Characterization of transgenic grapevine ectopically expressing plant defensin peptides

by

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Declaration

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Summary

Grapevine (*Vitis vinifera* L.) is one of the most important and widely grown food crops in the world. The cultivation and commercial production of this crop has, however, became highly dependent on the use of pesticides. One of the strategies to limit the use of chemicals is to harness the species natural defence mechanisms. This strategy requires the understanding of these plant defence mechanisms. Among the highly specialized defence mechanism of plants is the production of specific antimicrobial peptides, called plant defensins. These peptides are small, basic, positively charged and cysteine-rich with a potent broad range of antimicrobial activity. The plant defensins form a vital part of the innate immune system of plants and are widely distributed throughout the plant kingdom. Several plant defensins have been isolated and predominantly characterized for their *in vitro* antifungal activity. However, other biological activities, such as heavy metal tolerance, ion channel blocking, α-amylase and protease inhibition and modulators of growth and development have also been attributed to these peptides. Some well-studied defensins have been described in literature in terms of their three-dimentional structure, antimicrobial *in vitro* functions/activities, their mode(s) of action, as well as their applications. Limited information, however, is available on their broader potential impacts on plant growth and development and more specific, non-defence related stress-mitigating functions within their host plants. Furthermore, little information exists on grapevine plant defensins. Although 79 defensin-like genes (DEFL) have been identified in the reference genome, only four grapevine plant defensins have been isolated and characterised to date for potential antifungal activities.

The goal of this study was therefore to evaluate potential *in planta/in vivo* functions of plant defensins in grapevine. In this study functional characterisation studies were performed on plant defensins overexpressed in grapevine. Genotypical screens were conducted on uncharacterised transgenic populations of two *V. vinifera* cultivars (Sultana and Red Globe), overexpressing three different defensin peptides (*Heliophilia coronopifolia* antifungal peptides 1 and 4 (Hc-AFP1, Hc-AFP4) and *Raphanus sativus* antifungal peptide 2 (Rs-AFP2)) to assess transgene integration and expression. These analyses revealed unique transgenic lines for the transgenic populations expressing the plant defensins Hc-AFP1 and Rs-AFP2, with the majority of these lines expressing the transgene. Although the presence of the transgene was confirmed for the transgenic *V. vinifera* (cv. Sultana) Hc-AFP4 and *V. vinifera* (cv. Red Globe) Hc-AFP4 lines, they did not exhibit any transgene expression and was not included in the growth, or biotic and abiotic stress phenotypical analyses. A previously characterised population of Vvi-AMP1 overexpressed in Sultana was also included in this study.

Two *in silico* approaches were used to contextualise the functional characterisation studies. The first was to compare the different peptides in terms of their sequence similarities, as well as deduced structural features. The Rs-AFP2 peptide's crystal structure has been resolved and the structure-function *in silico* analyses made use of the data available for this peptide. The Hc-AFP1, Hc-AFP4 and Rs-AFP2 peptides showed more similarities in sequence and structure compared to the Vvi-AMP1 defensin peptide. The majority of the sequence and structural differences between Hc-AFP1, Hc-AFP4 and Rs-AFP2 and the Vvi-AMP1 peptide resided in the conserved λ-core motif that is known to be important in determining the antifungal activities of plant defensin peptides.

The second *in silico* approach was to use existing gene expression data in grapevine to evaluate where (in which organs and tissues) and under which (stressful) conditions grapevine defensin genes show differential expression patterns. Using the Corvina gene atlas, it was shown that under

normal non-stressed conditions, DEFL genes were expressed in all grapevine organs and tissues at various developmental stages. Furthermore, using available microarray data, it was found that some defensins responded to biotic stress such as *B. cinerea* infection, although the Vvi-AMPs did not respond. Similarly, strong upregulation was found in response to *Planococcus ficus* (mealybug) infestation and in response to abiotic stress such as locally applied heat stress and leaf dehydration, with Vvi-AMP1 showing the strongest upregulation to the latter.

Guided by the results of the *in silico* gene expression analysis, the *in vivo* functions of plant defensin peptides in grapevine were evaluated by analysis of the transgenic grapevines, overexpressing plant defensin peptides Hc-AFP1, Rs-AFP2 and Vvi-AMP1 in terms of growth, as well as biotic and abiotic stress. From these phenotypical observations it was evident that genotypical background (Sultana versus Red Globe) had a strong effect on several of the phenotypes observed. Furthermore, some of the observed phenotypes were peptide-specific, whereas in most other instances all peptides caused the same type of response to a particular stress, but with varying strength of the response, or some differences in mechanism. In terms of growth, the transgenic populations only showed mild phenotypes and no overt stunting or abnormalities were observed. Some plant lines, however, showed slower growth and root inhibition *in vivo* and these observed growth alterations were possibly a result of higher metabolic load on the plants due to the overexpression of the peptides, an aspect that deserves further study.

The transgenic populations were evaluated for their *in vivo* functions towards biotic stress through evaluating their defence phenotypes against two fungal pathogens, namely the necrotrophic fungus, *Botrytis cinerea* and the biotrophic fungus *Erysiphe necator,* as well as the insect pest, *Planococcus ficus* (mealybug). None of the transgenic plant lines displayed a resistant defence phenotype towards *B. cinerea*, whereas all the transgenic lines showed enhanced resistance towards the biotrophic powdery mildew fungus through an increased penetration resistance mechanism. Some plant lines also displayed programmed cell death (PCD) associated resistance, especially the transgenic plants that contained the Rs-AFP2 construct. PCD is associated with the mechanism of action of the Rs-AFP2 peptide. All tested plant lines also showed promising results towards the soft scale insect *P. ficus,* all reducing the infestation significantly, making this the first report of an *in planta* anti-insect activity of plant defensins Hc-AFP1, Rs-AFP2 and Vvi-AMP1.

The transgenic populations were also evaluated for their *in vivo* functions towards abiotic stress by subjecting plants to an active drying experiment and evaluating their intrinsic water use efficiencies (WUE). The majority of the plant lines all demonstrated an increase in intrinsic WUE, but the Red Globe Rs-AFP2 plant line showed a decrease in intrinsic WUE, which can at least partly possibly be linked to the reduced growth parameters demonstrated for this plant line, specifically the reduction in root growth.

In conclusion, this study contributed to the current understanding of how plant defensins function *in vivo,* confirming growth impacts, antifungal activities, anti-insect activity and a role in water stress management. Furthermore, we gained a vital insight into the *in vivo* functions of grapevine plant defensins.

This thesis is dedicated to
Brad, my brother and parents

Biographical sketch

Helmien Barkhuizen was born in Bellville, South Africa, on 2 June 1988. She matriculated from Bellville High in 2006. In 2007 Helmien enrolled for a BSc-degree in Molecular Biology and Biotechnology at Stellenbosch University, which she obtained in 2009. In 2010 she received a HonsBSc-degree in Biochemistry after which she graduated with an MSc (*cum laude*) in Wine Biotechnology at the Institute of Wine Biotechnology at Stellenbosch University in 2013.

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Preface

This thesis is presented as a compilation of chapters. Each chapter is introduced separately and is written according to the style of the journal of Plant Physiology.

Chapter 1	**General Introduction and project aims**
Chapter 2	**Literature review** Plant defensin peptides and their role in defence and development.
Chapter 3	**Research results** *In silico* comparison of the sequence similarities and structural features of plant defensins Hc-AFP1, Hc-AFP4, Vvi-AMP1 and Rs-AFP2.
Chapter 4	**Research results** Genetic, phenotypic and physiological characterization of seven transgenic grapevine ectopically expressing plant defensin peptides.
Chapter 5	**Research results** Challenging of defensin expressing transgenic grapevine populations and their controls with *Botrytis cinerea, Eryshipe necator* and *Plannococcus ficus* to describe and characterize potential defense phenotypes.
Chapter 6	**Research results** Phenotyping of transgenic grapevine ectopically expressing Vvi-AMP1, Hc-AFP1 and Rs-AFP2 plant defensin peptides for their response to drought stress
Chapter 7	**General discussion and conclusion**

Table Contents

List of Abbreviations and Acronyms

Ace-AMP1	*Allium cepa* antimicrobial peptide 1
Ah-AMP1	*Asteraceae. hippocastanum* antifungal peptide 1
Ah-PDF1.b	*Arabidopsis halleri* plant defensin 1.1b
At-PDF1.1	*Arabidopsis thaliana* plant defensin 1.1
At-PDF2.3	*Arabidopsis thaliana* plant defensin 2.3
BhDef1	*Brassica hybrid* defensin 1
BhDef2	*Brassica hybrid* defensin 2
CADEF1	*Capsicum annuum* defensin 1
CAL1	Cadmium accumulation in leaf 1
CaMV	Cauliflower mosaic virus
CaMca1p	*Candida albicans* metacaspase 1
CSαβ	Cysteine stabilized αβ motif
Ct-AMP1	*Clitoria. ternatea* antimicrobial peptide 1
CWI	Cell wall integrity
DEF2	Defensin 2
DEFL	Defensin like
Dm-AMP1	*Dahila merckii* antimicrobial peptide 1
EGAD1	*Elaeis guinesis* abnormality defensin 1
ETI	Effector triggered immunity
GAST1	Gibberellic Acid-Stimulated Transcript 1
GlcCer	Glucosylceramide
GLRaV3	Grapevine leaf-roll associated virus 3
GPL	Glycosyl phosphatidyl inositol
Ha-Def1	*Helianthus annuus* defensin 1
Hc-AFP1	*Heliophilia coronopifolia* antifungal peptide 1
Hc-AFP4	*Heliophilia coronopifolia* antifungal peptide 4
HOG1	High osmolality glygerol 1
Hs-AFP1	*Heuchera sanguinea* antifungal peptide 1
Hs-AFP1	*Heuchera sanguinea* antifungal peptide 2
IPC	Inositol-p-ceramide
LRR	Leucine rich repeat
MALDI-TOF	Matrix-assisted laser depolarization/ionization time of flight mass spectrometry
MAPK	Mitogen activated protein kinase
MICP	Mannose-inositol-P-ceramide
$M(IP)_2C$	Mannose (inositol-P)$_2$-ceramide

MRSA	Methicillin-resistant *Staphylococcus aureus*
MtDef4	*Medicgo truncatula* defensin 4
MtDef5	*Medicgo truncatula* defensin 5
MS	Mass spectrometry
MsDef1	*Medicago sativa* defensin 1
NaD1	*Nicotiana alata* defensin 1
NaD2	*Nicotiana alata* defensin 2
NB	Nucleotide binding
NBS	Nucleotide binding site
NMR	Nuclear resonance magnetic spectroscopy
PA	Phosphatidic acid
PAMP	Pathogen associated molecular patterns
PAR	Photosynthetic active radiations (PAR)
Pcc	*Pectobacterium carotovorum* subsp. *Carotovorum*
PCD	Programmed cell death
PDB	Protein data bank
PDF1	Plant defensin type 1
PhD1	*Petunia hybrida* defensin 1
PhD2	*Petunia hybrida* defensin 2
PI	Phosphatidylinositol
PIP	Phosphatidylinositol phosphate
PIP_2	Phosphatidylinositol bisphosphate
PIP_3	Phosphatidylinositol triphosphate
PL	Phospholipid
Psd1	*Pisum sativum* defensin 1
PsDef1	*Pinus sylvestris* plant defensin 1
PTI	PAMP-triggered immunity
REN4	Resistance to *Erysiphe necator* 4
ROS	Reactive oxygen species
RPW8.1	Resistance to Powdery mildew 8.1
Rs-AFP1	*Raphanus sativus* antifungal peptide 1
Rs-AFP2	*Raphanus sativus* antifungal peptide 2
Rs-AFP2	*Raphanus sativus* antifungal peptide 3
Rs-AFP2	*Raphanus sativus* antifungal peptide 4
RUN1	Resistance to *Uncinula necator* 1
StSN2	*Solanum tuberosum* snakin-2
TAD1	*Triticum aestivum* defensin 1
Tfgd2	*Trigonella foenum-graecum* defensin 2

TPP3	Tomato pistil predominant 3 defensin
VaD1	*Vigna angularis* defensin 1
VrD1	*Vigna radiate* defensin 1
VuD1	*Vigna unguiculata* defensin 1
Vvi-AMP1	*Vitis vinifera* antimicrobial peptide 1
Vvi-AMP2	*Vitis vinifera* antimicrobial peptide 2
Vvi-AMP3	*Vitis vinifera* antimicrobial peptide 3
Vvi-AMP4	*Vitis vinifera* antimicrobial peptide 4
Vvi-PEN1	*Vitis vinifera* penetration 1
WT	Wild type
WUE	Water use efficiency
ZmES4	*Zea mays* embryo sac 4

Chapter 1

INTRODUCTION AND PROJECT AIMS

GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Plants are constantly challenged by invading pathogens and pests (Terras et al. 1995; Broekaert et al. 1995). Diseases in plants lead to major production and economic losses in agricultural industries worldwide. This poses a major threat to food security worldwide. Therefore, the protection of crops against plant diseases plays an important role in the growing demand for food quality and quantity (Savary et al. 2012).

Grapevine is one of the most important food crops worldwide. The international organization of vine and wine (OIV) 2016 statistical report estimated that grapevine is cultivated on over 7.5 million hectares of land worldwide. This includes the cultivation for fresh grapes, dried grapes, wine, juices and musts (OIV 2017). The majority of vines used for the production of these products are mostly *Vitis vinifera* L. cultivars, originating from the *Eurasian* grape species. Due to centuries of domestication, human selection for production and quality traits, as well as vegetative propagation of the plant materials, the modern cultivars used for commercial production have become highly susceptible to pathogens and pests. Grape growing therefore requires the frequent use of preventative spaying programs (Qiu et al. 2015). This not only increases the production costs of the crop, but it poses health threats to the producers, the consumer and the broader environment (Wilson and Tisdell 2000; Aktar et al. 2009; Carvalho 2017).

Modern and sustainable viticulture aims to limit the use of chemicals by using alternative strategies. One of these strategies includes the conventional breeding for resistance. The introductions of resistance genes form North American and wild Chinese *Vitis* species to produce interspecific “French-American” and “Chinese-French” hybrids respectively have been explored. Despite the significant economic and environmental benefits of this strategy, commercial adoption has been limited due to possible unwanted recessive traits and the perceived reduction in wine quality (Qiu et al. 2015) and the loss of the marketable varietal names. Selecting for quantitative traits controlling both resistance and fruit quantity and quality is a very difficult task (Donald et al. 2002). Although advances in genotyping have supported more efficient marker-based breeding programs, significant challenges still remain. Moreover, marker-based breeding typically generate large populations of plants that need phenotyping in addition to genotyping; the screening of a large amount of plants requires high cost infrastructure, specifically for high throughput phenotyping.

