein tested, and induced cell death as predicted, although it did not cover the entire infiltrated area (Fig. 3.10A). Quantification of the results show that SsSSP induced cell death in about 40% of the infiltration area and is significantly different to the empty vector (Fig. 3.10B). The strength of the wounding response induced by infiltration with empty vector is shown to be about 20% of the infiltration area (Fig. 3.10B). Although, it should be considered that this quantification method is a percentage of the infiltration area and this area was quite small in comparison to the size of the syringe which causes the wounding response. This may be a reason for the strong level of mechanical damage observed. These results illustrate that SsSSP induces cell death in the *S. sclerotiorum* host plant *O. basilicum*.

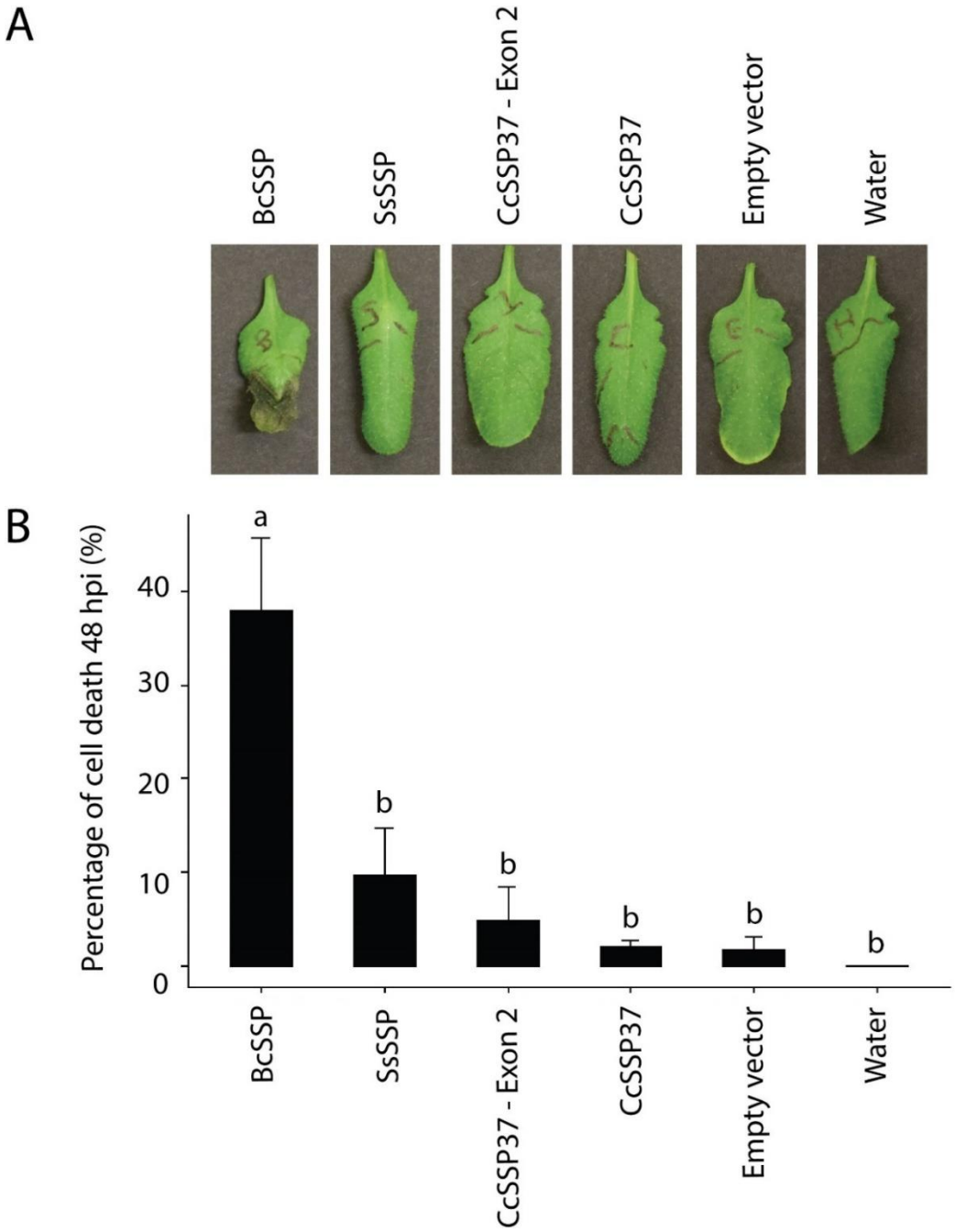


Figure 3.9. *Arabidopsis thaliana* leaves infiltrated with cell-free culture filtrate of recombinant *Pichia pastoris* expressing CCL-SSPs and CcSSP37 – Exon 2.

A – 8-week-old *A. thaliana* leaves 48 h post-infiltration (hpi). Infiltration area is marked with black pen. Left to right leaves were infiltrated with; BcSSP, SsSSP, CcSSP37 – Exon 2, CcSSP37, empty vector, and water. **B** – Percentage of leaf area exhibiting cell death 48 hpi. Cell death area was measured with ImageJ 1.52a software, and data analysed with Ri386 v3.5.1 software. Tukey tests were performed to a significance level of P = 0.01. Lines above the bars represent standard error, and bars with different letters are significantly different to each other. Images are representative and sample sizes are as follows; BcSSP, SsSSP, CcSSP37, empty vector, water (n = 9), CcSSP37 – Exon 2 (n = 6).

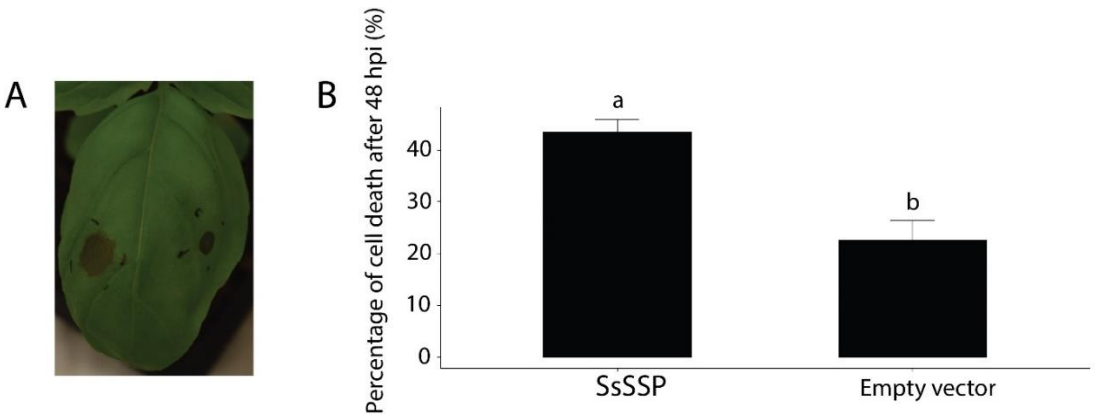


Figure 3.10. *Ocimum basilicum* (basil) leaves infiltrated with cell-free culture filtrate of recombinant *Pichia pastoris* expressing SsSSP and empty vector.
A – Basil leaf infiltrated with SsSSP (left) and empty vector (right) at 48 h post-infiltration (hpi). Infiltration area is marked with black pen. **B** – Percentage of leaf area exhibiting cell death following infiltration with SsSSP and empty vector. Percentage of cell death was measured using ImageJ 1.52a software, and the data analysed using Ri386 v3.5.1. Tukey tests were done to a significance of P = 0.01. The letters above the bars illustrate that the two bars are significantly different to one another. The lines above the bars represent standard error. Image is representative (n = 8).

3.12 SsSSP does not cause cell death when infiltrated into six *S. sclerotiorum* host plants

It has been shown that SsSSP can induce cell death on the *S. sclerotiorum* host plant *O. basilicum*. I suggest that SsSSP will also induce cell death in other host plants of *S. sclerotiorum*. The cell-free culture filtrate of *P. pastoris* recombinantly expressing SsSSP and other CCL-SSPs was infiltrated into *S. sclerotiorum* host plants *Raphanus sativus* (Radish), *Brassica campestris* (Pak Choi), *Phaseolus vulgaris* (Bean), *Triticum aestivum* (Wheat), and *Beta vulgaris var Cruenta* (Beetroot) (Saharan & Mehta, 2008). It was found that infiltration of neither SsSSP, nor empty vector, induced any cell death in Radish, Pak Choi, or Beetroot (Fig. 3.11A, B, C). Bean leaves were infiltrated with SsSSP, BcSSP, CcSSP37, empty vector, and water. It was found that all CCL-SSPs and the empty vector induced cell death (Fig. 3.11C). This was likely due to PAMPs, such as cell wall fragments being present in the culture filtrate and activating plant immunity, as the empty vector elicited a response while water did not. Wheat was infiltrated with empty

vector, BcSSP, SsSSP, and CcSSP37 – Exon 2. It was found that SsSSP induced cell death erratically; necrosis was observed in 5 of 12 leaves tested (Fig. 3.11D). The inconsistency of these results suggest further work is required before any conclusions can be made about the ability of SsSSP to induce cell death in *T. aestivum*. These results show that culture filtrate of recombinant *P. pastoris* expressing SsSSP, does not induce cell death on the six host plants of *S. sclerotiorum* tested.