An important aspect of identifying potential resistance genes/mechanisms against pathogens and pests is the understanding of the natural defence mechanisms of the species. If putative resistance

genes are identified, functional analysis of the isolated genes is still required to confirm potential roles in the intended plant species. This thesis aims to make progress in this regard and will focus on functional characterisation of several defensin peptides in grapevine cultivars.

Plant defensin peptides form part of a large family of antimicrobial peptides and are widely distributed throughout the plant kingdom. The first plant defensins were isolated form wheat and barley endosperm and was originally classified as new members of the thionin family, however, further investigation into their structures revealed a closer relation to that of the insect and mammalian defensins, and they were renamed as 'Plant defensins' (Colilla et al. 1990; Mendez et al. 1990; Bruix et al. 1993; Terras et al. 1995). Plant defensins have a broad range of antimicrobial activity and are usually expressed in the peripheral barriers between tissue types of plant organs where they act as protective antimicrobial barriers (Terras et al. 1995). The expression of these peptides occur during normal plant growth and development, but can also be induced in response to fungal infection or mechanical wounding (Osborn et al. 1995; Terras et al. 1995; Broekaert et al. 1995; Thevissen 1997; Thomma et al. 2002; Lay and Anderson 2005).

A very large number of peptides have been isolated and characterised *in vitro* (mostly in culture-based methods) against a wide range of pathogens (Terras et al. 1992a; Broekaert et al. 1995). The modes of action of some of these peptides have also been studied, leading to a few generalised models of interactions. These peptides exert their mode of action through the initial step of binding to a specific target on the fungal membrane. These membrane targets have been identified as two groups of fungal lipid targets, namely sphingolipids and phospholipids. Upon binding to these membrane targets, the peptides are then either internalized or can exert their toxic antifungal activity form outside the fungal cell. Among plant defensin peptides, several mechanisms to kill fungal cells have been elucidated. These include the production of reactive oxygen species (ROS), apoptosis, membrane permeabilization, impairment of mitochondrial function, interference with divalent cation homeostasis, calcium channel blocking, cell cycle arrest, cell wall stress and septum mislocation (Cools et al. 2017; Parisi et al. 2018).

Knowledge of the production patterns in the hosts, the subsequent successful purification of the peptides and intensive protein and activity characterisations lead to crystallised structures of defensins, providing scope for structure-function analysis (Bruix et al. 1993; Fant et al. 1998; Almeida et al. 2002; Lay et al. 2003; Liu et al. 2006; De Medeiros et al. 2010; Meindre et al. 2014; Omidvar et al. 2016; Khairutdinov et al. 2017).

Although plant defensins are best known for their antifungal activities, these peptides have been reported to also potentially play a role in growth and development. For example, roles in flower

development and fertilization have been reported (Tregear et al. 2002; Allen et al. 2008; Stotz et al. 2009; Amien et al. 2010).

In grapevine, 79 defensin-like genes (DEFL) were identified in the genome (Giacomelli et al. 2012). To date only four plant defensin peptides have been isolated and characterized from *V. vinifera*. The first grapevine defensin, *V. vinifera* antimicrobial peptide 1 (Vvi-AMP1) (De Beer and Vivier, 2011) was isolated and characterised and shown to be expressed exclusively in the berry tissue from the onset of ripening (refer to Table 1.1 for a summary of the information gathered on this peptide). In addition to Vvi-AMP1, three more defensins from grapevine (Vvi-AMP2, Vvi-AMP3 and Vvi-AMP4) have been isolated and characterised. Vvi-AMP2 and Vvi-AMP4 were demonstrated to be specifically expressed in the grapevine inflorescences and inhibited *B. cinerea in vitro,* with Vvi-AMP2 being more potent (Nanni et al. 2014). Vvi-AMP3 was specifically expressed in the seeds at the pre-veraison stage (Giacomelli et al. 2012; Nanni et al. 2014). All of the *V. vinifera* plant defensins analysed so far grouped to the non-morphogenic type of defensins, inhibiting the growth of fungi without altering the morphology of the treated fungal hyphae.

In general, defensin peptides are less well studied for their *in vivo* or *in planta* roles and therefore the intended study will specifically focus on *in planta* phenotypes that can be correlated to expression of plant defensins. Grapevine was chosen as the plant host for this study, since relatively little knowledge is available for grapevine defensins and the Institute for Wine Biotechnology (IWBT) where the study will be conducted has a record of studying plant defensins and has several resources available to facilitate the study.

1.2 PROJECT AIMS AND OBJECTIVES

Against this background, the aim of this study was to evaluate potential *in vivo* functions of four different plant defensins in grapevine. The approach was to use (existing) transgenic grapevine populations that overexpress different plant defensins as resources to perform genetical and phenotypical characterisation experiments to evaluate a range of potential *in planta* impacts of the defensins.

The four chosen plant defensins were: *Heliophilia coronopifolia* antifungal peptides 1 and 4 (Hc-AFP1, Hc-AFP4), *Raphanus sativus* antifungal peptide 2 (Rs-AFP2) and Vvi-AMP1. Table 1.1 summarises the data that was already amassed on these peptides. It is clear that the four peptides have all been shown to have strong antifungal activities *in vitro* against a range of fungal pathogens (Table 1.1). The peptides differed in several aspects, according to what was described before, specifically with regards to their morphogenetic abilities. Hc-AFP1, Hc-AFP4 and Rs-AFP2 all inhibited the growth of fungal hyphae by altering the morphology of the hyphae, whereas Vvi-AMP1 is classified as a non-morphogenetic defensin. The mode of action of all four peptides are

associated with membrane permeabilization, however the mode of action of Hc-AFP1, Hc-AF4 and Vvi-AMP1 has not been investigated to the same extent as that of Rs-AFP2, where much more is known about the underlying mechanisms of the mode of action. Furthermore, only Vvi-AMP1 and Rs-AFP2 have been studied in different plant hosts after overexpression (Table 1.1). The homologous overexpression of Vvi-AMP1 in grapevines lead to enhanced resistance towards the powdery mildew fungus through a programmed cell death resistance mechanism (Du Plessis, 2012). Rs-AFP2 has been overexpressed in a number of host species with enhanced resistance reported against a range of pathogens. The majority of these studies confirmed enhanced antifungal activities with *in vitro* assays of transgenic plant extracts and some with *in planta* infection assays.

The following resources supported the planned study:

The grapevine transformation and regeneration platform of the IWBT previously generated the following transgenic grapevine populations that were available to this study:

- Characterised lines:
    - *V. vinifera* cv. Sultana population, overexpressing Vvi-AMP1
- Putative and uncharacterised lines:
    - *V. vinifera* cv. Sultana, and *V. vinifera* cv. Red Globe lines expressing Hc-AFP1 from *H. coronopifolia;*
    - *V. vinifera* cv. Sultana, and *V. vinifera* cv. Red Globe lines expressing Hc-AFP4 from *H. coronopifolia*;
    - *V. vinifera* cv. Sultana and *V. vinifera* cv. Red Globe lines expressing Rs-AFP2 from *R. sativus.*

Against this backgroung: the specific objectives of this project were as follows:

1. *In silico* analysis of the chosen defensins to compare their structural features.
2. *In silico* analysis of the expression patterns of antimicrobial peptide encoding genes in *V. vinifera*, using publicly available data to create a context for defensin expression in grapevine organs and in reaction to stimuli.
3. Genetic characterization of the uncharacterised transgenic populations of *V. vinifera* transformed with the Hc-AFP1, Hc-AFP4 and Rs-AFP2 encoding genes to confirm their transgenic status, analyse gene integration patterns, as well as defensin gene expression in the transgenes, in comparison with the untransformed controls.
4. The selection and maintenance of a number of transgenic lines expressing Hc-AFP1, Hc-AFP4, Rs-AFP2, Vvi-AMP1, and their controls to form part of phenotyping experiments.

Table 1.1 Summary of prior work on the peptides that form part of this study.

	***In vitro* analysis**				***In planta* analysis**			
Defensin peptide and source	**Purified**	**Confirmed activity**	**Morphogenic / Non-morphogenic**	**Mode of action**	**Host**	**Confirmed activity**	**Mode of action**	**References**
Vvi-AMP1 (*Vitis vinifera*)	Yes	*Fusarium oxysporum* *F.solani* *Botrytis cinerea* *Verticillium dahliae*	Non-morphogenic	Permeabilization	Tobacco Grapevine	- *E. necrator*	- PCD**	(De Beer and Vivier 2008; Tredoux 2011; Du Plessis 2012)
Hc-AFP1 (*Heliophila coronopifolia*)	Yes	*F. solani* *B. cinerea*	Morphogenic	Permeabilization	-	-	-	(De Beer and Vivier 2011; Barkhuizen 2013)
Hc-AFP4 (*Heliophila coronopifolia*)	Yes	*F. solani* *B. cinerea*	Morphogenic	Permeabilization	-	-	-	(De Beer and Vivier 2011; Barkhuizen 2013)
Rs-AFP2 (*Raphanus sativus*)	Yes	*Alternaria brassicola* *A. pisi* *Collectotrichum lindemuthianum* *F. culmorum* *F. oxysporum* *Nectria haematococca* *Phomba betae* *Pyricularia oryzae* *Trichoderma hamatum* *Nectria haematococca* *P. infestans* *Septoria nodorum* *Pyrenophora tritici-repent*	Morphogenic	Permeabilization, ROS*, Apoptosis,Ca2+ influx, K+ efflux,	Rice Tobacco Apple Tomato Canola	*Magnaporthe oryzae* *Rhizoctonia solani* *A. longpipes* *Pseudomonas syringe* pc. t*abaci* *A. longpipes* *P. syringe* pc. t*abaci* *A. solani* *F. oxysporum* *Phytohtora infestans* *Rhisoctonia solani* *A. solani*	- - - - - - - - - - -	(Terras et al. 1992a, b, 1995; Bondt et al. 1998; Parashina et al. 2000; Jha and Chattoo 2010)

*ROS –Reactive oxygen species

**PCD – Programmed cell death

An *in silico* analysis performed previously (Du Plessis 2012) with publically available micro-array data showed that grapevine defensin-like (DEFL) genes co-expressed with genes involved in developmental processes, as well as in response to biotic and abiotic stimuli. Importantly, the data suggested that some DEFL genes were highly regulated by tissue-specificity, while others were driven by cultivar specificity. Grapevine DEFL genes were demonstrated to transcriptionally respond in reaction to salinity stress, water stress and in response to exogenous abscisic acid treatment. Furthermore, these DEFL genes were shown to be putatively co-expressed in grapevines infected with the leaf roll virus, GLRaV3 as well as grapevines infected with Bois noir phytoplasma. Moreover, these DEFL genes co-expressed with various genes involved in anti-insect activities, suggesting that grapevine DEFL genes play a role in plant innate immunity against pathogens and possibly pests, particularly during insect herbivory and associated diseases.

Using the information gathered in this prior analysis of Du Plessis (2012), the following traits was selected to form part of the phenotyping of the transgenic populations:

a. Differences in morphology and growth (vegetative organs);
b. Defence phenotypes, when challenged with biotrophic and necrotrophic fungal pathogens;
c. Defence phenotypes against an insect pest (the soft scale insect, *Planococcus ficus*, vector for leafroll virus 3); and
d. Abiotic resistance phenotypes against simulated drought stress.

The results linked to these aims are presented in the research Chapters 3-6, following a literature review in Chapter 2. The outcomes of this study are concluded upon in Chapter 7.

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Chapter 2

Literature review

Plant defensin peptides and their role in defence and development

2.1 Introduction

Plant defensin peptides are ubiquitous throughout the plant kingdom. With their broad range of potent antimicrobial activities, these peptides form a vital part of the sophisticated innate immune system of plants. These peptides are constitutively expressed in the peripheral cells of different plant organs in order to supply a first line of defence to their plant hosts towards invading pathogens. Plant defensin peptides can be induced upon pathogen invasion and mechanical wounding. As single gene products, these peptides are rapidly produced with low energy cost to their hosts (Terras et al. 1992a, 1993; Osborn et al. 1995; Broekaert et al. 1995). In addition to their potent antimicrobial activities, some plant defensins have been reported to also play a role in the growth and development of plants (De Zélicourt et al. 2007; Allen et al. 2008; Stotz et al. 2009a, b; Amien et al. 2010; Mondragón-Palomino et al. 2017; Parisi et al. 2018).

This review describes and discusses plant defensin structure, localization, biological functions, antifungal modes of action, as well as what is known of their broader involvement in plant stress (biotic and abiotic).

2.2 Plant defensins

2.2.1. A short introduction on defensin peptide discovery, classification and the defensin super-families

Broekaert and colleagues first introduced the term “plant defensin” in 1995 after the first members of the plant defensin superfamily were isolated form wheat and barley endosperm (Broekaert et al. 1995). These peptides were initially classified as a novel subclass of the thionin family (γ-thionins) (Colilla et al. 1990; Mendez et al. 1990; Gachomo et al. 2012). However, subsequent isolation and identification of other γ-thionins-like proteins in other plant families, along with structural information that showed these proteins to be more similar in structure and function to that of insect and mammalian defensins (Bruix et al. 1993; Terras et al. 1995; Broekaert et al. 1995) motivated the renaming to plant defensins.

Several classification methods have been described and implemented for plant defensin peptides over the years. Initially, a broad classification was made based on the antifungal activity of plant defensins and the different effects that these peptides had on fungal growth (Broekaert et al. 1995). Morphogenic and non-morphogenic plant defensins were for example described where morphogenic plant defensins was characterized by inhibition of growth with distinct morphological changes on the treated hyphae. This included the multiple budding and swelling of germ tubes and hyperbranching of the hyphae. Well characterized members of this group include peptides from *Brassicaceae, such as Raphanus sativus* antifungal peptide 1 (Rs-AFP1) and Rs-AFP2, *Hydrangea sanguine* antifungal peptide 2 (Terras et al. 1992a; Osborn et al. 1995; Broekaert et al. 1995). The non-morphogenic plant defensins are characterized by their ability to cause growth

inhibition without the induction of visible morphogenic effect on treated hyphae. Representative members of this group include plant defensins from *Asteraceae*, including *Dahlia merckii* antimicrobial peptide 1 (Dm-AMP1); *Asteraceae hippocastanum* antimicrobial peptide 1 (Ah-AMP1) and *Clitoria. ternatea* antimicrobial peptide 1 (Ct-AMP1) (Terras et al. 1992a; Osborn et al. 1995; Broekaert et al. 1995). This classification method however proved to be sub-optimal as it was only based on the antifungal activity of plant defensins and did not account for any of the other many biological activities that some plant defensins possess (Van der Weerden and Anderson 2013).

Later, a further classification that is still used today was put forward by Harrison et al. (1997) (Figure 2.1). This early method of classification was based on the amino acid composition of the mature defensin domain. With the dramatic increase in new defensin peptides being discovered and their sequences becoming available, only some of the consensus sequences “rules” and groupings remained intact in this classification system and the subgroups originally described are no longer sufficient to classify all known plant defensins (Van der Weerden and Anderson 2013). Regardless of this and the fact that other classification methods have been proposed, this classification method by Harrison et al (1997) that primarily focuses on the amino acid composition of the mature defensin domain remains to be the most preferred method of classification.

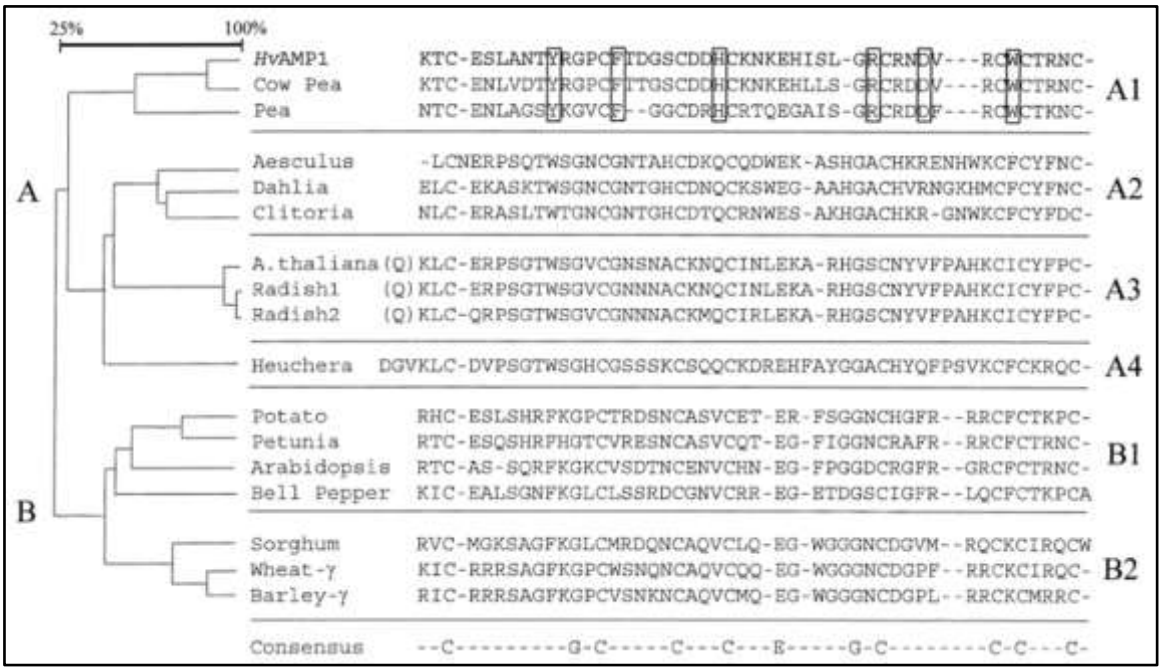

Figure 2.1. Dendrogram of 17 plant defensin sequences representing the traditional classification method proposed by Harrison et al., (1997). Plant defensin peptides are organised based on sequence similarities and divided in subgroups A1-B1 (Reproduced form Harrison et al. (1997) with permission from CSIRO Publishing).

Plant defensins originated form a vast and diverse evolutionary group of proteins called the *cis*-defensin superfamily. This superfamily is classified by the *cis*-orientation of the disulphide bonds in a defensin-characteristic cysteine-stabilized α-helix motif (Figure 2.2). This superfamily not only

consists of plant defensins, but also includes antimicrobial defensins form fungi and invertebrates. This *cis*-defensin superfamily all have a conserved core scaffold, the cysteine-stabilized α-helix motif (CSαβ), with the main differences residing in the length of the loops between the conserved cysteines and the locations of additional disulfides, if any (Shafee et al. 2016, 2017; Parisi et al. 2018). In contrast, the vertebrate α-, β-, θ-, and invertebrate big defensins form part of the *trans*-defensin superfamily. Although these *trans*-defensins share many features with the *cis*-defensins, they remain evolutionary unrelated. The *trans*-defensins lack the CSαβ motif, while the CC cysteine spacing constrains the disulfides. This results in the orientation of disulfides to be in opposite directions and bonding to different secondary elements occur (Figure 2.2) (Shafee et al. 2016, 2017; Parisi et al. 2018).

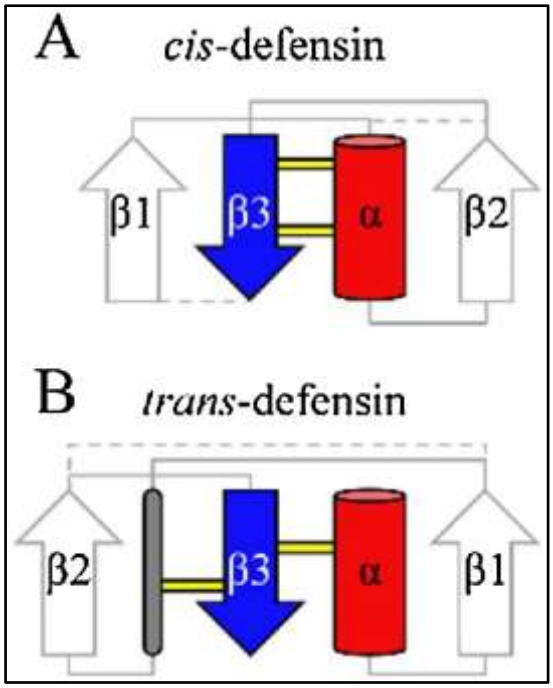

Figure 2.2. The conserved disulfide bridges of the two defensin superfamilies. (A) The disulfide bridges of the *cis*-defensin superfamily. (B) The disulfides of the *trans*-defensin superfamily. The conserved disulfide bonds are indicated in yellow and the non-conserved disulfide bonds are indicated in dashed lines. Figure from Parisi et al. (2018).

2.2.2. Plant defensin structure and the importance of structural motifs for function

Plant defensin encoding genes can be divided in two major groups depending on the structure of the mature transcript. In the first group the transcript encodes a polypeptide that can be divided into two components. The first part is translated to form the signal peptide that is responsible for targeting the peptide to the extracellular space, whereas the second part would yield the mature peptide domain. In most cases, the signal peptide of plant defensins is acidic. In addition to targeting the peptide for secretion, the signal peptide also acts as an inhibitor of the biological activity of the defensin peptide until it's function is required (Carvalho and Gomes 2009).

In the second group, the transcript encodes an additional C-terminal pro-domain of approximately 33 amino acids (Lay and Anderson 2005). The C-terminal pro-domain has to date only been found in plant defensins isolated form solanaceous species, where they are constitutively expressed in the floral tissues and fruit (Lay and Anderson 2005). This C-terminal pro-domain is acidic in nature and is characterized by its high acidic and hydrophobic amino acid content. This net acidic charge of the pro-domain is able to counteract the net basic nature of the defensin domain, leading to an overall neutrally charged defensin peptide (Lay and Anderson 2005). Although several roles have been proposed for the C-terminal pro-domain, Lay et al (2014) determined that it was crucial and sufficient for vacuolar targeting that played a vital role in the detoxification of the defensin peptide as it moved through the secretory pathway.

The mature peptide domain is usually 45-55 amino acids in length and yields a basic peptide with a p*I* value around 9. The processed domain constructs a small, basic, cationic molecule with a molecular mass of 5-7 kDa (Broekaert et al. 1995; Lay and Anderson 2005; Aerts et al. 2008; Carvalho and Gomes 2009). Plant defensin sequence conservation is typically limited to eight cysteine residues in four intramolecular disulfide bridges, two glycine residues at positions 13 and 34, an aromatic residue at position 11, and a glutamic acid residue at position 29 (numbering relative to Rs-AFP2; Broekaert et al. 1995; Lay et al. 2003). This highly conserved scaffold enables the defensin peptide to tolerate hypervariable sequences, that is responsible for the diverse biological functions attributed to these peptides (Lacerda et al. 2014; Tam et al. 2015). The eight conserved cysteine residues are responsible for the characteristic disulfide bridges that play a vital role in the stabilization of the three dimensional structure of these peptides. These inter-chain disulfide bridges form the cysteine stabilizing αβ motif (CSαβ) that has been well documented in peptides that possess antimicrobial activity (Broekaert et al. 1995; Lay and Anderson 2005; Aerts et al. 2008; Carvalho and Gomes 2009).

Structural studies with crystallography and nuclear magnetic resonance (NMR) have been widely extended during the last few years and to date 15 three-dimensional (3D) structures of plant defensins are available in the Protein Data Bank (PDB) (Figure 2.3) (Meindre et al. 2014; Omidvar et al. 2016). These studies all showed that plant defensins adapt a compact globular structure comprised of three anti-parallel β-sheets (β_1 represents the amino acids from Lys^2 and Arg^6, β_2 from His^{33} to Tyr^{28} and β_3 from His^{43} to Pro^{50}) and one α-helix (from Asn^{18} to Leu^{28}) connected to the β-sheet by disulphide bonds, i.e., a cysteine-stabilized αβ, or CSαβ fold (Bruix et al. 1993; Fant et al. 1998; Almeida et al. 2002; Omidvar et al. 2016; Khairutdinov et al. 2017). Although plant defensins have very limited sequence homology, all members of this family adopt this global fold centred around the CSαβ motif. Two anti-parallel β-strands, β_2 and β_3, are joined by a loop (loop L3) to form a γ-core motif $GXCX_{3-9}C$ (X being any amino acid and G being a conserved glycine

residue) (Bloch and Richardson 1991; Bruix et al. 1993; Broekaert et al. 1995; Fant et al. 1998; Carvalho and Gomes 2009; De Medeiros et al. 2010).

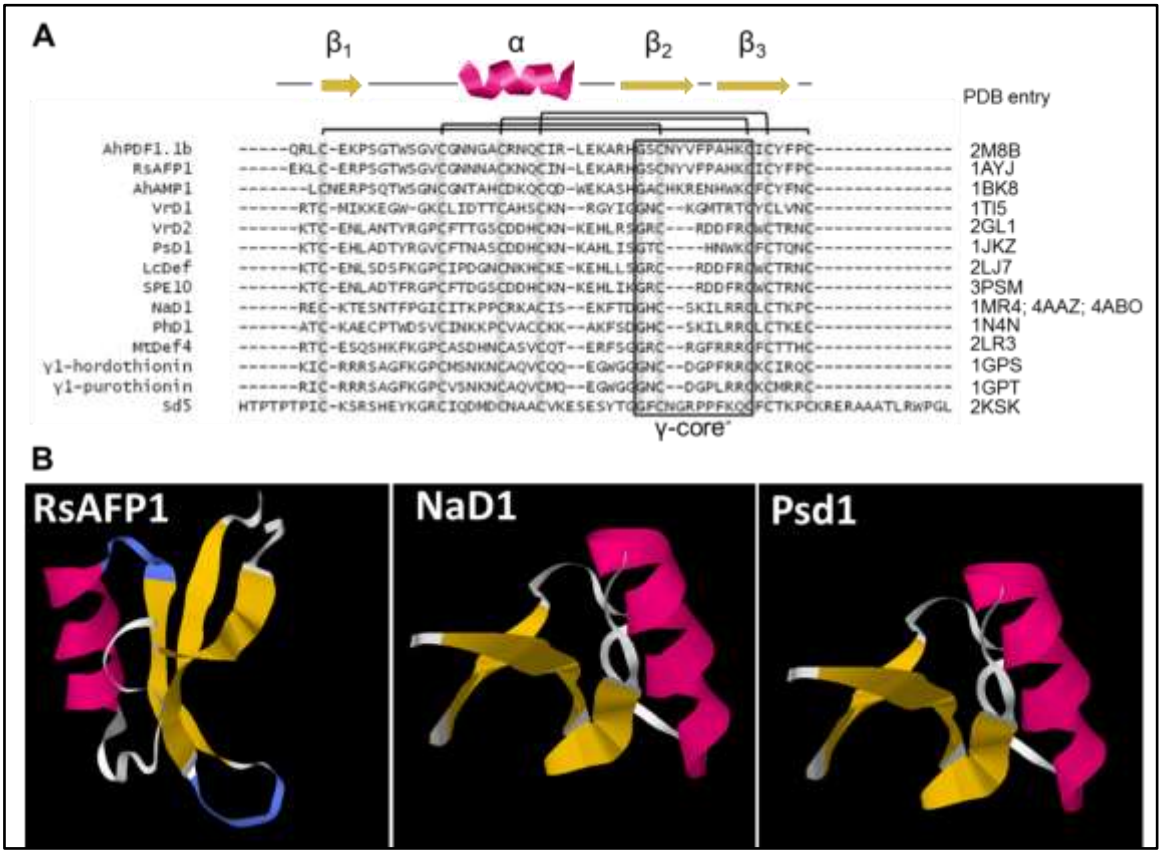

Figure 2.3 (A) Alignment of the 14 sequences of plant defensins for which the 3D structure was determined with their respective Protein Data Bank (PDB) entries. The γ-core motif, 4 disulfide bridges and the secondary elements are highlighted. (B) The secondary structures of representative plant defensins. The secondary structure is represented by different colours: pink-α-helix, yellow-β-strand and white- random coil (Reprinted (adapted) with permission from Meindre et al. 2014. Copyright (2014) American Chemical Society. The three dimensional structures were obtained from the PDB).

Some plant defensins contain five disulfide bonds. This has been described for the *Petunia hybrida* defensin 1 and 2 peptides (PhD1 and PhD2). The fifth disulfide bridge connects the α-helix and the β_1 strand, providing further stabilization of the defensin peptide structure. This additional disulfide bond, however, does not affect the typical three-dimensional structure of the defensin (Lay et al. 2003a; Lacerda et al. 2014).

Slight amino acid differences lead to some variability in the length of the loops and secondary structure, and ultimately subtle observable changes in calculated surface properties. These variations are in turn reflected by small conformational changes in the tertiary structures which are also reflected in the biological activities of the peptides (Lacerda et al. 2014; Meindre et al. 2014).

As mentioned above the cysteine stabilized αβ motif (CSαβ) is a highly conserved motif of plant defensin peptides (Cornet et al. 1995; Broekaert et al. 1995; Fant et al. 1998; Lay et al. 2003b). This motif consists out of one pair of cysteine residues spaced by a tripeptide sequence (Cys-X-X-X-Cys) in an α-helix and is cross-linked to two disulfide bridges to a second pair of cysteines,

separated by a single amino acid residue (Cys-X-Cys) in the C-terminal β-sheet (Kobayashi et al. 1991; Broekaert et al. 1995). This structural motif is also found in insect defensins and scorpion neurotoxins and is generally common to all peptides that possess antimicrobial activity (Almeida et al. 2002; Carvalho and Gomes 2009). Due to the relationship between the conserved three-dimensional structure and highly diverse biological functions, this motif has been suggested as a great scaffold for protein engineering (Yang et al. 2009; De Oliveira Dias and Franco 2015).