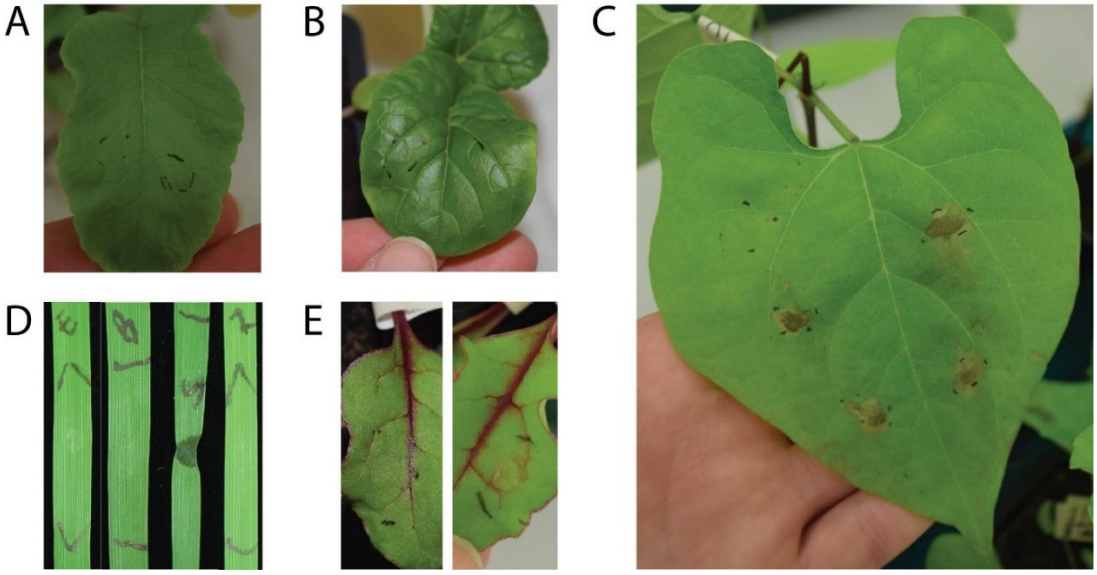


Figure 3.11. Leaves of *Sclerotinia sclerotiorum* host plants infiltrated with cell-free culture filtrate of recombinant *Pichia pastoris* expressing CCL-SSPs 48 h post-infiltration (hpi).
A – 5-week-old *Raphanus sativus* (Radish, pink beauty) infiltrated with empty vector on the left and SsSSP on the right (n = 5). **B** – 5-week-old *Brassica campestris* (Pak Choi, mini toy choi) infiltrated with empty vector on the left and SsSSP on the right (n = 9). **C** – 4-week-old *Phaseolus vulgaris* (Bean, climbing cobra) infiltrated from the left side down; water, empty vector, CcSSP37. From the right side down; BcSSP and SsSSP (n = 8). **D** – 4-week-old *Triticum aestivum* (Savannah wheat) infiltrated left to right with empty vector, BcSSP, SsSSP, and CcSSP37 – Exon 2. Infiltration with SsSSP induced cell death in 5 samples. SsSSP-induced cell death is shown in the figure. All other CCL-SSPs, including the empty vector, did not induce any cell death response. Images are representative and sample sizes are as follows; SsSSP (n = 12), BcSSP (n = 7), empty vector (n = 8), CcSSP37 – Exon 2 (n = 5). **E** – 5-week-old *Beta vulgaris var Cruenta* (Beetroot, action F1 hybrid) infiltrated with empty vector on the left and SsSSP on the right leaf (n = 4). All plants were assessed for cell death and photographed 48 hpi. Representative images are shown.

4 Discussion

CCL-SSPs are candidate effector proteins of the necrotrophic fungus *Ciborinia camelliae*. These proteins were first identified by Denton-Giles (2014) after the transcriptome was sequenced and the secretome predicted. The CCL-SSP family, which emerged from amino acid identity analysis as a highly supported clade in *C. camelliae*, has 73 unique members. Of these, 72 were shown to be expressed during infection. Notably, the CCL-SSPs are not unique to *C. camelliae*. Indeed, a single homolog of the CCL-SSP family has shown to be encoded by the genomes of the closely related necrotrophs, *Botrytis cinerea* and *Sclerotinia sclerotiorum*, (BcSSP and SsSSP, respectively). CCL-SSPs were predicted to be effector candidates due to their high cysteine content, small size and signal peptide, and because they are under positive selection. The role of both SsSSP and BcSSP as putative effector proteins was supported by infiltration of the recombinant proteins into highly susceptible *Camellia* hybrid 'Nicky Crisp', where they induced cell death. Ten of the 73 *C. camelliae* CCL-SSPs (CcSSPs) were also tested in the same way; however, no cell death was induced after infiltration apart from a small amount induced by CcSSP92. Amino acid sequence identity of the CcSSPs to SsSSP and BcSSP (Fig. 1.2) suggests that the *C. camelliae* CCL-SSPs would also share the same function; however, this is yet to be proven (Denton-Giles, 2014).

4.1 Characterisation of the CCL-SSPs from *C. camelliae, B. cinerea,* and *S. sclerotiorum*

In this study, two CcSSPs were investigated; CcSSP92 and CcSSP37. A majority of this study focuses on CcSSP37 because this protein was chosen to be used in the region swap design. Of the 73 CcSSPs, CcSSP92 and CcSSP37 are the most similar in amino acid sequence identity to SsSSP and BcSSP. CcSSP92 is 76% identical to BcSSP and 75% identical to SsSSP, while CcSSP37 is 55% identical to BcSSP and 58% identical to SsSSP (Meijer et al., 2018). CcSSP37 was used for the region swap rather than CcSSP92 because of the previous finding that CcSSP92 induces very weak cell death in *Camellia* 'Nicky Crisp' petals (Denton-Giles, 2014). Although, it should be noted that this weak cell death was not replicated in this study (Fig 3.1). It was shown in this study that CcSSP37 does

not induce cell death in *Camellia* 'Nicky Crisp' petals (Fig. 3.4A), *Nicotiana benthamiana* leaves (Fig 3.5A), or *Arabidopsis thaliana* leaves (Fig. 3.9A). It was also shown that CcSSP92 does not induce any cell death when infiltrated into *Camellia* 'Nicky Crisp' petals (Fig. 3.1). The results that CcSSP37 does not induce cell death has been shown in a previous study, where CcSSP37 was infiltrated into *Camellia* 'Nicky Crisp' petals and *N. bethamiana* leaves and no cell death was induced (Denton-Giles, 2014).

Similar infiltrations of SsSSP and BcSSP were performed in *Camellia* 'Nicky Crisp' petals, where these proteins were shown to induce strong cell death. The SsSSP-induced cell death covers the entire infiltration area and BcSSP-induced cell death covers almost the entire infiltration zone (Fig. 3.4A). Quantification of this cell death discovered a significant difference in the strength of cell death between SsSSP and BcSSP, where SsSSP induced a stronger level of over 75% cell death per infiltration area, while BcSSP induced over 50% cell death per infiltration area (Fig. 3.4C). Examination of the infiltrated petals from 0 hpi to 12 hpi also suggests that SsSSP may induce cell death more rapidly than BcSSP, as while both induced visible cell death 2 hpi, the cell death induced by SsSSP was stronger at this time point and covered a greater area of the infiltration zone (Fig. 3.4B). Further evidence that SsSSP and BcSSP may induce cell death to a different extent, was the discovery that BcSSP can induce a strong level of cell death in *A. thaliana* leaves, while SsSSP does not (Fig. 3.9A). SsSSP was also shown to induce a medium level of cell death in *Ocimum basilicum* leaves which did not completely cover the infiltration area (Fig. 3.10A), although BcSSP was not tested. Interestingly, in a previous study where BcSSP and SsSSP where infiltrated into *Camellia* 'Nicky Crisp' petals, no differences in cell death-inducing capability was reported (Denton-Giles, 2014). These results illustrate that while SsSSP and BcSSP belong to the CCL-SSP family and share high amino acid sequence identity, it is possible that they can trigger cell death to different extents. The vastly different responses upon infiltration into *A. thaliana* leaves suggests that these two proteins may target different molecules or receptors, or that perhaps they have different affinities to their targets. More work is required to determine how these two proteins induce cell death and in what manner before it can be suggested how they differ. Meanwhile, these results support my hypothesis that different CCL-SSPs can differentially induce cell death in distinct plant species.

4.2 Evolutionary studies and gene duplication to produce large effector families suggest that some *C. camelliae* CCL-SSPs must have retained cell death-inducing activity

While there is vast evidence to support SsSSP and BcSSP as effector proteins that induce cell death, the function of *C. camelliae* CcSSPs remains elusive. It has been hypothesised that the *CCL-SSP* family underwent lineage-specific gene expansion via gene duplication to become so large (Denton-Giles, 2014). Phylogenetic analysis of five host-specific lineages of *Blumeria graminis* also identified gene duplications within the genome, and these were more frequent, along with deletions, in candidate effector gene families. This was believed to be due to the enrichment of transposable elements surrounding effector candidate genes (Menardo, Praz, Wicker, & Keller, 2017). Similar to what has been found for the *CCL-SSP* family (Denton-Giles, 2014), the same study also identified that repeated gene duplications of single genes in a lineage had generated large clades of effector gene families (Menardo et al., 2017).

It was hypothesised that host-driven selection pressure is the cause for the expansion of the CCL-SSP family in the host-specific pathogen *C. camelliae*, as *S. sclerotiorum* and *B. cinerea*, which are broad host pathogens, have only one CCL-SSP homologue (Denton-Giles, 2014). Host range has been shown to have an effect on deletion or duplication occurrence in the phylogenetic history of plant pathogens. A recent study of species in the *Colletotrichum acutatum* species complex and other related species, discovered lineage specific deletions of CAZymes (carbohydrate active enzymes) and proteases in species with small host ranges, while those with large host ranges were found to have duplications of the same genes. These broad host pathogens were also shown to have a contraction of lineage-specific proteins predicted to be effectors (Baroncelli et al., 2016). This data suggest that host selection pressure is a force that affects protein content of pathogens, and that broad-host pathogens use more non-specific virulence proteins such as CAZymes rather than effector proteins.