Another highly conserved structural motif found throughout disulphide containing antimicrobial peptides is the γ-core motif. This motif comprises two antiparallel β-sheets with an interposed turn region. The consensus sequence of the γ-core motif is Gly-X-Cys(X_{3-9})Cys and in some peptides this sequence is also orientated in reverse. This multidimensional signature structural motif takes part in one to four disulfide bonds and is conserved among disulfide-containing antimicrobial peptides throughout different biological kingdoms and reflects an ancient evolutionary relationship. Non-antimicrobial peptides failed to achieve this three-dimensional γ-core motif seen in antimicrobial peptides. This motif therefore integrates hallmark physiochemical properties that in turn facilitate and define antimicrobial function (Yount and Yeaman 2004; Yount et al. 2007).

Sagaram et al. (2011) showed that the γ-core motif defines the unique antifungal properties of each defensin peptide. This was demonstrated when the γ-core motif of *Medicago sativa* defensin 1 (MsDef1) was replaced with that of *M. truncatula* defensin 4 (MtDef4) in order to determine the effect of this substitution on the biological activity of MsDef1 on *Fusarium graminearum* and ultimately the role of the γ-core motif in the antimicrobial activity of plant defensin peptides (Sagaram et al. 2011). In addition, to the important role of the cysteine residues in the γ-core, Sagaram et al. (2011) showed that the positively charged amino acids and hydrophobic side chains present in the γ-core loop were essential for antifungal activity, since the γ-core motif of MtDef4 alone was sufficient for antifungal activity (Sagaram et al. 2011). More structure-function studies demonstrated the importance of the positively charged residues at the second β-turn and their role in antimicrobial activity. Spelbrink et al. (2004) demonstrated that the Arg_{38} was critical for the antifungal activity of MsDef1 (Spelbrink et al. 2004).

Within the γ-core motif, the positively charged RGFRRR loop connecting the β2 and β3 was also linked to biological activity as this hexapeptide of MtDef4 alone was sufficient to cause significant membrane permeabilization. This was also demonstrated to play a role in the ability of MtDef4 to enter the fungal cell and it was suggested that this loop acts as a translocation signal necessary for the internalization of the peptide (Sagaram et al. 2011, 2013).

2.3 The (biotic) defense roles of plant defensins

The first antimicrobial activity of plant defensins was reported by Terras et al. (1992). From then on numerous plant defensins have been isolated and various biological activities for these plant defensins have been reported in terms of defence against fungi, bacteria and insects (refer to Table 2.1 for a summary).

Table 2.1. Summary of the biological activity profiles that have been identified for some plant defensin peptides**.**

Biological activity	Examples	Plant source	Reference
Antifungal agents	Rs-AFP1-4	*Raphanus sativus*	(Terras et al. 1992a, 1995)
	SD2	*Helianthus annuus*	(Urdangarín et al. 2000)
	Psd1	*Pisium sativum*	(Almeida et al. 2000)
	Ps-Def1	Pinus sylvestris L.	(Hrunyk et al. 2017)
	At-PDF2.3	*Arabidopsis thaliana*	(Vriens et al. 2016b)
	Hs-AFP1	*Heuchera sanguine*	(Terras et al. 1992b; Osborn et al. 1995)
	Dm-AMP1	*Dahlia merckii*	(Terras et al. 1992b; Osborn et al. 1995)
	Ah-AMP1	*Asculus hippocastanum*	(Terras et al. 1992b; Osborn et al. 1995)
	Ct-AMP1	*Clitoria ternatea*	(Terras et al. 1992b; Osborn et al. 1995)
	PvD1	*Phaseolus vulgaris*	(Games et al. 2008)
	NaD1	*Nicotiana alata defensin*	(Van der Weerden et al. 2008)
	Vvi-AMP1	*Vitis vinifera*	(De Beer and Vivier 2008)
	Vvi-AMP2	*Vitis vinifera*	(Nanni et al. 2014)
	Hc-AFP1-4	*H.coronopifolia*	(De Beer and Vivier 2011)
	TPP3	*Lycopersicon esculentum cv. Zhongshu*	(Stotz et al. 2009a)
	Ap-Def1	*Adenanthera pavonina*	(Soares et al. 2017)
	NsD7	*Nicotiana suaveolems*	(Kvansakul et al. 2016)
	Mt-Def5	*Medicago truncatula*	(Islam et al. 2017)
Antibacterial agents	Dm-AMP1	*Dahlia merckii*	(Osborn et al. 1995)
	Ah-AMP1	*Asculus hippocastanum*	(Osborn et al. 1995)
	PsDef1	*Pinus sylvestris*	(Kvansakul et al. 2016)
	HisXarJ1-1	*Caspsicum genus*	(Guillén-Chable et al. 2017)
	Ct-AMP1	*Clitoria ternatea*	(Osborn et al. 1995)
	Rs-AFP2	*Bacillus megaterium*	(Terras et al. 1992a)
	Bh-Def14	*Brassica hybrid*cv Pule	(Kaewklom et al. 2016)
	WCBAFP	*Phaseolus vulgaris* cv. 'white cloud bean'	(Wong et al. 2006)
	Br-AFP2	*Brassica rapa*	(Terras et al. 1993)
	MtDef5	*Medicago truncatula*	(Velivelli et al. 2018)
Insecticidal α-Amylase inhibitors	VrCRP	*Vigna radiata*	(Chen et al. 2002)
	VuD1	*Vigna unguiculata*	(Pelegrini et al. 2008)
	Slα1-3	*Sorghum bicolor*	(Bloch and Richardson 1991; Osborn et al. 1995)

Table 2.1. Continued

Biological activity	Examples	Plant source	Reference
	SIα1-2	*Sorghum bicolor*	(Bloch and Richardson 1991; Osborn et al. 1995)
	VrD1	*Vigna radiata*	(Lin et al. 2007)
	PsDef1	*Pinus sylvestris*	(Khairutdinov et al. 2017)
Protease inhibitor	5495 Da plant defensin	*Crassia fistula*	(Wijaya et al. 2000)
Ion channel blockers	γ1- and γ2-zeothionins	*Zea mays*	(Kushmerick et al. 1998)
	MsDef1	*Medicago sativa*	(Ramamoorthy et al. 2007b)
Protein translation inhibitors	γ-hordothionin	*Hordem vulgare*	(Mendez et al. 1990; Carvalho and Gomes 2009)
	ω-hordothionin	*Hordem vulgare*	(Mendez et al. 1990; Carvalho and Gomes 2009)

2.3.1 Antibacterial activities: Although the principle antimicrobial activity of plant defensins is primarily observed against fungi, some bacteria, especially the Gram positive bacteria are also inhibited (Osborn et al. 1995; Lay and Anderson 2005; Stotz et al. 2009a; Carvalho and Gomes 2009). The majority of studies have been directed towards the antifungal activity of plant defensins and for that reason far less is known about the antibacterial activity of plant defensin peptides. Interestingly, the defensin from gymnosperms, *Pinus sylvestris* plant defensin 1 (PsDef1) was not only found to be active against Gram positive bacteria but had a dose dependent activity towards the Gram negative bacteria *Pectobacterium carotovrum* and *Pseudomonas fluorescens* (Khairutdinov et al. 2017). Some defensins have shown to be both antifungal and antibacterial. These defensins include *Vigna angularis* defensin 1 (VaD1), Ct-AMP1, Dm-AMP1 and Ah-AMP1 (Osborn et al. 1995; Chen et al. 2005; De Oliveira Carvalho and Gomes 2011).

Little is known about the mechanism of this antibacterial activity of most plant defensins however a recent study by Velivelli et al. (2018) demonstrated that the antibacterial activity of the bi-domain *M. truncatula* defensin 5 (MtDef5) exerts its antibacterial activity through a multistep mechanism where it interacted with the outer surface of the bacteria, followed by the permeabilization of the bacterial membrane. These peptides then translocate into the cells where they were bound to negatively charged bacterial DNA. Velivelli et al (2018) proposed that these peptides kill bacterial cells by either inhibiting DNA synthesis and/or transcription (Velivelli et al. 2018).

2.3.2 Anti-insect activities: Several defensin peptides have been discovered that have the ability to inhibit the α-amalyse activity of the insect gut. This is an important feature of some plant defensins since these insects not only harm plants by feeding on them, but are also the vectors of

some plant viruses. An example of such a virus is the grapevine leaf-roll associated virus 3 (GLRaV3) that is known to be transmitted through the grafting of infected material and more importantly through mealybug and soft scale insect vectors (Douglas and Krüger 2008).

The gymnosperm plant defensin, PsDef1 was showed to have insect α-amylase enzyme inhibiting activity and antibacterial activity (Khairutdinov et al. 2017). Furthermore, *Vigna radiate* defensin 1 (VrD1) inhibited the α-amalyse activity of the mealworm beetle (*Tenebrio molitor*) (Chen et al. 2004, 2005; Lin et al. 2007; Hu et al. 2018), whereas the *Vigna unguiculata* defensin 1 (VuD1) from cowpea inhibited α-amylases from gut extracts of the pest insects Bean weevil (*Acanthoscelides obtectus*) and the Mexican bean weevil (*Zabrotes subfasciatus*) (Pelegrini et al. 2008). These defensins also showed low inhibitory activity towards mammalian α-amylases from porcine pancreas and human saliva, highlighting its specificity for insect α-amylases. Some plant defensins have also shown to have protease inhibitory activity, namely the inhibition of trypsin. One such plant defensin was isolated from seeds of *Cassia fistula* (Wijaya et al. 2000).

2.3.3 Antifungal activity of plant defensins and their mechanism of antifungal action

Although plant defensins possess an enormous multiplicity of biological activities, the antimicrobial activity of plant defensins is mainly observed against fungal pathogens. These peptides possess powerful antifungal activity against an enormous variety of fungal species (Lay and Anderson 2005; Carvalho and Gomes 2009; Cools et al. 2017a).

Plant defensins are not only active towards plant fungal pathogens, but also against human fungal pathogens such as *Candida albicans, C. krusei,* and model yeast, *Saccharomyces cerevisiae* (bakers and brewer's yeast) (Lay and Anderson 2005; Carvalho and Gomes 2009; Stotz et al. 2009b; Cools et al. 2017a). Although defensins are active against a broad range of fungal pathogens, the inhibitory activity and the potency of this activity is dependent on the specific fungus and plant defensin peptide pairing (Terras et al. 1992b, 1993; Osborn et al. 1995; Broekaert et al. 1995; Aerts et al. 2008; Carvalho and Gomes 2009; Stotz et al. 2009b; Wilmes et al. 2011; Cools et al. 2017a).

The antifungal activity of plant defensins is associated with membrane permeabilization as a result of membrane interaction (Thevissen et al. 1996). Plant defensins have the ability to induce a range of rapid membrane responses that interfere with the divalent cation homeostasis of the fungal cell. These membrane responses are the result of the peptide binding to a specific membrane target (De Samblanx et al. 1997; Thevissen 1997). Another membrane response is the alkalinisation of medium and the induction of membrane potential changes (Thevissen et al. 1996; Thevissen 1997). These studies confirmed the importance of membrane target sites in terms of defensin mode-of-action.

The antifungal activity of plant defensins is strongly antagonized by cations (Terras et al. 1992b, 1993; Osborn et al. 1995; Almeida et al. 2000). The activities of some plant defensins, such as the *R. sativus* defensins are more strongly antagonised by the presence of divalent cations compared to monovalent cations (Terras et al. 1992b), whereas in the case of MsDef1, the antagonistic effect is metal specific (Spelbrink et al. 2004). It has been proposed that these cations may interfere with the binding of the peptide to its membrane target through a mechanism where an increase in medium ionic strength results in the cations and peptide to compete for the putative receptors (De Samblanx et al. 1997).

Two different types of membrane permeabilization have therefore been distinguished. The first type of membrane permeabilization is called cation-sensitive permeabilization, as it is antagonized by cations. Moreover, it is associated with high concentrations of plant defensin peptides (10 to 40 μM) and a strong permeabilization that is usually detected within 30-60 minutes after the addition of the defensin peptide. The second type of permeabilization is termed cation resistant permeabilization and is only slightly affected by cations. It occurs at very low defensin peptide concentrations (0.1 to 1 μM) that typically correlate with the concentrations required to cause fungal growth inhibition and can only be detected after two to four hours of incubation (Thevissen et al. 1999).

2.3.3.1. Mechanism of action of antifungal defensin peptides

The binding of a plant defensin to its membrane target is the first step in the antifungal mode of action. These specific membrane targets were discovered in 1997 by Thevissen et al. (1997) through the use of radiolabelled HsAFP1 ([^{35}S]Hs-AFP1) and testing its binding specificity towards the hyphae of *Neurospora crassa* and microsomal membrane preparations. These results revealed that the binding of Hs-AFP1 to its target was specific, irreversible and had the ability to be saturated. Moreover, this binding could be competed for with excess unlabelled Hs-AFP1, as well as structurally related peptides. The structurally related peptides, however, had a weaker competition compared to the unlabelled Hs-AFP1. Furthermore, peptides that were structurally unrelated were unable to cause displacement. The binding of the radiolabelled Hs-AFP1 was also reduced in the presence of cations (Thevissen 1997). The high affinity binding sites were eventually identified as fungal lipid targets, namely sphingolipids and phospholipids (PLs) (Poon et al. 2014; Muñoz et al. 2014; Baxter et al. 2015).

Since the discovery of the sphingolipids and phospholipids as the specific membrane targets of plant defensin peptides, major advances have been made in determining the specific sphingolipid and phospholipid target for several defensin peptides, as well as the discovery of the sensitivity genes that were involved in the biosynthesis of these membrane components. Most of these

studies involved the use of yeast deletion mutants without containing the specific enzymes responsible for the synthesis of the specific sphingolipid these plant defensins interact with. However, due to the significant role phospholipids play in fungal cellular processes, it is impossible to perform deletion studies with these lipids in order to determine if they are possible defensin membrane targets. Instead binding to functionally important lipids are determined through the use of protein-lipid overlay assays.