The data shown here is consistent with the idea that the ten tested CcSSPs did not induce cell death, and likely many of the other untested CcSSPs, have lost the ability to cause cell death because of the *Camellia* host adapting to these proteins to become resistant. However, I suggest that a small number of the CcSSPs might still have cell death-inducing functions that susceptible *Camellia* species have not yet adapted to. The large expansion

of the CCL-SSP family, and the fact that they are predicted to be under positive selection (Denton-Giles, 2014), suggests that CCL-SSPs are acquiring mutations to combat ever changing and adapting host resistance, and potentially to target different *Camellia* species. How positive selection and evolution can be beneficial to a pathogen was illustrated in a recent study of the rice blast fungus *Magnaporthe oryzae*. *M. orysae* produces an avirulence protein, AVR-Pii, which is recognised by the rice resistance protein, Pii to activate ETI and plant immunity. The *Pii* resistance gene has been used in China for over 20 years as a control method against this fungus. These researchers tested 454 field isolates for presence of the *AVR-Pii* avirulence gene in their genome. A total of 82 isolates were found to carry *AVR-Pii*, and of the three halotypes identified, one was shown to be virulent. These results suggest the effector protein AVR-Pii is under positive selection to enable acquisition of mutations and generation of a halotype which is no longer recognised by the resistance protein Pii (L. Lu et al., 2019*).*

4.3 The protein sequence encoded by Exon 2 of SsSSP is essential for cell death-inducing activity

The importance of positive selection to overcome host resistance further supports my hypothesis that some CcSSPs can induce cell death. I decided to investigate how SsSSP activates cell death. If the cell death-inducing activity of SsSSP was governed by a specific domain or amino acid residues, it could be used as a way of predicting cell death-inducing function activity in CcSSPs, should they contain the same region or amino acid residues. This case where the effector protein of interest (SsSSP) has a homologue that is not capable of inducing cell death in the same host (CcSSP37) provides a unique opportunity to perform domain swaps. The experimental design of domain swapping is that two similar proteins have domains switched between one another to generate chimeric proteins (Brutus, Sicilia, Macone, Cervone, & De Lorenzo, 2010). A study which exploited the same scenario of having a functional and non-functional homologue investigated the tomato immune receptor Ve1. Domain swaps between *Verticillium dahlia* effector Ve1, and the non-functional homologue Ve2 provided useful information about the function of various domains of Ve1 (Fradin et al., 2014). Domain swaps have been used recently to elucidate the function of effector proteins in other studies (Matić,

Pegoraro, & Noris, 2016; Mesarich et al., 2016), illustrating that it is a method that could be useful to determine which regions of SsSSP determines cell death-inducing function.

I decided to investigate which coding region of SsSSP confers cell death activity through domain swaps. Although, for this study I have named this methodology as "region swaps", as no domains have been identified in any CCL-SSPs so far. Region swaps were generated between SsSSP and CcSSP37. These two proteins are very similar, being 58% identical in sequence identity, and like all CCL-SSPs, have the same ten conserved cysteine residues (Denton-Giles, 2014). Four different regions were chosen for their amino acid variability and swapped between *CcSSP37* and *SsSSP* to generate eight chimeric genes (Fig. 3.2). These genes were then recombinantly expressed in *P. pastoris* and the culture filtrate containing the chimeric protein of interest infiltrated into *Camellia* 'Nicky Crisp' petals. Out of the eight chimeric proteins, only one induced cell death; CcSSP37 – Exon 2 (Fig. 3.4A). This chimeric protein contains the region encoded by Exon 1 of *CcSSP37*, and the region encoded by Exon 2 of *SsSSP*. This experiment discovered that the protein region encoded by Exon 2 of *SsSSP* is essential for SsSSP cell death-inducing ability. This was also confirmed by the lack of cell death induced by SsSSP – Exon 2, which is the reciprocal swap (Fig. 3.4A). Therefore, these results support my hypothesis that a specific region of SsSSP can be identified that confers cell death-inducing ability.

The discovery that only one chimeric region-swapped protein induces cell death generates several questions. Is the loss of cell death activity in the region swaps SsSSP – Mid 1, SsSSP – Mid 2, and SsSSP – C term a cause of an essential domain being disrupted, or is it due to loss of stability of the protein? There is also the possibility that the entire region encoded by Exon 2 is required for activity. There are examples where the domain required for activity of a fungal effector is quite large. The *B. cinerea* effector BcSpl1, a cerato-platanin protein, was suggested by the generation of truncated proteins lacking conserved regions, that a large 40 amino acid long central region was required for necrosis-inducing ability. This region was also found to produce two surface-exposed loops in the structure of the protein (Frias et al., 2014).

Another recent discovery of a very large functional domain of an effector protein was in *C. fulvum* effector Avr4. Avr4 has been shown to bind chitin through a chitin-binding domain (ChBD) that requires three disulphide bonds for stability and function (van den

Burg et al., 2003). The x-ray crystal structure of *Pseudocerospora fuligena* Avr4 (PfAvr4) provided more information about how Avr4 binds chitin through the ChBD (Kohler et al., 2016). However, it wasn't until x-ray crystallography of *C. fulvum* Avr4 (CfAvr4) in complex with chitohexaose was performed that it was found that the ChBD extends the entire length of the protein (Hurlburt et al., 2018). Considering CfAvr4 is 105 amino acids in length after removal of the signal peptide (Joosten, Vogelsang, Cozijnsen, Verberne, & De Wit, 1997), this makes the ChBD a very large domain, and therefore, not impossible for the entire region encoded by Exon 2 of SsSSP to be essential for cell death-inducing activity.

There have also been cases where a functional domain of an effector is a lot smaller. The *Ustilago maydis* effector Pit2, after alignments with orthologs in related fungal species, was found to contain a 14-amino acid conserved sequence motif. Mutation of the motif resulted in loss of virulence, and the motif alone as a synthetic peptide could inhibit proteases (which is the putative function of Pit2) (Mueller et al., 2013). CCL-SSPs also have two conserved sequence motifs. There is a 15-amino acid conserved motif at the N terminus, just after the signal peptide (Fig. 3.2A). However, the result of the region swaps shows that this motif, while it may be important for function, is not the determining factor for cell death-inducing activity, as it is part of the region encoded by Exon 1. There is also a small three-amino acid motif at the end of the coding region named "Mid 2" (Fig. 3.2A). However, this motif also isn't likely to be the essential region determining cell death activity, as the region swap CcSSP37 – Mid 2 did not induce cell death (Fig. 3.4A).

The possibility that the entire region encoded by Exon 2 of *SsSSP* is required for cell death-inducing activity has been discussed above. However, it is also possible that the other chimeric proteins did not induce cell death because their chimeric structure was unstable. In a recent study of effector protein Avr4, domain swaps were made between the sequences of Avr4 proteins that do not induce Cf-4 mediated cell death (*Cercospora apii* CaAvr4 and *Cercospora beticola* CbAvr4), and Avr4 proteins that do induce cell death (*C. fulvum* CfAvr4 and *Dothistroma septosporum* DsAvr4). The researchers then performed point mutations to try and determine which residues within the domain they isolated, are essential for Cf-4 recognition and activation of the hypersensitive response. They discovered that mutating proline residue 87 (Pro87) to arginine eliminated any cell

death in tomato leaves and concluded that this single amino acid must be essential for Cf-4 recognition (Mesarich et al., 2016).

However, in a more recent study, they mutated the same residue Pro87 to alanine and discovered no change, as the protein still activated cell death in tomato. These researchers suggested that it is not the amino acid Pro87 that is essential, but for a small aliphatic residue to be in this position (Hurlburt et al., 2018). They suggest that mutating Pro87 to arginine in the previous study (Mesarich et al., 2016) was disrupting the structural integrity of the protein so that it was degraded by proteases in the plant before it was recognised by Cf-4 (Hurlburt et al., 2018). This hypothesis was supported by the discovery that four residues in Avr4 that could possibly be targets for recognition by Cf-4, make the protein more susceptible to proteolytic degradation when these residues were mutated. The researchers concluded that these residues are likely not targets for recognition by Cf-4 but are important for the structure of the protein and preventing degradation by proteolysis (Hurlburt et al., 2018). Therefore, the lack of cell death-inducing activity of the other chimeric proteins could be a result of an unstable structure, where these proteins are being degraded by host proteases before they can induce cell death.

These new discoveries tie back to previous research and provide new insights into fungal evolution and adaption to host resistance. It was discovered 22 years ago that some strains of *C. fulvum* that do not induce a Cf-4 mediated hypersensitive response (HR) contain various point mutations in the *Avr4* gene. These isoforms of Avr4 that don't activate HR were shown to be unstable, and the researchers suggested that production of Avr4 proteins that are susceptible to proteolysis but do not activate host immunity, is a strategy this fungus is implementing to overcome host resistance (Joosten et al., 1997). This hypothesis was further supported by a more recent study which investigated 133 *C. fulvum* isolates from Japan. It was found that the *Avr4* genes of these isolates contained many unique mutations such as frame shift mutations, transposon insertions, point mutations and deletions. These mutated and unique versions of the *Avr4* gene were cloned and expressed through a modified binary Potato Virus X–based vector in tomato plants to determine whether the proteins could still activate Cf-4 mediated HR. Interestingly, many of these mutated Avr4 proteins were discovered to not activate HR, although they maintained their chitin-binding ability. This supported the hypothesis that acquisition of

mutations in Avr4 is an evolution strategy of *C. fulvum* to avoid host detection by producing unstable Avr4 proteins and therefore overcoming resistance (Iida et al., 2015).

While these discoveries discussed above were made in a biotrophic fungus that produces avirulent effector proteins, it could be possible that necrotrophic fungi such as *C. camelliae* can acquiring such mutations for the opposite function. It has been hypothesised previously that necrotrophic pathogens could be hijacking plant immune systems to benefit their own development and survival, as an HR response provides available nutrients to necrotrophic pathogens which can only acquire nutrients from dead cells (Lorang et al., 2012). This has been further supported by the discovery of plant genes usually associated with resistance (NBS-LRR genes) that actually confer susceptibility to necrotrophic pathogens, such as the *Pc* gene that mediates susceptibility to *Periconia circinate* (Nagy & Bennetzen, 2008), the *Tsn1* gene that confers susceptibility to ToxA of *Stagonospora nodorum* and *Pyrenophora tritici-repentis* (Faris et al., 2010), the *Scs6* susceptibility gene of barley which mediates susceptibility to *Cochlibolus sativus* (Leng et al., 2018), and the well-established Victoria oat blight interaction where the *PvLOV* gene causes susceptibility to *Cochliobolu victoria* (Lorang et al., 2018). Therefore, it is a possibility that the expansion and subsequent positive selection of *CCL-SSP* genes is an evolutionary strategy to produce effectors that activate HR to facilitate disease.

4.4 SsSSP cell death-inducing activity is weakened in *A. tumefaciens* transient transformation assays

The original expression system that was used to investigate cell death-inducing function of chimeric region-swapped proteins was *P. pastoris.* Recombinant expression by *P. pastoris* is a well-established method (Cregg et al., 2018) and has been used successfully by other researchers to characterise effector proteins (Frias et al., 2011; S. Lu, Faris, Sherwood, Friesen, & Edwards, 2014; Y. Zhang, Liang, Qiu, Yuan, & Yang, 2017), and other plant or pathogen proteins (Palomares, Monsalve, Rodríguez, & Villalba, 2002; S. B. Zhang et al., 2019). This system was determined as successful in this study as infiltrations of *P. pastoris*-expressed proteins in *Camellia* 'Nicky Crisp' petals resulted in cell death from SsSSP, BcSSP and CcSSP37 – Exon 2 (Fig. 3.4A). However, *Camellia* 'Nicky Crisp' petals are only available at certain times of the year, so it was investigated whether these region-swapped chimeric proteins and CCL-SSPs could induce cell death

in *N. benthamiana.* These results were successful and confirmed that *N. benthamiana* could be used a model plant for this system (Fig. 3.5A), therefore supporting my hypothesis that activity of CCL-SSPs can be modelled in a different system. However, when infiltrated into *N. benthamiana* there was a greater background response, evidently not a result of mechanical damage as it was prevalent in the empty vector control and all other infiltrations, but not from the water control.

I suggest that infiltrating these proteins with the *P. pastoris* culture filtrate rather than as purified protein resulted in the background response observed. Proteomic analysis of *P. pastoris* has shown that 75 different proteins are secreted while undergoing recombinant expression (Huang et al., 2011). Along with the possibility that any one of these proteins might activate an immune response in *N. benthamiana*, it is also highly likely that during the process of producing culture filtrate and then spinning it down to remove cells, yeast cell wall fragments could be released into the culture filtrate. Yeast cell walls contain chitin, which is a well-known PAMP that elicits PTI (Huaping, Xiaohui, Lunying, & Junsheng, 2017; Yamada et al., 2016). Therefore, it is possible that the "background" of the culture filtrate; being the vast quantity of proteins other than CCL-SSPs which are also being secreted by *P. pastoris*, evident from the Tricene-SDS-PAGE performed (Fig. 3.3C), could be the cause of the background response observed, and may be masking inducers of weak cell death. Therefore, I decided to move into a different expression system.

I decided to use *A. tumefaciens* transient transformation assays (ATTAs) to test the effect of CCL-SSP and chimeric region-swapped proteins in *N. benthamiana* leaves. ATTAs are a common method that has been utilized by many, such as for the characterisation of candidate effectors of *Puccinia striiformis* f sp *tritici* (Petre et al., 2016) and to discover that the *Phytophthora infestans* effector PexRD54 antagonises a host autophagy cargo receptor to mediate disease (Dagdas et al., 2016). More recently, ATTAs have been used to characterise the effectors RipAW and RipAR of *Ralstonia solanacearum* and their role in suppressing host PTI (Nakano et al., 2017), and to investigate the location and delivery of the *Phytophthora infestans* effectors Pi04314 and EPIC1 (S. Wang et al., 2017). Therefore, there is sound evidence that the ATTA system would be a useful tool for characterising the cell death activity of CCL-SSPs.

It was my hypothesis that this new expression system would result in the same or greater level of cell death from expression of SsSSP. Instead, these ATTAs produced very weak responses in *N. benthamiana* leaves of leaf discolouration and small areas of cell death in the positive control, INF1 – T (Fig. 3.8A). Another study that observed very weak to zero cell death from ATTAs found that it wasn't until they co-expressed their ATTAs with silencing suppressor *A. tumefaciens* P19 that they observed cell death (Meijer et al., 2018). Therefore, it could be possible that if P19 was co-expressed with chimeric region-swapped and *CCL-SSP* genes, a stronger response could be observed. Because the responses were so weak, I decided to photograph the leaves under UV light, where any cell death could be identified by the fluorescence of phenolic compounds which are released from the central vacuole upon cell death (Koga et al., 1988). This method is quite sensitive and was effective in illustrating differences in cell death levels between different proteins. This method of observing fluorescence in whole leaves was devised by Hunziker (2018) and has not been replicated often, although there are several examples where autofluorescence has been examined at the microscopy level (Meijer et al., 2018; Sørensen, Labouriau, & Hovmøller, 2017).

My other hypothesis was that by using ATTAs, background responses would be reduced. However, this was not the case as leaf discolouration and high intensities of fluorescence were observed from the empty vector negative control in half of the samples (Fig. 3.8B). These undesirable responses from the empty vector affected all other proteins tested because, not only did it suggest that any cell death induced could be the result of the assay rather than activity of the protein, but also quantification of fluorescence intensities was normalized to the empty vector on each leaf (Fig. 3.8C). These results are not the only cases where ATTAs have induced undesired responses. Similar results of leaf discolouration have been observed in a recent study where the researchers also experienced "undesired side-effects" from ATTAs, such as leaf discolouration and also elevated expression of pathogenesis-related (PR) proteins (Zhou, Cox, & Kearney, 2017). The discovery that callose and lignin were deposited in cells after ATTAs is further evidence that infiltration of *A. tumefaciens* could be activating host immunity (Le Mauff et al., 2017). Another study where leaf discolouration was observed suggested that perhaps these effects are the result of stress rather an immune response (Pruss, Nester, & Vance, 2008). However, this hypothesis was refuted after a study was performed on the transcriptome, extracellular proteome, and active secretome of leaves infiltrated with *A.*

tumefaciens and discovered a steady increase in expression of immunity-related genes at the sacrifice of photosynthesis-related gene expression, explaining why so many researchers have observed discolouration of infiltrated leaves (Grosse-Holz et al., 2018). These results illustrate that infiltration of *A. tumefaciens* can affect the plant and likely activates host immunity. Therefore, the undesirable results from ATTAs in my research is not specific to the experimental conditions used in my research.

4.5 A N-terminal tag may be more effective for use with CCL-SSPs than a C-terminal tag

The transition to ATTAs presented the opportunity to add a tag to CCL-SSPs and chimeric region-swapped proteins. A tag was not added to CCL-SSPs that were expressed in *P. pastoris* because previous work had shown that addition of a c-Myc 6xHis-tag to the C terminus of SsSSP and BcSSP reduced cell death activity. Tagged SsSSP was observed to induce cell death in a delayed manner (8 hpi opposed to 2 hpi) and cell death did not extend to the entire infiltration area. While tagged BcSSP failed to induce any cell death at all (Denton-Giles, 2014). Therefore, the CCL-SSPs and region-swapped CCL-SSPs were produced without a tag. However, the Tricene-SDS-PAGE gel could not provide any evidence that the CCL-SSPs and chimeric region-swapped proteins were being expressed or were present at equal concentrations (Fig. 3.3C). Protein concentration has been shown to be important for activity of effector proteins, as several effector proteins have been shown to be dose-dependent, such as the *B. cinerea* effector BcSpl1 (Frias et al., 2011), *Puccinia striiformis* f. sp. *Tritici* effector PstSCR1 (Dagvadorj et al., 2017), *Xanthomonas orysae* pv. *Oryzicola* effector Tal2a (Hummel, Wilkins, Wang, Cernadas, & Bogdanove, 2017), and also the *S. sclerotiorum* effector Sscp1 (Yang et al., 2018). Therefore, addition of a tag might allow protein identification, which could determine whether CCL-SSPs and chimeric region-swapped proteins are being expressed and if these proteins are dose-dependent.