Sphingolipids are essential components of eukaryotic membranes and take part in functions like mediating cell adhesion/recognition, serving as lipid moieties for glycosyl phosphatidyl inositol (GPI)-anchored proteins and play a vital role in intracellular vesicle transport, signalling, heat stress responses, Ca^{2+} homeostasis and transport of GPI-anchored proteins (Thevissen et al. 2000a; Olsen and Jantzen 2001; Warnecke and Heinz 2003). These lipids contain a backbone of ceramide together with a polar head group. Depending on the type of polar head group, these sphingolipids are classified in phosphosphingolipids and glycosphingolipids (Reviewed in Merrill et al. 1997). The phosphosphingolipids can be further subdivided into three major classes namely, inositol-p-ceramide (IPC), mannose-inositol-P-ceramide (MIPC) and mannose (inositol-P)$_2$-ceramide M(IP)$_2$C, with M(IP)$_2$C being the most abundant sphingolipid in *S. cerevisiae* (Patton and Lester 1991; Hechtberger et al. 1994; Daum et al. 1998; Cools et al. 2017a). M(IP)$_2$C was identified as the membrane target for Dm-AMP1 (Thevissen et al. 2000a, 2003b) and *Arabidopsis thaliana* plant defensin 2.3 (At-DEF2.3) (Vriens 2015) (Table 2.1). Interestingly, the binding of Dm-AMP1 to (M(IP)$_2$C) showed that the presence of equimolar concentrations of ergosterol enhanced the binding of Dm-AMP1 to (M(IP)$_2$C). This corresponds to the fact that sterols are associated with sphingolipids in fungal membranes (Bagnat et al. 2000; Thevissen et al. 2003b).

The second group of sphingolipids, glycosphingolipids contain a sugar or ionisable head group with the glucose-containing glucosylceramide (GlcCer) being the most common glycosphingolipids in fungi (Fernandes et al. 2016). This sphingolipid was discovered to be the target for Rs-AFP2 (Table 2.2). This was identified through the use of deletion mutant strains of *Pichia pastoris* and *C. albicans* devoid of the gene *gcs*, encoding the enzyme UDP-glucose:ceramide glucosyltransferase, that is responsible for the catalysis of the final step in GlcCer biosynthesis (Thevissen et al. 2004). GlcCer was also identified as the target of and Ms-Def1 (Ramamoorthy et al. 2007a) and Psd1 (Table 2.2) (De Medeiros et al. 2010; Neves de Medeiros et al. 2014).

Phospholipids (PLs) are another one of the major components of eukaryotic membranes. These lipids are present in low concentrations and despite their low abundance, they play vital regulatory roles in cellular signalling, cytoskeletal rearrangements and membrane trafficking (Phan et al. 2015). PLs consist out of a glycerol backbone linked to two fatty acids and a polar head group. As with sphingolipids, the polar head group defines the various species of PLs, and include

phosphatic acid (PA), phosphatidylserine, phosphatidylglycerol and phosphoinositides. Phosphoinositides can further be classified into: phosphatidylinositol (PI), phosphatidylinositol phosphate (PIP), phosphatidylinositol bisphosphate (PIP_2) and phosphatidylinositol triphosphate (PIP_3) (Cools et al. 2017a). Phospholipids have been identified as the membrane targets of NaD1 (Poon et al. 2014), *Nicotiana alata* defensin 2 (NaD2) (Payne et al. 2016), the tomato pistil predominant 3 defensin (TPP3) (Baxter et al. 2015), Ms-Def1 and Mt-Def4 (Sagaram et al. 2013) (Table 2.2).

Interestingly, a specific plant defensin can interact with various lipids. This has been reported for Psd1; this pea defensin can bind to both phospholipid and the sphingolipid GlcCer (Neves de Medeiros et al. 2014). This phenomenon has also been demonstrated for NaD1, NaD2, MsDef1, MtDef4 (Sagaram et al. 2013; Cools et al. 2017a).

Upon binding to the specific membrane target, plant defensins are either internalized by fungal cells, or exert their antifungal action from outside the cell. Rs-AFP2 is an example of a defensin peptide that is not internalized into to the fungal cell after binding to its membrane target (Thevissen et al. 2003a) (Table 2.2). In contrast, plant defensins that are internalized include NaD1 (Lay et al. 2012), Mt-Def4 (Sagaram et al. 2013), Hs-AFP1 (Cools et al. 2017b), Mt-Def5 (Islam et al. 2017) and Psd1 (Neves de Medeiros et al. 2014) (Table 2.2). Critical to the internalization of NaD1 is the oligomerization of NaD1 with its fungal membrane target, $PI(4,5)P_2$ that results in the destabilization of the membrane causing irrecoverable membrane disruption and thus providing defensins with entry into the fungal cell. The NaD1-$PI(4,5)P_2$ oligomer is comprised out of seven NaD1 dimers in a cationic grip conformation, bound to 14 PIP_2 molecules. The interaction between NaD1 and PIP_2 was shown to be mediated by a number of positively charged amino acids in the β_2-β_3 loop region of NaD1 that interacted with the negatively charged phosphor-head groups of $PI(4,5)P_2$ (Lay et al. 2012; Poon et al. 2014; Baxter et al. 2015; Payne et al. 2016). This cationic grip binding mechanism is conserved among class two solanaceous defensin peptides and was also observed for the tomato defensin TPP3 (Baxter et al. 2015). Moreover, it has been proposed that the RGFRRR motif within the γ-core motif of the Mt-Def4 defensin is the translocation signal required for the internalization of the protein (Sagaram et al. 2013).

Linked to the modes of action discovered for several plant defensins, several mechanisms of fungal cell killing have been confirmed (refer to Table 2.2 for a summary, as well as Parisi et al. (2018) for a recent review). In some instances, several of these mechanisms contribute in combination to the peptide's mode of action, for example the membrane permeabilization, production of reactive oxygen species (ROS) and induced apoptosis of the Rs-AFP2 peptide. The mode of fungal cell killing will be discussed for four well-studied peptides, namely Rs-AFP2, NaD1, Ms-Def1 and Mt-Def4.

Examples of antifungal peptides

Rs-AFP2: Upon binding to its fungal membrane target, namely GlcCer, Rs-AFP2 induces the rapid increase in K^+ efflux and Ca^{2+} uptake, as well as the production of ROS (Figure 2.4A). The production of ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (-OH) are a consequence of normal aerobic respiration. The production of these radicals results in the damage of fungal proteins, lipids and DNA leading to the induction of programmed cell death, or apoptosis (Ames *et al*., 1993; Aerts *et al*., 2007). Apoptosis in context of defence is an evolutionary conserved process whereby a cell commits suicide in order to protect the organism by eliminating dangerous, infected or damaged cells (De Brucker *et al*., 2011). This Rs-AFP2-induced apoptosis occurs through the activation of fungal caspases independent of *Candida albicans* metacaspase 1 (CaMca1p). Furthermore, Rs-AFP2 activates the MAPK signalling pathways of *F. graminearum* and induces cell damage by activating the cell wall integrity (CWI) pathway. In the human fungal pathogen *C. albicans*, Rs-AFP2 induced the accumulation of ceramides in the plasma membrane, more specifically phytoC24-ceramide that affected septin formation and localization that impaired the yeast to form hyphae. All these induced mechanisms ultimately lead to fungal cell death (Figure 2.4A) (Terras et al. 1992a; Osborn et al. 1995; Thevissen et al. 1999, 2004; Nett and Andes 2006; Aerts et al. 2007; Thevissen et al. 2012; Vriens et al. 2015, 2016a; Parisi et al. 2018).

NaD1: This peptide dimerizes to bind its fungal membrane target, $PI(4,5)P_2$ that is located on the inner leaflet of the plasma membrane. This binding results in oligomerization that in turn leads to membrane destabilization. NaD1 is internalized into the fungal cell where it interacts with intracellular targets. These interactions lead to the production of ROS, permeabilization of the plasma membrane and granulation of cytoplasm that all ultimately lead to fungal cell death. The osmotic and oxidative stress experienced by the fungal cell leads to the further activation of the high osmolality glygerol 1 (HOG1) pathway in an attempt to cope with the adverse effects by the NaD1 peptide (Lay et al. 2003b; Van der Weerden et al. 2008; Van Der Weerden et al. 2010; Muñoz et al. 2014; Bleackley et al. 2016; Payne et al. 2016; Parisi et al. 2018). The mechanism of action of NaD1 is summarized in figure 2.4B.

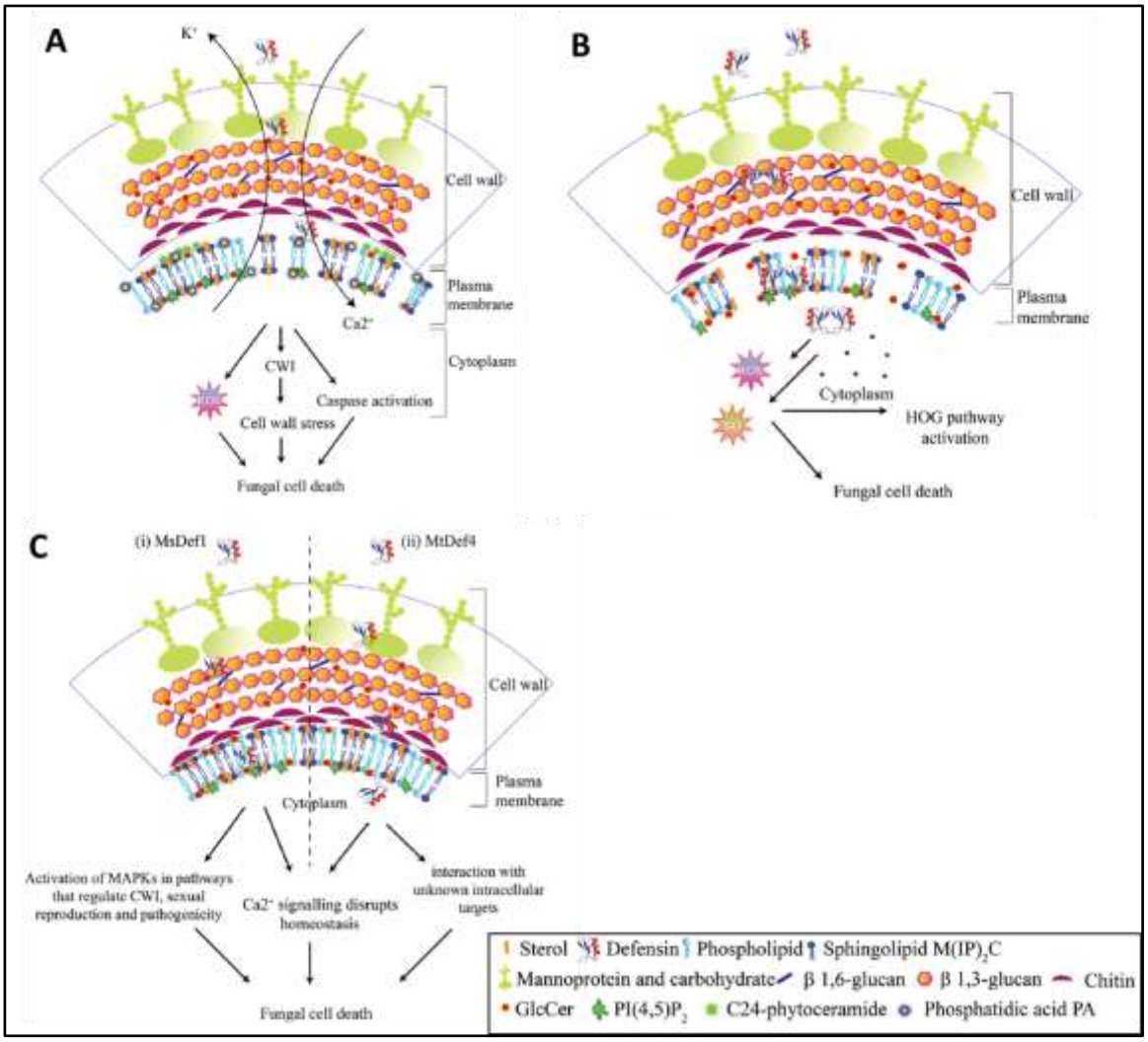

Figure 2.4. The proposed mechanism of antifungal action of plant defensins (A) Rs-AFP2, (B) NaD1 and (C) Ms-Def1 and Mt-Def4 (Parisi et al. 2018).

Ms-Def1a and Mt-Def4: Upon binding to the membrane target GlcCer, **Ms-Def1** disrupts Ca^{2+} signalling and Ca^{2+} gradients resulting in the characteristic morphological abnormalities known to be caused by morphological defensins. This defensin peptide activates two MAPK signalling pathways involved in the regulation of the CWI pathway, sexual reproduction and pathogenicity (Figure 2.4C) (Gao et al. 2000; Ramamoorthy et al. 2007b; Allen et al. 2008; Sagaram et al. 2011; Muñoz et al. 2014; Parisi et al. 2018). In contrast, **Mt-Def4** is a non-morphogenic defensin. This peptide binds to phosphatidic acid (PA) in the fungal membrane that leads to the disruption of the Ca^{2+} signalling and Ca^{2+} gradients in in the fungal cell resulting the inhibition of hyphal growth and fusion and ultimately cell death (Figure 2.4C) (Sagaram et al. 2011, 2013; Muñoz et al. 2014; El-mounadi et al. 2016; Parisi et al. 2018).

2.3.4. Exploiting plant defensins in plant disease control

Through the knowledge gained in the past years through structure function studies, synthetic hybrid peptides based on natural defensin peptides have been synthesised in order to increase

their activity, modify the specific targets, increase the specificity to a target or to decrease possible cytotoxicity (Ageitos et al. 2017).

Due to the long polypeptide backbone (~50 amino acids), a conserved C-terminal cysteine residue and the complex three-dimensional fold comprising of eight cysteine residues paired into four disulfide bridges, these peptides have always been a chemical synthesis challenge. However, recently Meindre et al. (2014) reported the first total chemical synthesis of the plant defensin AhPDF1.1b.

The complex and unique oxidative folding was achieved by using a procedure based on thermodynamically controlled disulphide shuffling in the presence of reduced and oxidized glutathione. MALDI-TOF MS analysis of the peptide confirmed the accuracy of this chemical synthesis method. Moreover, the activity of the peptide was found to be more potent to that of the recombinant peptide (Meindre et al. 2014).

Several synthetic defensin peptides with potent antimicrobial activity have been synthesised through the knowledge of the known regions vital to their antimicrobial activity. Derivatives from the natural defensin peptides *Brassica hybrid* defensin 1 (BhDef1) and *Brassica hybrid* defensin 2 (BhDef2) were synthesised based on the known regions vital to the antimicrobial activity of defensin peptides Rs-AFP1 and 2; *Pisum sativum* defensin 1 (Psd1); Ms-Def1; NaD1 and Mt-Def4. Interestingly, these synthesized synthetic peptides showed antibacterial activity towards the Gram positive bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Salmonella* Thypi (Kaewklom et al. 2016). Similarly, synthetic peptides derived from β_2-β_3 loop region of Rs-AFP2 consisting only of 19 amino acids were found to be almost as potent as Rs-AFP2 (Schaaper et al. 2001).