Tags are often used so that after recombinant expression, the protein can be proven as successfully expressed, the protein concentration can be identified, and in some cases the protein can be purified. Protein presence can be confirmed by a variety of methods. A recent study used the *P. pastoris* expression system for the analysis of plant protein β-1,3-glucanase, and purified this protein by an anion exchange resin to confirm that the

protein was present (S. B. Zhang et al., 2019). More traditional methods of confirming protein production require addition of a tag, such the 6 x His epitope, which was used to purify *B. cinerea* effector protein BcSpl1 after expression in *P. pastoris* (Y. Zhang et al., 2017). Given the results of previous research using a C-terminal c Myc 6xHis tag (Denton-Giles, 2014), and the necessity of the region encoded by Exon 2 of *SsSSP* for cell death-inducing activity (Fig. 3.4A, 3.5A), it is possible that the C-terminal tag was interfering directly with the region of SsSSP that is required for cell death activity. It has also been shown that the region encoded by Exon 1 of SsSSP is not the factor determining cell death-inducing activity. Therefore, a N-terminal 3 x FLAG tag was added to the genes to be expressed through the ATTA system (Fig. 3.6A).

While the issues with the ATTAs has been previously discussed, I believe that there is some evidence from the ATTA experiment that merits further investigation of an N-terminal tag. Firstly, the tagged SsSSP – T was observed to induce a strong intensity of fluorescence and was the only protein other than the positive control INF1 – T to induce fluorescence significantly different to the empty vector (Fig. 3.8C). An unexpected result was that the untagged SsSSP did not induce strong cell death and was observed to induce fluorescence at an intensity lower than the empty vector. While the issues with the empty vector has been previously discussed, it is quite possible that an N-terminal tag will not affect cell death-inducing activity like what was observed from the C-terminal tagged CCL-SSPs (Denton-Giles, 2014). I believe that these results, while they are unable to provide any substantial evidence about the addition of an N-terminal tag, are promising and warrant further investigation.

5 Conclusions

5.1 The protein region encoded by Exon 2 of SsSSP is essential for cell death-inducing activity

The results of this study have further characterised the cell death-inducing activity of CCL-SSPs. It was shown that the protein region encoded by Exon 2 of SsSSP is essential for cell death-inducing activity. This was illustrated through generation and infiltration of chimeric region-swapped proteins containing regions from SsSSP and CcSSP37. The chimeric region-swapped protein CcSSP37 – Exon 2 was able to induce cell death, while all other chimeric proteins did not. Therefore, the protein region encoded by Exon 2 of SsSSP is essential for cell death-inducing activity and supports my hypothesis that a specific region of SsSSP could be identified that confers ability to induce cell death.

5.2 SsSSP and BcSSP may induce cell death to differing extents

SsSSP and BcSSP cell death-inducing activity was shown to be active on not only the *C. camelliae* host *Camellia* 'Nicky Crisp' petals, but also *N. bethamiana* leaves. SsSSP was also shown to induce a moderate amount of cell death on basil leaves. Interestingly, it was found that BcSSP induced strong cell on *A. thaliana* leaves, while SsSSP did not. These results were not the first in this study to suggest that SsSSP and BcSSP may induce cell death to a different extent. Quantification of cell death from infiltrations of SsSSP and BcSSP into *Camellia* 'Nicky Crisp' petals showed a significant difference in percentage of cell death induced

by the two proteins, and the results also suggested that SsSSP induced cell death more rapidly than BcSSP. These results support my hypothesis that different CCL-SSPs can induce cell death in distinct plant species. Further work is required to determine whether the molecular mechanisms of these two proteins differ.

5.3 The *P. pastoris* expression system produced the best results for characterisation of CCL-SSPs

Several methods for testing the cell death-inducing activity of CCL-SSPs were investigated in this study. It was found that the clearest results were observed from using the recombinant *P. pastoris* expression system to infiltrate *Camellia* 'Nicky Crisp' petals. Although there was a drawback was that the genes used for this experiment were not tagged and were not observed through Tricene-SDS-PAGE. *A. tumefaciens* transient transformation assays were also performed in *N. benthamiana* to test the cell death-inducing activity of CCL-SSPs. However, it was found that the empty vector control induced unexpected results and rendered the experiment inconclusive. Therefore, these results illustrate that the *P. pastoris* system is the most effective for characterisation of cell death-inducing activity of CCL-SSPs and supports my hypothesis that a different system could be found to test activity of CCL-SSPs.

5.4 Future directions

The chimeric region-swapped CCL-SSPs showed that the region encoded by Exon 2 of SsSSP is required for cell death-inducing activity. However, all other chimeric region-swapped proteins did not induce cell death, and it was not determined from this study whether this was a result of these proteins not having cell death-inducing activity, an inadequate concentration of protein present, or compromised stability of chimeric proteins. A C terminus tag was added to newly generated chimeric region-swapped genes however, the very weak responses were observed from ATTAs and the undesirable responses induced by the empty vector control meant it could not be confirmed whether this tag is suitable for use with these proteins.

Therefore, I suggest a repeat of the ATTAs with tagged chimeric region-swapped genes be performed with more replicates. I would suggest co-infiltrating with *A. tumefaciens* P19, as previous studies have found that its use has strengthened observed cell death (Meijer et al., 2018). I also suggest testing BcSSP with this method also, to determine whether BcSSP and SsSSP induce cell death in the same manner. Western blots should also be performed on protein extracted from infiltrated leaves, to determine whether the tagged proteins can be detected.

After western blots are performed, I suggest analysing the concentration of CCL-SSPs being expressed. Differing protein concentrations was discussed in this study as a factor which could be accounting for the apparent difference in cell death-inducing strength of BcSSP and SsSSP, and the lack of cell death induced by CcSSPs tested.

I also suggest that these tagged proteins be tested in the host plant *Camellia* 'Nicky Crisp'. This could be done by first performing apoplastic washes with *N. benthamiana* leaves infiltrated with recombinant *A. tumefaciens* carrying the tagged chimeric region-swapped genes, then infiltrating the apoplastic fluid into *Camellia* 'Nicky Crisp' petals to test for cell death-inducing activity. *C. camelliae* grows exclusively in the apoplastic space for a significant period of the infection period, and CCL-SSPs are putative apoplastic proteins (Denton-Giles, 2014), therefore they should be present in the apoplastic fluid.

It would also be advantageous to know the mechanisms of how SsSSP induces cell death. From previous reports of the activity of effector proteins, it is likely that SsSSP interacts with another protein to induce cell death, such as a plant receptor. Therefore, I suggest performing co-immunoprecipitation assays with SsSSP and BcSSP to determine what proteins they interact with.

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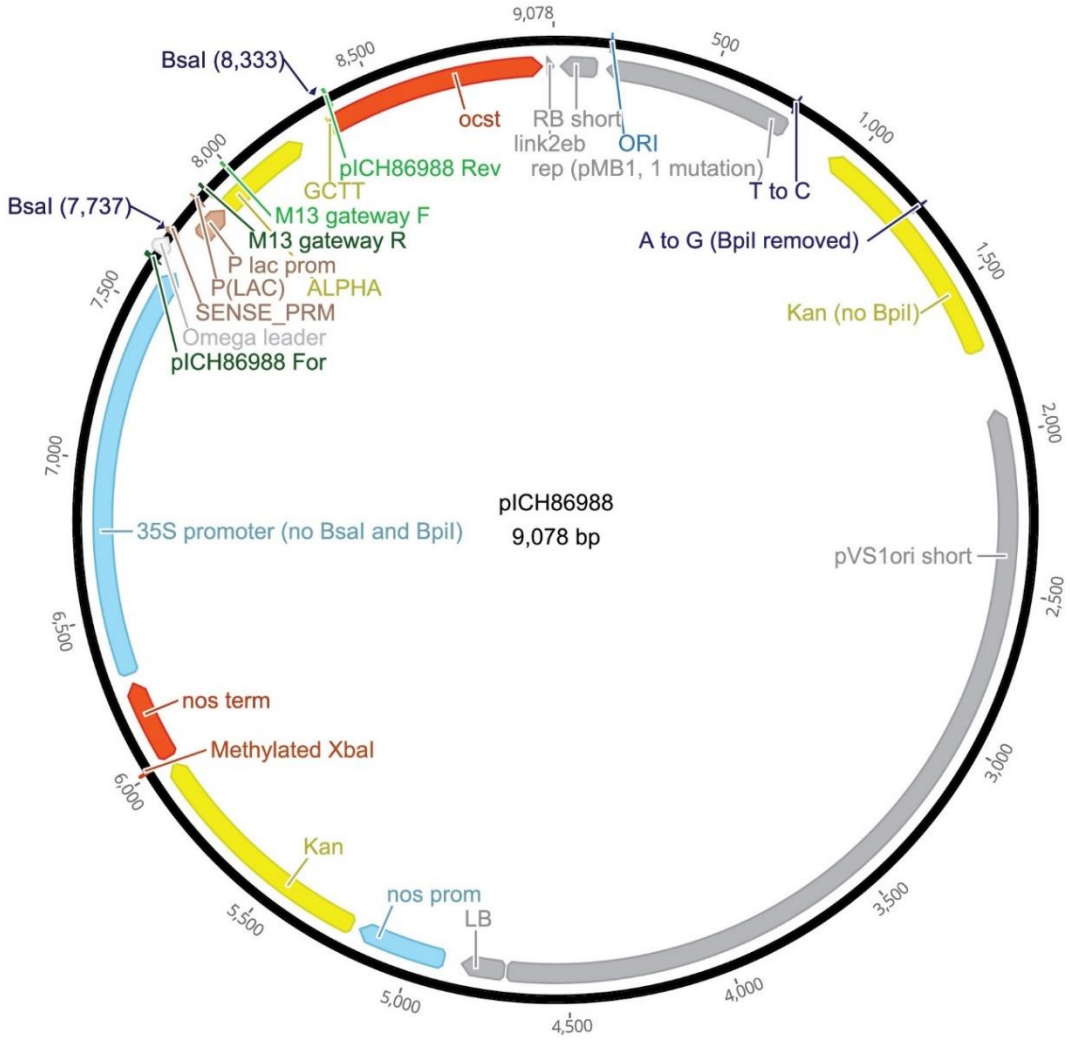
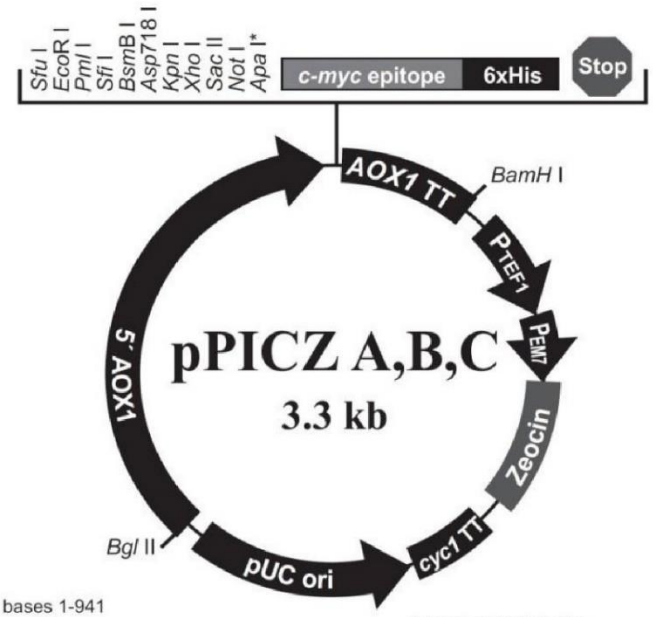
7 Appendices

Appendix 7.1. pICH86988 vector map



Appendix 7.2. pPICZA vector map



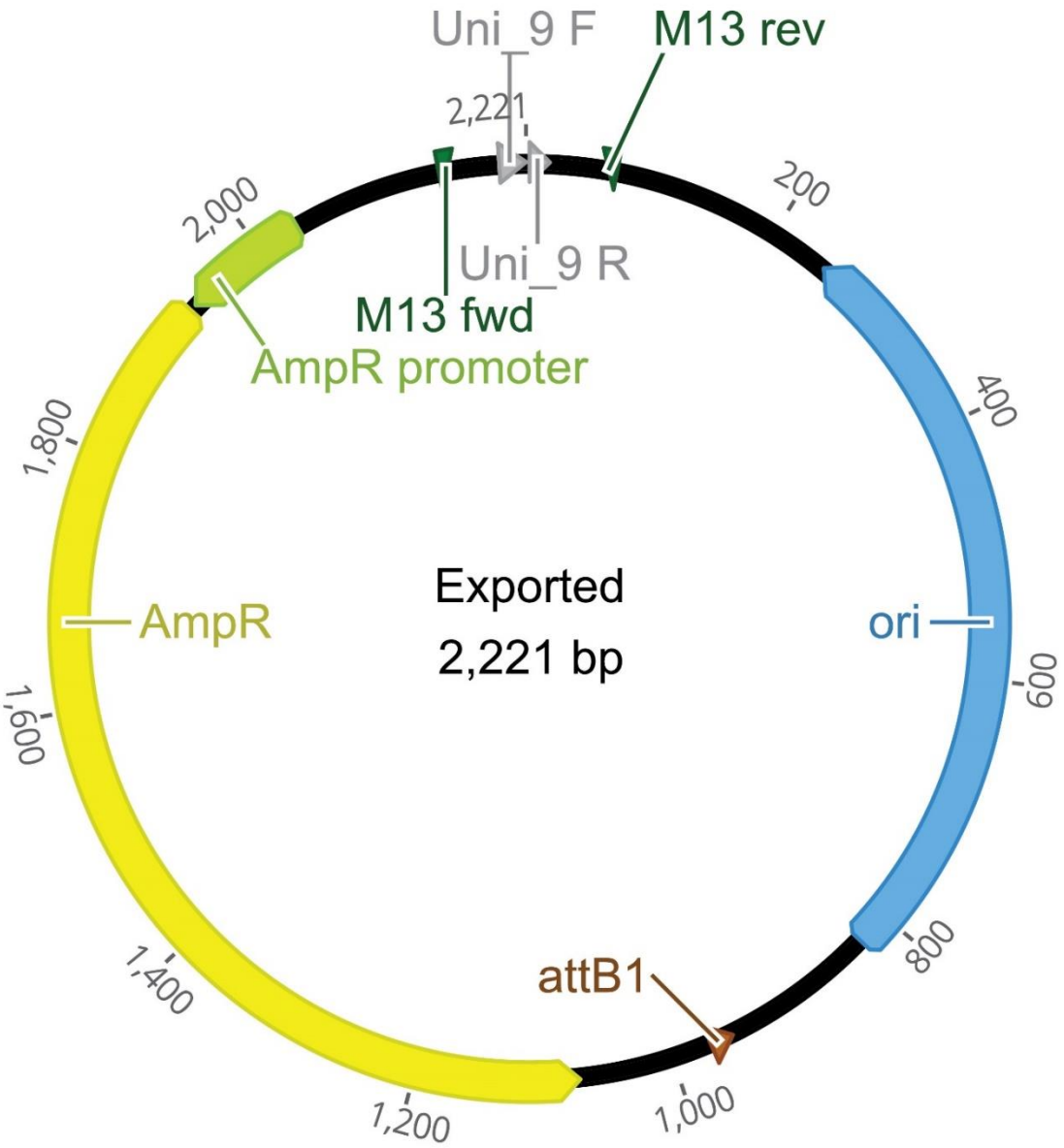
Comments for pPICZ A:
3329 nucleotides

5´ *AOX1* promoter region: bases 1-941
5´ end of *AOX1* mRNA: base 824
5´ *AOX1* priming site: bases 855-875
Multiple cloning site: bases 932-1011
c-myc epitope tag: bases 1012-1044
Polyhistidine tag: bases 1057-1077
3´ AOX priming site: bases 1159-1179
3´ end of mRNA: base 1250
AOX1 transcription termination region: bases 1078-1418
Fragment containing TEF1 promoter: bases 1419-1830
EM7 promoter: bases 1831-1898
Sh ble ORF: bases 1899-2273
CYC1 transcription termination region: bases 2274-2591
pUC origin: bases 2602-3275 (complementary strand)

* The restriction site between *Not* I and the *myc* epitope is different in each version of pPICZ:

Apa I in pPICZ A
Xba I in pPICZ B
*Sna*B I in pPICZ C

Appendix 7.3. pTwist Amp High Copy vector map



Appendix 7.4. Nucleotide coding sequences of genes used in this study and the appropriate plasmid used for expression

Plasmid	Gene name	Sequence
pPICZA	*CcSSP37*	ATGGTTAAGTTTATCTCTCTTGCTCTTTTCGTCATCGCA TCTGCCGTCGCCGTTCAGGCTTGTACCTTCTGTCAGTGT GAGTTTTCAGATGGTGCCCATTGTTGCGTTTACTCTTCC ACAGCATTCGATCAAATTAACTGTAAGGACGTCTGCTC TAAAGCTCACAGAGCCGACGGTAAATCCTACGGTACC GGTGCTCCAGGAACTGCATGTTCAGCTGGTGGAAAATA TCAATGCACTAACGCCTGGACAAGTTTGGATAGAACTC CTTGTTACAGAATGGACGAACCATTCTCACCTAATAGT CCACAGCCT
	BcSSP	ATGGTCAGAATCTCCTCTATCGTTATGTTTTTCCTTGCC AGTGCCCTTTCCGTCCAGGCTTGTACCTATTGCCAGTGT GAGTTCTCTAACGGAGATCATTGTTGCGTTTACTCCGA CGCCGAAATTGGTAACTTGGATTGTCCAACCTATTGCG CCAATGCACACAGAGCAGACGGAGCTGCCGGTGGAGG TACTGCTTGTGCAGCTGGTGGTAAATACAAATGCGCTT CAGCCTTTACTGCTCTTGATAGAACACCTTGTTATAAG CAA
	SsSSP	ATGGTCAGAATCGCATCTATCGCATTGTTCTTTATCGC ATCCGCTCTTTCCGTTCAAGCCTGCACCTATTGTCAGTG TGAGTTTTCTAACGGTGGACATTGTTGCGTTTACTCCG ATGCTGAAATCGGTAACTTGGACTGTAATAAGTACTGC GCAAACGCTCACAGAGCTGATGGTACTAATGGAGTCG GTGGACCAGGAACAGCCTGTGCTGCCGGTGGAGCCTA CAAGTGCGCAACCATCCCAGAGTCACTTGACAGAACTC CTTGTTATAAACAA
	CcSSP37 – Exon 2	GAATTCACTATGGTTAAGTTTATCTCTCTTGCTCTTTTC GTCATCGCATCTGCCGTCGCCGTTCAGGCTTGTACCTTC TGTCAGTGTGAGTTTTCAGATGGTGCCCATTGTTGCGTT TACTCTGATGCTGAAATCGGTAACTTGGACTGTAATAA GTACTGCGCAAACGCTCACAGAGCTGATGGTACTAATG GAGTCGGTGGACCAGGAACAGCCTGTGCTGCCGGTGG AGCCTACAAGTGCGCAACCATCCCAGAGTCACTTGACA GAACTCCTTGTTATAAACAATAATTGGTCCCTAGAGGA TCTGGGCCC
	CcSSP37 – Mid 1	GAATTCACTATGGTTAAGTTTATCTCTCTTGCTCTTTTC GTCATCGCATCTGCCGTCGCCGTTCAGGCTTGTACCTTC TGTCAGTGTGAGTTTTCAGATGGTGCCCATTGTTGCGTT TACTCTGATGCTGAAATCGGTAACTTGGACTGTAATAA GTACTGCGCAAACGCTCACAGAGCCGACGGTAAATCC TACGGTACCGGTGCTCCAGGAACTGCATGTTCAGCTGG TGGAAAATATCAATGCACTAACGCCTGGACAAGTTTGG ATAGAACTCCTTGTTACAGAATGGACGAACCATTCTCA