Plant defensin peptides with their potent antifungal activity are very attractive candidates to be used for genetic engineering strategies. These peptides generally possess low eukaryotic cell toxicity. Furthermore, these peptides are single gene product enabling the plant to deliver these molecules rapidly and without excessive energy (Osborn et al. 1995; Thomma et al. 2002). Several plant defensins have been used to generate transgenic crops with enhanced resistance towards economically important pathogens (Table 2.3).

Transgenic pepper plants expressing a defensin peptide isolated form pepper displayed strong resistance towards the causal agent of anthracnose disease, *Colletotrichum gloeosporioide* (Seo et al. 2014). The radish defensin peptide, Rs-AFP2 has been used to generate several transgenic plants of several crops namely tomato, tobacco, rice, canola and apple. All of these plants displayed enhanced disease resistance towards a wide range of fungal and bacterial pathogens

(Terras et al. 1992a; Parashina et al. 2000; Jha and Chattoo 2010). Although plant defensins have been transformed into a wide variety of host plants, only one study exists with grapevine as host plant. The *Vitis vinifera* antimicrobial peptide 1 (Vvi-AMP1) was ectopically expressed in *V. vinifera* (cv. Sultana) and presented enhanced resistance towards the biotrophic powdery mildew grapevine fungus, *E. necator* (Du Plessis 2012).

Although a wealth of studies has reported on genetically engineered transgenic plants with enhanced resistance through the expressing plant defensins, the majority of these studies have only reported on the resistance phenotype of these transgenic plants with limited information regarding the growth phenotype of the transgenic plants. Stots et al. (2009) reported that transgenic tomato plants expressing the tomato defensin, Defensin 2 (Def2) had smaller leaves and fruits with defects in seed production compared to the untransformed controls (Stotz et al. 2009a). Another report showed that the expression of NaD1 in cotton plants had an toxic effect on the growth and fertility of the transgenic plants (Anderson et al. 2009).

Table 2.2 A summary of modes of action of well-studied plant defensins (adapted form Cools et al. 2017a)

Defensin	Species of origin	Fungal membrane target	Oligomeri-zation	Uptake/target in fungi?	Antifungal mode of action/fungal cell killing	References
Ms-Def1	*Medingo sativa*	GlcCer, $PI(3,5)P_2$ *	Unknown	Unknown	Disruption of calcium homeostasis, membrane permeabilization, inhibition conidial germination and cell fusion, *MAPK* (CWI pathway)	(Gao et al. 2000; Ramamoorthy et al. 2007b; Allen et al. 2008; Sagaram et al. 2011; Muñoz et al. 2014)
NaD1	*Nicotiana alata*	$PI(4,5)P_2$ *	Oligomer of 7 dimers with $PI(4,5)P_2$'s	Yes	Membrane permeabilization, ROS and NO production (oxidative stress), *MAPK* (HOG pathway)	(Lay et al. 2003b; Van der Weerden et al. 2008; Van Der Weerden et al. 2010; Muñoz et al. 2014; Bleackley et al. 2016; Payne et al. 2016)
NaD2	*Nicotiana alata*	PA*	Unknown	Unknown	Unknown	(Bleackley et al. 2016)
TPP3	*Solanum lycopersicum*	$PI(4,5)P_2$ *	Dimers	Unknown	Membrane permeabilization	(Baxter et al. 2015; Payne et al. 2016)
Rs-AFP2	*Raphanus sativus*	GlcCer	Dimers and tetramers	No	ROS, metacaspase-independent apoptosis, ceramide accumulation, cell wall stress and septin mislocalization, Ca2+ influx, K+ efflux, membrane permeabilization, *MAPK* (CWI pathway)	(Terras et al. 1992a; Osborn et al. 1995; Thevissen et al. 1999, 2004; Aerts et al. 2007; Thevissen et al. 2012; Vriens et al. 2016a)
Rs-AFP1	*Raphanus sativus*	-	Dimers and tetramers	Unknown	Unknown	(Terras et al. 1992b; Fant et al. 1998)
Dm-AMP1	*Dahlia merckii*	$M(IP)_2C$, ergosterol	Unknown	Unknown	Ca^{2+} influx, K^+ efflux, membrane permeabilization	(Osborn et al. 1995; Thevissen et al. 1996, 1999, 2000b, 2003b; Aerts et al. 2011)

Table 2.2 (cont.)

Defensin	Species of origin	Fungal membrane target	Oligomerization	Uptake/target in fungi?	Antifungal mode of action/fungal cell killing	References
Hs-AFP1	*Heuchera sanguinea*	PA*	Unknown	Yes, possibly mitochondria	ROS, apoptosis, mytochondrial dysfunction, membrane permeabilization, *MAPK* (HOG and CWI pathway)	(Osborn et al. 1995; Thevissen 1997; Aerts et al. 2011; Vriens et al. 2015; Cools et al. 2017b)
Mt-Def4	*Medingo truncatula*	PA*	Unknown	Yes, (partially) energy dependent	Disruption of calcium homeostasis, membrane permeabilization, inhibition conidial germination and cell fusion	(Sagaram et al. 2011, 2013; Muñoz et al. 2014; El-mounadi et al. 2016)
Mt-Def5	*Medingo truncatula*	PI3P, PI4P, PI5P, $PI(3,5)P_2$, $PI(4,5)P_2$, ,PA, PI, PS	Oligomers	Yes, Multiple targets	Membrane permeabilization, ROS,	(Islam et al. 2017; Velivelli et al. 2018)
At-PDF2.3	*Arabidopsis thaliana*	$M(IP)_2C$	Unknown	Unknown	K^+ channel inhibitor	(Vriens 2015; Vriens et al. 2016b)
Psd1	*Pisum sativum*	GlcCer, ergesterol	Unknown	Yes / cyclin F	Cell cycle arrest, K^+ channel inhibitor	(Almeida et al. 2000, 2002; Lobo et al. 2007; De Medeiros et al. 2010; Neves de Medeiros et al. 2014)
SPE10	*Pachyrrhizus erosu*	Unknown	Dimers	Unknown	Unknown	(Song et al. 2011)
PvD1	*Phaseolus vulgaris*	Unknown	Unknown	Unknown	Membrane permeabilization, ROS and NO production (oxidative stress)	(Mello et al. 2011)
Ap-Def1	*Adenanthera pavonina*	Unknown	Unknown	Unknown	Membrane permeabilization, chromatin condensation, caspase activation, apoptosis and ROS	(Soares et al. 2017)
NsD7	*Nicotiana suaveolems*	PA*	Oligomer	Unknown	Membrane permeabilization	(Kvansakul et al. 2016)

CWI: Cell wall integrity; GlcCer: Glucocylceramide; HOG: High osmolarity/glygerol; M(IP)2C: Mannosyldiinositolphosphorylceramide; NO: Nitrogen oxide; PA: Phosphatidic acid; PDB: Protein data bank; PI(4,5)P2: Phosphatidylinositol (4,5) bisphosphate; ROS: Reacvite oxygen species.

Table 2.3. Transgenic plants expressing plant defensin peptides with conferred resistance towards economically resistant fungal pathogens (adapted form Goyal and Mattoo 2014)**.**

Plant defensin	Source	Host plant	Target pathogen/pest	References
Natural peptides				
Rs-AFP2	*Raphanus Sativus*	Rice	Fungal: *Magnaporthe oryzae;* Rhizoctonia solani	(Jha and Chattoo 2010)
		Tobacco	Fungal: *Alternaria longpipes*	(Terras et al. 1995)
			Bacterial: *Pseudomonas syringe* pc. t*abaci*	
		Apple		
		Tomato	Fungal: *Alternaria solani; Fusarium oxysporum; Phytohtora infestans; Rhisoctonia solani*	(Parashina et al. 2000)
		Canola	Fungal: Alternaria solani	
Psd1	*Pisum sativum*	Canola	Fungal: *Leptosphaeria maculans*	(Wang et al. 1999)
BSD1	*Brassica campestris L. ssp. pekinensis*	Tobacco	Fungal: *Phytohtora parasitica*	(Swathi Anuradha et al. 2008)
BjD	Mustard	Tobacco	Fungal: *Fusarium moniliform; P. parasitica*	
		Peanut plants	Fungal: *Cercospora arachidicol; Pheoisariopsis personata*	
Wasabi defensin	*Wasabi*	Rice	Fungal: Magnaporthe grisea	(Kanzaki et al. 2002)
		Melon	Fungal: *Fisarium oxysporym; A. solani*	(Ntui et al. 2010)
alfAFP	*Medicago sativa (alfalfa)*	Potato	Fungal: *V. dalhiae*	(Gao et al. 2000)
		Tomato	Fungal: *Rhizoctonia solanacearum*	(Chen et al. 2006)
Ms-Def1	*Medicago sativa*	Tomato	Fungal: Fusarium oxysporum	(Abdallah et al. 2010)
PhDef1 and PhDef2	*Petunia hybrida*	Banana	Fungal: *F. oxysporum*	(Ghag et al. 2012)
Sm-AMP-D1	*Stellaria media*	Banana	Fungal: *F. oxysporum*	(Ghag et al. 2014)

Table 2.3. (cont.)

Plant defensin	Source	Host plant	Target pathogen/pest	Reference
NaD1	*Nicotiana alata*	Cotton	Fungal: *F. oxysporum*	(Gaspar et al. 2014)
NmDef02	*Nicotiana megalosiphon*	Potato	Fungal: *Phytohtora infestans*	(Portieles et al. 2010)
TAD1	*Triticum aestivum*	Wheat	Fungal: *Typhula ishikariensis; Fusarium graminearum*	(Sasaki et al. 2016)
JcDef	*Jatropha curcas*	Tobacco	Fungal: *Rhizoctonia solani*	(Wang et al. 2017)
Vvi-AMP1	*Vitis vinifera*	Grapevine	Fungal: *Erisiphe necrator*	(Du Plessis 2012)
Dm-AMP1	*Dahlia merckii*	Papaya	Oomycetes:*Phytophthora palmivora*	(Zhu et al. 2007)
		Rice	Fungal: *Magnaporthe oryzae; R. solani*	(Carvalho and Gomes 2009)
BrD1	*Brassa rapa*	Rice	Insect:Brown planthopper (*Nilaparvata lugens)*	(Choi et al. 2009)
Modified/synthetic peptides				
D4E1		Tobacco	Fungal: *Aspergillus flavus; Verticilium dalhia*	(Cary et al. 2000)
BP100		Rice	Fungal: *F. verticilliodes*	(Nadal et al. 2012)
			Bacteria: *Dickeya chrysanthemi*	
BTD-S	*Baboon θ-defensins*	*Arabidopsis thaliana*	Fungal: *Verticilium dalhia*	(Cary et al. 2000)
D4E1		Tobacco	Fungal: *Collecotrichum destructivum*	(Rajasekaran et al. 2005)
		Cotton		
Tfgd2-Rs-AFP2	*Trigonella foenumgraecum*	Peant	Fungal: *Cercospora arachidicola; Phaeoisariopsis personata*	(Bala et al. 2016)

2.4 Role of plant defensins during growth and development

The impact of defensins on the developmental processes are to date still poorly explored and understood. However, most of these proposed functions suggest protective roles in either protecting the host plants in their vulnerable flowering and/or reproductive stages, or protecting the host plants form invading parasitic plants.

Plant defensin peptides have been isolated form all organs and tissues of plants (leaves, pods, tubers, fruitages, roots, flowers, and rind) (Table 2.4). The expression of these peptides can be consecutive throughout all organs or organ specific. These peptides are usually expressed in peripheral layers, between plant organs and in stomatal cells where they form the first barrier to pathogen attack. Moreover, the expression of these peptides can also be induced upon pathogen attack and mechanical wounding (Broekaert et al. 1995; Finkina and Ovchinnikova 2018).

Radish seed defensins, Rs-AFP1 and Rs-AFP2, are expressed in high levels in the in the middle lamellae of the cell walls of the different seed tissues (Terras et al. 1995). These peptides are released after and during germination when the seed coat is disrupted, releasing these peptides into the immediate environment. This supresses the fungal growth in the immediate environment of the seedling, enhancing its chances for survival (Terras et al. 1995).

Several plant defensins are exclusively expressed the floral organs (Table 2.4), reflecting the protective role plant defensins play in regards to the reproductive organs (Tregear et al. 2002). The *Nicotiana alata* defensin 1 (NaD1) is exclusively expressed in the anthers, pistils, ovaries and petals of the tobacco flowers with no expression in any of the other plant organs. This peptide is expressed in the outer layers of the sepals and petals and in the surrounding tissues of the pollen and pollen tubes (Lay et al. 2003a).

Some plant defensins in turn are specifically expressed in fruits, protecting the ripening fruits when they are most vulnerable to pathogen attack. One such defensin is the bell pepper defensin peptide J1 that is only detectable in the bell pepper at the orange and ripe stages in high concentrations (Meyer et al. 1996).

These expression and localization patterns of plant defensins further emphasize the important role these peptides play in the protection of plants. Although plant defensins are renowned for their antimicrobial activity, various side activities involved in growth and development have also been described for these peptides; a few examples will be highlighted below.

Table 2.4. Summary of the expression and localization patterns of some plant defensin peptides in different plant organs.

Plant organ	Defensin peptide	Native host	References
Leaves	Rs-AFP1	*R. sativus*	Terras et al. 1995b
	PtH-St1	*Solanum tuberosum*	Moreno et al. 1994
	Hc-AFP3	*Heliophilia coronopifolia*	De Beer and Vivier 2011
Fruit	Vvi-AMP1	*V. vinifera*	De Beer and Vivier 2008
	J1	*Capsicum annuum*	Meyer et al. 1996b
	Hc-AFP2	*H. coronopifolia*	De Beer and Vivier 2011
Flowers	NaD1	*Nicotiana alata*	Lay et al. 2003a
	DEF2	*Solanum lycopersicon*	Stotz et al. 2009a
	EGAD1	*Elaeis guinesis*	Tregear et al. 2002
	ZmES4	*Zea mays*	Amien et al. 2010
	Vvi-AMP2	*V. vinifera*	Nanni et al. 2013
	PhD1-2	*P. hybrida*	Lay et al. 2003a
	PtH-St1	*Solanum tuberosum*	Moreno et al. 1994
	SD2	*Helianthus annus*	Urdangarín et al. 2000
	Hc-AFP1	*H. coronopifolia*	De Beer and Vivier 2011
Seeds	Rs-AFP1-2	*R. sativus*	Terras et al. 1995b
	Dm-AMP1	*D. merckii*	Broekaert et al. 1995
	Ct-AMP1	*C. ternatea*	Broekaert et al. 1995
	Ah-AMP1	*A. hippocastanum*	Broekaert et al. 1995
	Hc-AFP4	*H. coronopifolia*	De beer and Vivier 2011
Roots	Hc-AFP1-4	*H. coronopifolia*	Weiller et al. 2016
	Ha-DEF1	*Helianthus annus*	De Zélicourt et al. 2007

Defensins that influence flower development and fertilisation events: The tomato defensin, Defensin 2 (DEF2) is abundantly expressed in immature flower buds during the meiotic stage (Stotz et al. 2009a). This plant defensin has a vital function in the male reproductive development, possibly in developmental signalling. This was revealed when the phenotypes of DEF2-silenced tomato plants were studied and shown to display a pollen viability defect similarly to late-acting sporogenous male sterile mutants (Stotz et al. 2009a). Furthermore, a defensin from palm oil, *Elaeis guinesis* abnormality defensin 1 (EGAD1) was also shown to play a role in flower development (Tregear et al. 2002).