		CCTAATAGTCCACAGCCTTAATTGGTCCCTAGAGGATC TGGGCCC
	CcSSP37 – Mid 2	GAATTCACTATGGTTAAGTTTATCTCTCTTGCTCTTTTC GTCATCGCATCTGCCGTCGCCGTTCAGGCTTGTACCTTC TGTCAGTGTGAGTTTTCAGATGGTGCCCATTGTTGCGTT TACTCTTCCACAGCATTCGATCAAATTAACTGTAAGGA CGTCTGCTCTAAAGCTCACAGAGCCGACGGTACTAATG GAGTCGGTGGACCAGGAACAGCCTGTGCTGCCGGTGG AGCCTACAAGTGCACTAACGCCTGGACAAGTTTGGATA GAACTCCTTGTTACAGAATGGACGAACCATTCTCACCT AATAGTCCACAGCCTTAATTGGTCCCTAGAGGATCTGG GCCC
	CcSSP37 – C term	GAATTCACTATGGTTAAGTTTATCTCTCTTGCTCTTTTC GTCATCGCATCTGCCGTCGCCGTTCAGGCTTGTACCTTC TGTCAGTGTGAGTTTTCAGATGGTGCCCATTGTTGCGTT TACTCTTCCACAGCATTCGATCAAATTAACTGTAAGGA CGTCTGCTCTAAAGCTCACAGAGCCGACGGTAAATCCT ACGGTACCGGTGCTCCAGGAACTGCATGTTCAGCTGGT GGAAAATATCAATGCGCAACCATCCCAGAGTCACTTG ACAGAACTCCTTGTTATAAACAATAATTGGTCCCTAGA GGATCTGGGCCC
	SsSSP – Exon 2	GAATTCACTATGGTCAGAATCGCATCTATCGCATTGTT CTTTATCGCATCCGCTCTTTCCGTTCAAGCCTGCACCTA TTGTCAGTGTGAGTTTTCTAACGGTGGACATTGTTGCG TTTACTCCTCCACAGCATTCGATCAAATTAACTGTAAG GACGTCTGCTCTAAAGCTCACAGAGCCGACGGTAAATC CTACGGTACCGGTGCTCCAGGAACTGCATGTTCAGCTG GTGGAAAATATCAATGCACTAACGCCTGGACAAGTTTG GATAGAACTCCTTGTTACAGAATGGACGAACCATTCTC ACCTAATAGTCCACAGCCTTAATTGGTCCCTAGAGGAT CTGGGCCC
	SsSSP – Mid 1	GAATTCACTATGGTCAGAATCGCATCTATCGCATTGTT CTTTATCGCATCCGCTCTTTCCGTTCAAGCCTGCACCTA TTGTCAGTGTGAGTTTTCTAACGGTGGACATTGTTGCG TTTACTCCTCCACAGCATTCGATCAAATTAACTGTAAG GACGTCTGCTCTAAAGCTCACAGAGCTGATGGTACTAA TGGAGTCGGTGGACCAGGAACAGCCTGTGCTGCCGGT GGAGCCTACAAGTGCGCAACCATCCCAGAGTCACTTG ACAGAACTCCTTGTTATAAACAATAATTGGTCCCTAGA GGATCTGGGCCC
	SsSSP – Mid 2	GAATTCACTATGGTCAGAATCGCATCTATCGCATTGTT CTTTATCGCATCCGCTCTTTCCGTTCAAGCCTGCACCTA TTGTCAGTGTGAGTTTTCTAACGGTGGACATTGTTGCG TTTACTCCGATGCTGAAATCGGTAACTTGGACTGTAAT AAGTACTGCGCAAACGCTCACAGAGCTGATGGTAAAT CCTACGGTACCGGTGCTCCAGGAACTGCATGTTCAGCT GGTGGAAAATATCAATGCGCAACCATCCCAGAGTCAC

		TTGACAGAACTCCTTGTTATAAACAATAATTGGTCCCT AGAGGATCTGGGCCC
	SsSSP – C term	GAATTCACTATGGTCAGAATCGCATCTATCGCATTGTT CTTTATCGCATCCGCTCTTTCCGTTCAAGCCTGCACCTA TTGTCAGTGTGAGTTTTCTAACGGTGGACATTGTTGCG TTTACTCCGATGCTGAAATCGGTAACTTGGACTGTAAT AAGTACTGCGCAAACGCTCACAGAGCTGATGGTACTA ATGGAGTCGGTGGACCAGGAACAGCCTGTGCTGCCGG TGGAGCCTACAAGTGCACTAACGCCTGGACAAGTTTGG ATAGAACTCCTTGTTACAGAATGGACGAACCATTCTCA CCTAATAGTCCACAGCCTTAATTGGTCCCTAGAGGATC TGGGCCC
pTwist Amp High Copy (Entry plasmid) pICH86988 (destinat-ion plasmid)	*CcSSP37 - T*	GGTCTCGAATGGGATTTGTTCTCTTTTCACAATTGCCTT CATTTCTTCTTGTCTCTACACTTCTCTTATTCCTAGTAAT ATCCCACTCTTGCCGTGCCCAAAAT**GACTACAAGGAC CACGACGGTGACTACAAGGACCACGACATCGACTA CAAGGACGACGACGACAAG**TGTACCTTCTGTCAGTGT GAGTTTTCAGATGGTGCCCATTGTTGCGTTTACTCTTCC ACAGCATTCGATCAAATTAACTGTAAGGACGTCTGCTC TAAAGCTCACAGAGCCGACGGTAAATCCTACGGTACC GGTGCTCCAGGAACTGCATGTTCAGCTGGTGGAAAATA TCAATGCACTAACGCCTGGACAAGTTTGGATAGAACTC CTTGTTACAGAATGGACGAACCATTCTCACCTAATAGT CCACAGCCTTAGGCTTTGAGACC
	SsSSP - T	GGTCTCGAATGGGATTTGTTCTCTTTTCACAATTGCCTT CATTTCTTCTTGTCTCTACACTTCTCTTATTCCTAGTAAT ATCCCACTCTTGCCGTGCCCAAAAT**GACTACAAGGAC CACGACGGTGACTACAAGGACCACGACATCGACTA CAAGGACGACGACGACAAG**TGCACCTATTGTCAGTGT GAGTTTTCTAACGGTGGACATTGTTGCGTTTACTCCGA TGCTGAAATCGGTAACTTGGACTGTAATAAGTACTGCG CAAACGCTCACAGAGCTGATGGTACTAATGGAGTCGG TGGACCAGGAACAGCCTGTGCTGCCGGTGGAGCCTAC AAGTGCGCAACCATCCCAGAGTCACTTGACAGAACTCC TTGTTATAAACAATAGGCTTTGAGACC
	SsSSP	GGTCTCGAATGGGATTTGTTCTCTTTTCACAATTGCCTT CATTTCTTCTTGTCTCTACACTTCTCTTATTCCTAGTAAT ATCCCACTCTTGCCGTGCCCAAAATTGCACCTATTGTC AGTGTGAGTTTTCTAACGGTGGACATTGTTGCGTTTAC TCCGATGCTGAAATCGGTAACTTGGACTGTAATAAGTA CTGCGCAAACGCTCACAGAGCTGATGGTACTAATGGA GTCGGTGGACCAGGAACAGCCTGTGCTGCCGGTGGAG CCTACAAGTGCGCAACCATCCCAGAGTCACTTGACAGA ACTCCTTGTTATAAACAATAGGCTTTGAGACC
	CcSSP37 – Mid 1 – C term - T	GGTCTCGAATGGGATTTGTTCTCTTTTCACAATTGCCTT CATTTCTTCTTGTCTCTACACTTCTCTTATTCCTAGTAAT ATCCCACTCTTGCCGTGCCCAAAAT**GACTACAAGGAC CACGACGGTGACTACAAGGACCACGACATCGACTA**