The defensin-like protein *Zea mays* embryo sac 4 (ZmES4) from maize plays a vital role in fertilization of maize by mediating pollen tube growth arrest and burst. The expression of this plant defensin-like protein is organ specific, being exclusively expressed in the embryo sac (Amien et al. 2010). Furthermore, the expression of this peptide is down-regulated immediately after fertilization, suggesting a specific role in fertilization. Interestingly, chemically synthesized ZmES4 applied to maize pollen tube *in vitro*, lead to pollen tube burst within seconds. This mechanism is species specific as it could not be achieved with other plant species. The target of ZmES4 was identified as the pollen tube-expressed potassium channel. This channel opens after ZmES4 treatment, leading to K+ influx and sperm release after osmotic burst (Amien et al. 2010). The involvement of these

peptides in the development of flowering suggests a duel function in development and protection, as these peptides are known for their antimicrobial activities.

A large scale expression analysis of the differentially expressed genes during self-pollination, interspecific pollination and infection with *F. graminearum* in the pistil transcriptomes of *A. thaliana* and *A. halleri* revealed the upregulation of defensin-like genes (DEFL) in pistils when exposed to foreign pollen (Mondragón-Palomino et al. 2017). This suggests a role of these DEFL genes in the process of pollen tube rejection (incompatibility). These genes were not upregulated when *A. halleri* were treated with its own pollen or pollen from *A. lyrate*, as *A. halleri* recognized it as compatible. This self-pollination of *A. halleri* or pollination with *A. lyrata* pollen resulted in the down-regulation in DEFL genes together with cytoplasmic immune receptors, nucleotide binding site leucine rich receptors (NBS-LRRs) and programme cell death (PCD) protease AtCEP1, and a component of the nuclear pores required for innate immunity, MOS7. It is suggested that these genes are down-regulation as it might interfere with pollen tube growth (Mondragón-Palomino et al. 2017).

Defensins that influence root growth: Plant defensins MsDef1, MtDef4 and Rs-AFP2 from *M. sativa*, *M. tracatula* and *R. sativus* respectively all have inhibitory activities on root hair and root growth of germinating *A. thaliana* seeds (Allen et al. 2008; Parisi et al. 2018). The inhibition is achieved by the depolarization of the growing root tip and root hair. This inhibition is achieved over a similar concentration range as reported for antifungal activity. This activity is, however, species and tissue specific as the treatment of *M. truncatula* seeds with MsDef1, MtDef4 and Rs-AFP2 showed no negative growth effects. Furthermore, the extremely rapid and reversible effect of these defensins on the root and root hair growth suggest that the target is on the outer membrane and acts via external membrane components (Allen et al. 2008; Parisi et al. 2018).

Another defensin that showed inhibitory activity towards plant growth is the sunflower defensin *Helianthus annuus* defensin 1 (Ha-Def1), which has an inhibitory activity towards the growth of parasitic plants by causing cell death, suggesting a defensive role (De Zélicourt et al. 2007). Furthermore, the ectopic expression of DEF2 had a pleiotropic effect on plant growth. The growth of the transgenic plants was initially retarded but however caught up to the untransformed controls and surpassed their growth. The transgenic plants however had smaller leaves and grew more upright. Furthermore, sepals were smaller and the style length of the transgenic plants were significantly shorter (Stotz et al. 2009a, b).

2.5. Plant defensins and their role in abiotic stress responses

Plants can respond to combined abiotic and biotic stress conditions while simultaneously preserving vital resources for growth and development. This ability is the result of extensive

evolutionary changes that evolved over years. These responses involve a complex network at transcriptome, cellular and physiological level (Nguyen et al. 2014). Plant defensins have been reported to react to various abiotic environmental stimuli. These peptides seem to play an adaptive role in the plant in response to these abiotic environmental stimuli. Theses abiotic stimuli are summarized in Table 2.5 and some examples will be discussed.

Table 2.5. Abiotic stimuli that have been identified as inducers to the expression of plant defensins.

Abiotic stimulus		Plant defensin	Native plant host	Reference
Cold		PDF1	*Oxytropis* (Fabaceae)	Archambault and Strömvik 2011
		TAD1	*Triticum aestivum*	Koike et al. 2002
Drought		CADEF1	*Capsicum annuum*	Do et al. 2004
Heavy metal	Zinc	AhPDF1.1b	*Arabidopsis halleri*	Mirouze et al. 2006; Mith et al. 2015;
	Zinc	AtPDF1.1	*Arabidopsis thaliana*	Hsiao et al. 2017
	Cadmium	SDmod	*Helianthus annuus L* and *Spinacia oleracea* (Synthetic)	Mahnam et al. 2018
	Cadmium	CAL1	*Oryza sativa*	Luo et al. 2018
Salinity		CADEF1	*Capsicum annuum*	Do et al. 2004
Wounding		CADEF1	Capsicum annuum	Do et al. 2004
		PgD1	Picea glauca	Germain et al. 2012
Hormones (applied exogenously)	Jasmonic acid	PDF1.2	*Arabidopsis thaliana*	Thomma et al. 1998
		PgD1	*Picea glauca*	Germain et al. 2012
	Salilic acid	CADEF1	Capsicum annuum	Do et al. 2004
	Abscissic acid	CADEF1	Capsicum annuum	Do et al. 2004
		Tgas118	*Triticum aestivum*	Van den Heuvel et al. 2001
	Methyl Jasmonate	CADEF1	Capsicum annuum	Do et al. 2004
		PDF1.2	Arabidopsis thaliana	Thomma et al. 1998

Defensins and cold and heat stress: When plants are exposed to cold stress, the typical reaction of plants involve the up-regulating the hydrophilic late embryogenesis abundant proteins and down-regulating photosynthesis-related genes, carbohydrate metabolism, GDSL-motif lipase, hormone metabolism and oxidative regulation genes. Plant defensin peptides have also showed to be upregulated in response to cold stress. A transcriptomic analysis of two arctic plant species, *Oxytropis maydelliana* and *O. arctobia;* and temperate plants showed that the arctic plants, under arctic simulated growth conditions, expressed more of Plant Defensin type 1 (Pdf1) compared to temperate plants (Archambault and Strömvik 2011). In winter wheat it was shown that plant defensin, *Triticum aestivum* defensin 1 (TAD1) was involved in cold-temperature resistance. This peptide was expressed in high levels in the crown tissue of the winter wheat plant after only 24 hours of cold treatment. This high level of TAD1 was maintained throughout the cold stress treatment of 14 days. Furthermore, the expression of this defensin peptide could not be induced

through the treatment of other plant defensin peptides, or hormonal treatment with salicylic acid nor methyl jasmonate. The mode of action underlying this cold acclimation function is however unknown, since antifreeze activity could not be detected. It was proposed that TAD1 had a combined action of transferring low temperature-induced resistance against pathogens during winter hardening (Koike et al. 2002; De Oliveira Carvalho and Gomes 2011).

Little is known about the involvement of plant defensins during heat shock conditions; however a link has been made between plant heat shock responses and plant defensins in *Arabidopsis*. The Pdf1.2 defensin gene has been identified as the targets of the plant's heat shock factor dependent negative regulation during heat shock conditions (Kumar et al. 2009). This aspect however remains poorly studied.

Defensins and heavy metal tolerance in plants: Heavy metal pollutants pose a great threat to humanity as it can lead to the growth inhibition and death of organisms and plants (Järup 2003). The major metals found in polluted areas are zinc (Zn), lead (Pb), copper (Cu) and cadmium (Cd). All these metals can become toxic when they are present at high levels (Baker and Brooks 1989). A great number of higher terrestrial plants are able to tolerate these metals and grow in contaminant soil and are referred to as hyper-accumulating plants. This phenomenon is ubiquitous in the plant kingdom (Baker and Brooks 1989; Mirouze et al. 2006). Although the exact mechanism of metal hyper-accumulation is not yet known, it has been shown that transmitter proteins play a significant role in the accumulation, transportation and disposal of these metals in plants. Furthermore, it has also been shown that in some plant species, chelation and transmembrane transport are involved in their metal tolerance (Mirouze et al. 2006; Mahnam et al. 2017). In some plants species, defensins have been shown to be induced to act as metal binding peptides in reaction to heavy metal stress, thereby participating in the mitigation of the stress.
Arabidopsis halleri plant defensin 1.1b (Ah-PDF1.1b) is antifungal but also confers zinc tolerance to yeast and plant cells through direct binding with the zinc itself, or with some zinc requiring protein (Mirouze et al. 2006; Mith et al. 2015). Another *Arabidopsis* defensin gene conferring zinc tolerance is *A. thaliana* plant defensin 1.1 (At-PDF1.1) (Shahzad et al. 2013). This zinc tolerance function of At-PDF1.1 is involved in its defence towards the necrotrophic bacterium *Pectobacterium carotovorum* subsp. *Carotovorum* (Pcc) via an iron-deficiency-mediated defence response (Hsiao et al. 2017).

Recently a defensin-like protein, Cadmium accumulation in leaf 1 (CAL1) was identified in rice that positively regulates the Cd accumulation in rice. This defensin-like protein is preferentially expressed in root exodermises and xylem parenchyma cells. The expression of CAL1 was induced with exposure to Cd. CAL1 was showed to lower the cytosolic Cd concentration by directly binding to Cd and acting as a chelating agent, facilitating the secretion of Cd to extracellular spaces. The

discovery of this defensin-like protein (CAL1) has great potential to be used in Cd polluted soils for environmental remediation (Luo et al. 2018).

Defensins and drought stress: Another environmental condition that has been identified as an inducer of plant defensin activity is drought stress. As one of the major environmental threats to plants, drought stress affects the entire physiology of plants (Dal Santo et al. 2016). A plant defensin isolated form the leaves of the pepper plant *Capsicum annuum* defensin 1 (CADEF1) has been reported to be expressed in response to drought stress. The function of this defensin peptide in drought stress however still has to be determined. The expression of this peptide was also induced upon salinity stress with 200 mM NaCl for 12 hours and wounding (Do et al. 2004).

2.6. Concluding remarks

Plant defensin peptides are potent antimicrobial peptides that are ubiquitous in the plant kingdom. These peptides play an integral role in the innate immune system of plants, protecting their host form invading pathogens. Furthermore, these multifunctional peptides have been implicated in plant growth and development and in some cases abiotic stress. Although major advancements have been made in the elucidation of the mode of antifungal action of some of these peptides, the *in vivo/in planta* functions of these plant defensin peptides remain poorly understood. Since their discovery, the literature that reported on new isolated defensin peptides and their *in vitro* activities against biotic agents, or their structural motifs and structure-function relationships has grown significantly. Excellent progress has also been made in terms of unravelling mode-of action and consequential cell killing of the biological agents targeted, but significantly less is known about their alternative functions and how they operate as part of the integrated stress responses of plants, particularly with regards to potential protective roles against abiotic stresses. The *in vivo* functional roles of defensin peptides warrants more in-depth studies, particularly since several growth and developmental functions could be described for a number of plant defensins.

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Chapter 3

Research results

In silico comparison of the sequence similarities and structural features of plant defensins Hc-AFP1, Hc-AFP4, Vvi-AMP1 and Rs-AFP2.

The authors contributed as follows to the work presented: HB performed all analysis, data-interpretation and compiling of results under the guidance of MAV and MR; HB drafted and finalized the chapter with inputs from MAV and MR.

RESEARCH RESULTS

In silico comparison of the sequence similarities and structural features of plant defensins Hc-AFP1, Hc-AFP4, Vvi-AMP1 and Rs-AFP2.

3.1 INTRODUCTION

Plant defensin peptides are small (45-54 amino acids), basic, cationic and cysteine-rich peptides. Although they have a vast array of biological functions they are best known for their potent antifungal activity (Osborn et al. 1995; Broekaert et al. 1995; Thevissen 1997; Almeida et al. 2000).

The three dimensional structures of several plant defensins have been resolved through crystallography and nuclear magnetic resonance (NMR) spectroscopy. These peptides adopt a globular structure that consists out of an α-helix; three helical turns; and three anti-parallel β-sheets that are all stabilized by four disulphide bridges (Bruix et al. 1993; Fant et al. 1998; Almeida et al. 2002; Lay et al. 2003b; Liu et al. 2006; De Medeiros et al. 2010; Meindre et al. 2014; Omidvar et al. 2016; Khairutdinov et al. 2017). Within the three dimensional structure of plant defensins, structural motifs that are conserved have been proven to play an essential role in the activity of these peptides. The cysteine stabilized αβ motif (CSαβ) is one of these motifs and is generally found in all peptides with antimicrobial activity. This motif consists of one pair of cysteine residues spaced by a tripeptide sequence (Cys-X-X-X-Cys) in an α-helix and is cross-linked by two disulfide bridges to a second pair of cysteines, separated by a single amino acid residue (Cys-X-Cys) in the C-terminal β-sheet (Kobayashi et al. 1991; Cornet et al. 1995; Broekaert et al. 1995; Fant et al. 1998; Lay et al. 2003b). Another structural motif important for the antimicrobial activity of plant defensins is the γ-core motif (Gly-X-Cys(X_{3-9})Cys). Like the CSαβ motif, the γ-core contains highly specific conserved amino acid residues contributing to the secondary structure (Yount and Yeaman 2004; Yount et al. 2007). Furthermore, the γ-core has been reported to facilitate antimicrobial function and to be directly or indirectly involved in the interaction of a plant defensin with its membrane target (Sagaram et al. 2011). Different sequence conservations within the γ-core motif have been connected to some biological functions of plant defensins. The consensus sequence RGFRRR within the loop connecting the β_2 and β_3 strand was reported to act as a translocation signal necessary for the internalization of the *Medicago truncatula* defensin 4, MtDef4 (Sagaram et al. 2011, 2013). Moreover, four consecutive residues Val^{39}-Phe^{40}-Pro^{41}-Ala^{42} and an amide bond of the Phe^{40}-Pro^{41} in the β_2-β_3 loop adopts an unusual *cis* conformation that is linked to the zinc tolerance activity of some plant defensins (Meindre et al. 2014). All these reports highlight the important role the γ-core has on the biological activity of plant defensins.