		CAAGGACGACGACGACAAGTGTACCTTCTGTCAGTGT GAGTTTTCAGATGGTGCCCATTGTTGCGTTTACTCTGAT GCTGAAATCGGTAACTTGGACTGTAATAAGTACTGCGC AAACGCTCACAGAGCCGACGGTAAATCCTACGGTACC GGTGCTCCAGGAACTGCATGTTCAGCTGGTGGAAAATA TCAATGCGCAACCATCCCAGAGTCACTTGACAGAACTC CTTGTTATAAACAATAGGCTTTGAGACC
	SsSSP – Mid 1 – C term - T	GGTCTCGAATGGGATTTGTTCTCTTTTCACAATTGCCTT CATTTCTTCTTGTCTCTACACTTCTCTTATTCCTAGTAAT ATCCCACTCTTGCCGTGCCCAAAAT**GACTACAAGGAC** **CACGACGGTGACTACAAGGACCACGACATCGACTA** **CAAGGACGACGACGACAAG**TGCACCTATTGTCAGTGT GAGTTTTCTAACGGTGGACATTGTTGCGTTTACTCCTCC ACAGCATTCGATCAAATTAACTGTAAGGACGTCTGCTC TAAAGCTCACAGAGCTGATGGTACTAATGGAGTCGGT GGACCAGGAACAGCCTGTGCTGCCGGTGGAGCCTACA AGTGCACTAACGCCTGGACAAGTTTGGATAGAACTCCT TGTTACAGAATGGACGAACCATTCTCACCTAATAGTCC ACAGCCTTAGGCTTTGAGACC
	INF1 - T	ATGGGATTTGTTCTCTTTTCACAATTGCCTTCATTTCTT CTTGTCTCTACACTTCTCTTATTCCTAGTAATATCCCAC TCTTGCCGTGCCCAAAAT**GACTACAAGGACCACGACG** **GTGACTACAAGGACCACGACATCGACTACAAGGAC** **GACGACGACAAG**ACGGCCTGCACCTCCACCCAGCAAA CCGCTGCCTACGTCGCCCTCGTGAGCATCCTGTCGGAC GCGTCCTTCAACCAATGCGCGACGGACTCGGGCTACTC GATGCTGACGGCCACGGCGCTGCCCACGACGGACCAA TACAAGAAGATGTGCGCGTCGACGGCCTGCAACACCA TGATCACCAAGATCGTGTCGCTCAACCCGCCCGACTGC GACCTGACGGTGCCCACGAGCGGCCTGGTGCTCAACGT GTACTCGTACGCGAACGGCTTCTCGGCCAAGTGCGTGT CGCCGTAA
	14801 - T	ATGGGATTTGTTCTCTTTTCACAATTGCCTTCATTTCTT CTTGTCTCTACACTTCTCTTATTCCTAGTAATATCCCAC TCTTGCCGTGCCCAAAAT**GACTACAAGGACCACGACG** **GTGACTACAAGGACCACGACATCGACTACAAGGAC** **GACGACGACAAG**GCACCACTCCTTCCTCGCGATGCAT CAGCTGCCGACTCATTAACCTACTGCGCCGCCATCTCG GACACGATTTGCTCTTCGACCTCTGATCTCGCCTCAAC CGCCGTCGTCACATTGCCCGCTGGTTGGTACGGACATT TCGAGTGCTATGCTACTGTCGATGGAATAGACCGCTAC TTCGCAGGCCATCCGCCCATCGCCCCGGGATCCGGTCA GAGTCAGGTTTGCTGGGCGACTGCTTCTGCGCTCGGCC CTGCGCAGGGAGGAACAGGGAGACAGGCGTGCCTTAA ATCTCAACTAGCCAACTGCTGCGAGATGACGCATCTAA GCATCCAAGGCCTCCAGTGCCCAGCTCCGACCAACAA AGTTGCTGGTCTGGCCGCCGATACAACGTACACCTCCT GA

Underlined – Signal peptide. When in pPICZA plasmid it is the *P. pastoris* signal peptide, and when in pTwist Amp High Copy or pICH86988 it is PR1aSP.
Bold – 3 x FLAG tag.

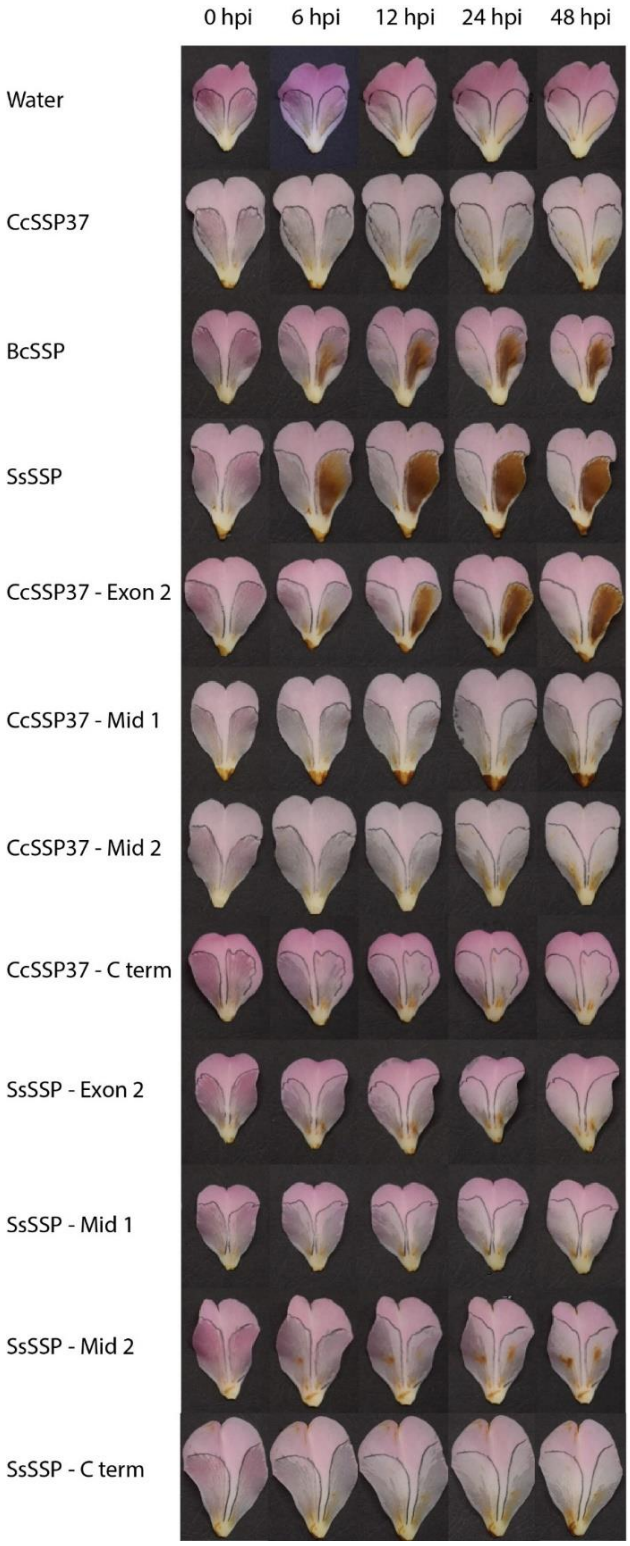
Appendix 7.5. Primer sequences and the expected PCR product sizes from amplification in each gene used in this study

Plasmid for use with	Primer	Primer sequence	PCR product size (bp)
pPICZA	AOX 5'	GACTGGTTCCAATTGACAAGC	Empty vector = 324
	AOX 3'	GCAAATGGCATTCTGACATCC	SsSSP = 558
			BcSSP = 549
			CcSSP37 = 594
			CcSSP37 - Exon 2 = 576
			CcSSP37 - Mid 1 = 612
			CcSSP37 - Mid 2 = 609
			CcSSP37 - C term = 579
			SsSSP - Exon 2 = 612
			SsSSP - Mid 1 = 576
			SsSSP - Mid 2 = 579
			SsSSP - C term = 609
pPICZA (3' gene specific)	AOX 5'	GACTGGTTCCAATTGACAAGC	
	Con1 - 3'	TCCTCTAGGGACCAATTATTGT	CcSSP37 - Exon 2 = 394

	Con2 - 3'	TCCTCTAGGGACCAATTATTGT	CcSSP37 - Mid 1 = 220
	Con3 - 3'	TTCAGCATCAGAGTAAACGC	CcSSP37 - Mid 2 = 338
	Con4 - 3'	CGTTAGTGCACTTGTAGGC	CcSSP37 - C term = 397
	Con5 - 3'	TAGGGACCAATTAAGGCTGT	SsSSP - Exon 2 = 425
	Con6 - 3'	GGAGGAGTAAACGCAACAAT	SsSSP - Mid 1 = 214
	Con7 - 3'	CGTAGGATTTACCATCAGCTC	SsSSP - Mid 2 = 284
	Con8 - 3'	TAGGGACCAATTAAGGCTGT	SsSSP - C term = 422
pTwist Amp High Copy	M13 - 5'	CAGGAAACAGCTATGAC	Empty vector = 103
	M13 - 3'	GTAAAACGACGGCCAG	SsSSP = 479
			SsSSP - T = 545
			CcSSP37 - T = 581
			CcSSP37 - Mid 1 - C term - T = 548
			SsSSP - Mid 1 - C term - T = 578
Any plasmid (gene specific)	PR1aSP - 5'	TAATATCCCACTCTTGCCGT	
	SsSSP - 3'	AGTTACCGATTTCAGCATCG	SsSSP = 102
	SsSSP - 3'	AGTTACCGATTTCAGCATCG	SsSSP - T= 168
	CcSSP37 - 3'	CTGTGGAAGAGTAAACGCAA	CcSSP37 - T = 156
	Con9 - 3'	CCAAGTTACCGATTTCAGCA	CcSSP37 - Mid 1 - C term - T = 171

	Con10 - 3'	AGCAGACGTCCTTACAGTTA	SsSSP - Mid 1 - C term - T = 189
pICH86988	PICH F'	AGGACACGCTCGAGTATAAG	Empty vector = 737
	PICH R'	CATGCGATCATAGGCTTCTC	SsSSP = 479
			SsSSP - T = 545
			CcSSP37 - T = 581
			CcSSP37 - Mid 1 - C term - T = 548
			SsSSP - Mid 1 - C term - T = 578

Appendix 7.6. Camellia 'Nicky Crisp' petals infiltrated with chimeric region swapped and CCL-SSP proteins recombinantly expressed in *P. pastoris* from 0 hpi to 48 hpi.



The left half of each petal was infiltrated with empty vector negative control and the right half was infiltrated with the treatment (labelled on the left side of the figure). Images are representative (n = 9).