Although the overall structure and structural features of plant defensins are highly conserved, these peptides share very little sequence homology. This high sequence diversity is thought to be responsible for the growing number of biological functions that have been attributed to these peptides (Thomma et al. 2003; Lay and Anderson 2005; Carvalho and Gomes 2009; Stotz et al. 2009). The traditional way of classifying these peptides has been based on amino acid composition of the mature defensin domain where certain characteristics of defensin activity can be associated with different subgroups. Plant defensins are divided in two subgroups A and B: subgroup A is further subdivided into A1-A4 and subgroup B into B1 and B2. Subgroups A3 and A4 both contain morphogenic plant defensins, meaning that all these peptides induce morphological changes in fungal hyphae. In contrast, subgroup A2 contains plant defensins that are all non-morphogenic defensins. These defensins inhibit the growth of defensins without causing any morphological changes on treated hyphae. Subgroup B typically house plant defensins that show more diverse biological activities, including antibacterial, α-amalyse and protein inhibitory activity (Terras et al. 1992, 1993; Osborn et al. 1995; Broekaert et al. 1995; Harrison et al. 1997).

The goal of this work was to use *in silico* analysis to characterise and compare the predicted structures of four plant defensins. These peptides will be functionally characterised in transgenic grapevine populations and the predicted structural comparisons will allow a theoretical framework as context when the potential functions of the peptides are explored in phenotyping experiments (Chapters 4-6 of this thesis). *Heliophila coronopifolia* antifungal peptide 1 (Hc-AFP1) and 4 (Hc-AFP4); the *Vitis vinifera* antimicrobial peptide 1 (Vvi-AMP1) and the *Raphanus sativus* antifungal peptide 2 (Rs-AFP2) were subjected to sequence analysis and homology modelling. According to the peptide classification system of Harrison et al. (1997), Vvi-AMP1 falls into subgroup B2, whereas the other three peptides group to subgroup A3. These groupings were maintained when comparing the peptides on sequence level. The four peptides were also compared to defensin-like (DEFL) sequences in the grapevine genome on the sequence level. Homology modelling showed that Hc-AFP1, 4 and Rs-AFP2 were structurally more related to one another compared to Vvi-AMP1, with the major structural differences between these peptides lying within the γ-core motif.

3.2 MATERIALS AND METHODS

3.2.1. Alignment analysis of plant defensin peptides Hc-AFP1, Hc-AFP4, Rs-AFP2 and Vvi-AMP1 and DEFL genes

All alignment analysis were performed using the MUSCLE algorithm (Edgar 2004) in CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark). Phylogenetic trees were created using CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark). Homology percentages of peptides with one another were determined with BLASTp and all peptide sequences were obtained from NCBI (refer to Table 3.1).

Table 3.1 Peptide and plant defensin sequences used in this analysis, with their source information.

Peptide / DEFL gene	Classification	Sequence	Accession/ Source
Hc-AFP1	A3	RYCERSSGTWSGVCGNSGKCSNQCQRLEGAAHGSCNYV FPAHKCICYYPC	GenBank: AER45489.1
Hc-AFP4	A3	QKLCERPSGTWSGVCGNNGACRNQCIRLERARHGSCNYV FPAHKCICYFPC	GenBank: AER45492.1
Rs-AFP2	A3	QKLCERPSGTWSGVCGNNNACKNQCINLEKARHGSCNYV FPAHKCICYFPC	GenBank: AAB22710.1
Vvi-AMP1	B1	RTCESQSHRFKGTCVRQSNCAAVCQT-EGFHGGNCRGFRRRCFCTKHC	(De Beer and Vivier 2008)
Vvi-AMP2	Unknown	RLCESQSHWFRGVCVSNHNCAVVCRNEHFVGGRCRGFR RRCFCTRNC	(Du Plessis 2012)
Vvi-AMP3	Unknown	RVCESQSHKFEGACMGDHNCALVCRNEGFSGGKCKGLR RRCFCTKLC	
DEFL 10	Unknown	QDPGSDCDFVGSCKNKADCAKPCGAKGHSPTAVLCVPNP NGGKRCCCIIA	
DEFL 21	Unknown	QQDGRCCKDHPKLGHCAPGKDDDPNGGKCWTYCITKCS KGGLCKKLSGGRHVCHCYC	
DEFL 52	Unknown	QGGXFCTVTEHFPGKCPSENLGCFIEMSGKYGASSMLHG CHCTQFXSDHTCACXAXCSPPL	
DEFL 59	Unknown	KEVKAARCMEVLDPNGCILPSCKQRCLQEKNGNGVCVPN RNGGYECICYYNC	
VviSnakin 2	Unknown	QPTTDGAGFCGLKCSKRCSQAAVLDRCMKYCGICCQECK CVPSGTYGNKHECPCYRDKKNSKGKPKCP	
VviSnakin 6	Unknown	TITEAPTPQPQQSTNGFPMHGVTQGSLHPQECAPRCTTRC SKTAYKKPCMFFCQKCCAKCLCVPPGTYGNKQFCPCYNN WKTKRGGPKCP	
VviSnakin 8	Unknown	SHGHGGHHYDQKNYGPGSLKSFQCPSQCSRRCGKTQYH KPCMFFCQKCCKKCLCVPPGYYGNKAVCPCYNNWKTKEG GPKCP	
VviSnakin 13	Unknown	SLVISNAEHSLTSVDESRDEVALHKKSHPRKINCSYACSRR CRKASRKNVCSRACKTCCKRCHCVPPGTYGNKNMCPCYA SLKTHGHKPKCP	
Rs-AFP1	A3	QKLCERPSGTWSGVCGNNNACKNQCINLEKARHGSCNYV FPAHKCICYFPC	GenBank: AAB22709.1
Dm-AMP1	A2	ELCEKASKTWSGNCGNTGHCDNQCKSWEGAAHGACHVR NGKHMCFCYFNC	UniProtKB/Swiss-Prot: P0C8Y4.1
NaD1	A2	RECKTESNTFPGICITKPPCRKACISEKFTDGHCSKILRRCL CTKPC	PDB: 4AAZ_A
Psd1	Unknown	KTCEHLADTYRGVCFTNASCDDHCKNKAHLISGTCHNWKC FCTQNC	UniProtKB/Swiss-Prot: P81929.2
MsDef1	A1	RTCENLADKYRGPCFSGCDTHCTTKENAVSGRCRDDFRC WCTKRC	UniProtKB - Q9FPM3
MtDef4	A2	RTCESQSHKFKGPCASDHNCASVCQTERFSGGRCRGFRR RCFCTTHC	PDB: 2LR3_A
Hs-AFP1	A4	DGVKLCDVPSGTWSGHCGSSSKCSQQCKDREHFAYGGA CHYQFPSVKCFCKRQC	UniProtKB/Swiss-Prot: P0C8Y5.1

3.2.2. Homology modelling of plant defensins Hc-AFP1, Hc-AFP4, Rs-AFP2 and Vvi-AMP1 and DEFL59

Secondary structure analysis and homology modelling of the mature peptide sequences were performed using the Swiss model server (https://swissmodel.expasy.org). A template search for each peptide query was conducted in the SWISS-MODEL template library in order to find the best templates for comparative modelling. The output templates of the template search were ranked according to the expected quality of the resulting predicted models. This was estimated by the Global Model Quality Estimate (GMQE) and Quaternary Structure Quality Estimate (QSQE). The GMQE and QSQE value is a tertiary structure level estimate of the expected model based on the target-template alignment. GMQE was expressed as a value between 0 and 1, where a higher number indicated a more reliable model (Waterhouse et al. 2018). QMEAN is a scoring function used once the models have been built. This quality estimate makes use of statistical potentials of mean force to produce quality estimates for both the entire structure (global) and local (per residue). This value is expressed as a Z-score value that provides an estimate of the degree of homology of the structural features observed in the model on a global scale. QMEAN Z-scores close to 0 indicate good agreement between the model structure and experimental structures of similar size (Benkert et al. 2011; Waterhouse et al. 2018). Templates were selected according to the sequence identity. Models were chosen based on the highest sequence identity, similarity and coverage of the query peptide sequence to the template sequence and the best GMQE and QMEAN Z-score values of their predicted models (Benkert et al. 2011; Bertoni et al. 2017; Bienert et al. 2017; Waterhouse et al. 2018). Rs-AFP2 was included in the homology modelling as a control to test the template selection method, as the three dimensional structure for this peptide had been determined through nuclear magnetic resonance spectroscopy (NMR) (Vriens et al. 2016). The NMR structure of Rs-AFP2 was obtained from the Protein Data Bank (PDB) and used for the comparative analysis of the constructed homology models. The models obtained were refined and analysed with YASARA structure (Krieger et al. 2002, 2004).

3.3 RESULTS AND DISCUSSION

3.3.1 Sequence characterization of plant defensins Hc-AFP1, Hc-AFP4, Rs-AFP2 and Vv-AMP1

Hc-AFP4 and Rs-AFP2 encode for 81-mer peptides, whereas Hc-AFP1 and Vvi-AMP1 respectively encode for 80- and 77-mer peptides (Table 3.2). Furthermore, sequence analysis confirmed that all the peptides contained a 29-mer signal peptide and had monoisotopic masses ranging between 5.48 and 5.73 kDa. All of these peptides are highly basic with predicted pI values between 8.50 (Hc-AFP1) and 9.37 (Vvi-AMP1).

An amino acid sequence alignment of the mature peptide regions for defensin peptides Hc-AFP1, Hc-AFP4, Rs-AFP2 and Vvi-AMP1 is shown in Figure 3.1 A. All of the defensins contained the

well-conserved eight cysteine residues involved in specific defensin folding, as well as the conserved glycine at positions 13 and 34, an aromatic residue, or tryptophan at position 11, and a glutamic acid at position 29.

Table 3.2: Structural parameters of the defensin peptides in this study.

Defensin	Signal peptide (amino acids)	Mature Peptide (amino acids)	MW (Da)	pI	Reference
Hc-AFP1	1-29	30-80	5479,32	8.50	De Beer and Vivier, 2011
Hc-AFP4	1-29	30-81	5731,61	8.94	De Beer and Vivier, 2011
Rs-AFP2	1-29	30-81	5731,63	9.08	Expassy tool
Vvi-AMP1	1-29	30-77	5495	9.37	De Beer and Vivier, 2008

It is clear from figure 3.1A and Table 3.3 that Rs-AFP2 and Hc-AFP4 shared the highest sequence homology (92%), followed by Hc-AFP1 and Hc-AFP4 with 80% sequence homology. Hc-AFP1, Hc-AFP4 and Rs-AFP2 formed a unique group within the phylogenetic tree in figure 3.1B. Within this unique group, Hc-AFP4 and Rs-AFP2 were the most similar to each other with a well-supported connecting node with a bootstrap value of 100. Hc-AFP4 and Rs-AFP2 are both seed defensins, whereas Hc-AFP1 is expressed in the stem and flowers of *H. coronopifolia* plants (Please refer to Table A3.1 in Addendum A to this chapter for a summary of features of these and other well-characterised defensins). The high level of shared homology between these three peptides can be expected as they all belong to the *Brassicacea* family (Broekaert et al. 1995). Furthermore, they all have membrane permeabilization activities that cause morphological changes to fungal hyphae, placing them in the A3 subgroup of morphogenic defensin peptides (Terras et al. 1992, 1993; Harrison et al. 1997). These peptides also all shared an identical consensus sequence of the γ-core motif, although Hc-AFP1 differed in a few amino acids in the adjoining β2 and β3 sheets.

Vvi-AMP1 however shared low sequence homology with Hc-AFP1 and Hc-AFP4, and no significant sequence homology could be detected between Vvi-AMP1 and Rs-AFP2, using the BlastP analysis. Vvi-AMP1 formed its own group within the phylogenetic tree in figure 3.1.B. It does not induce morphological changes on treated hyphae and is classified as a non-morphogenic defensin (De Beer 2008; De Beer and Vivier 2011) and belongs to subgroup B1 that are known to be non-morphogenic defensin peptides (Terras et al. 1992, 1993; Harrison et al. 1997). Furthermore, form Table 3.1 and figure 3.1 we see that Vvi-AMP1 has a completely different amino acid sequence in its consensus sequence of the γ-core motif compared to that of Hc-AFP1, Hc-AFP4 and Rs-AFP2. The γ-core motif determines the type of antifungal activity of plant defensins. When the γ-core motif of morphogenic defensin MsDef1 was replaced with that of the non-morphogenic defensin MtDef4, the altered peptide became non-morphogenic. Furthermore, this substitution also changed the membrane target, indicating that this motif is directly or indirectly

involved in the interaction of plant defensin peptides with their membrane targets (Sagaram et al. 2011).

The four defensin sequences were compared to a selection of seven (refer to Table 3.1) well characterized plant defensin peptides, with known modes of actions and the results are presented in a phylogenetic tree (Figure 3.2B). Hc-AFP4 and Rs-AFP2 shared high sequence homology with Rs-AFP1 and formed a unique group with a well presented node (bootstrap value of 100) that in turned grouped with Hc-AFP1. Rs-AFP2 is a very well characterized plant defensin peptide and its mode of action involves the binding of a specific fungal membrane target namely glucosylceramide (GlcCer) (Table A3.1, Addendum A). MtDef4 was the closest related peptide to the Vvi-AMP1 amino acid sequence (Figure 3.2), forming a unique grouping with a well-supported node (bootstrap value of 99), followed by NaD1 with a less well supported node (bootstrap value of 30). The γ-core motifs of MtDef4 and Vvi-AMP1 only differed with a single amino acid at position 35. Although MtDef4 and NaD1 possess different membrane targets, they, together with Vvi-AMP1 are all non-morphogenic defensins (Table A3.1, Addendum A). When considering the known activities of the different defensins, it is clear that all the peptides exhibit *in vitro* activity against similar fungal species (refer to the summaries prepared in Addendum A Table A3.1). The well characterised Rs-AFP2 peptide has been tested against many more fungal species, but the four peptides have all shown activity against *Botrytis cinerea* and *Fusarium* species (all peptides were tested at least against these pathogens).

Table 3.3. Sequence homology percentages among defensin peptides used in this study (determined with BLASTp).

Defensin peptide	Hc-AFP1	Hc-AFP4	Rs-AFP2	Vvi-AMP1
Hc-AFP1	100%	80%	76%	37%
Hc-AFP4	80%	100%	92%	30%
Rs-AFP2	76%	92%	100%	NS
Vvi-AMP1	37%	30%	NS	100%

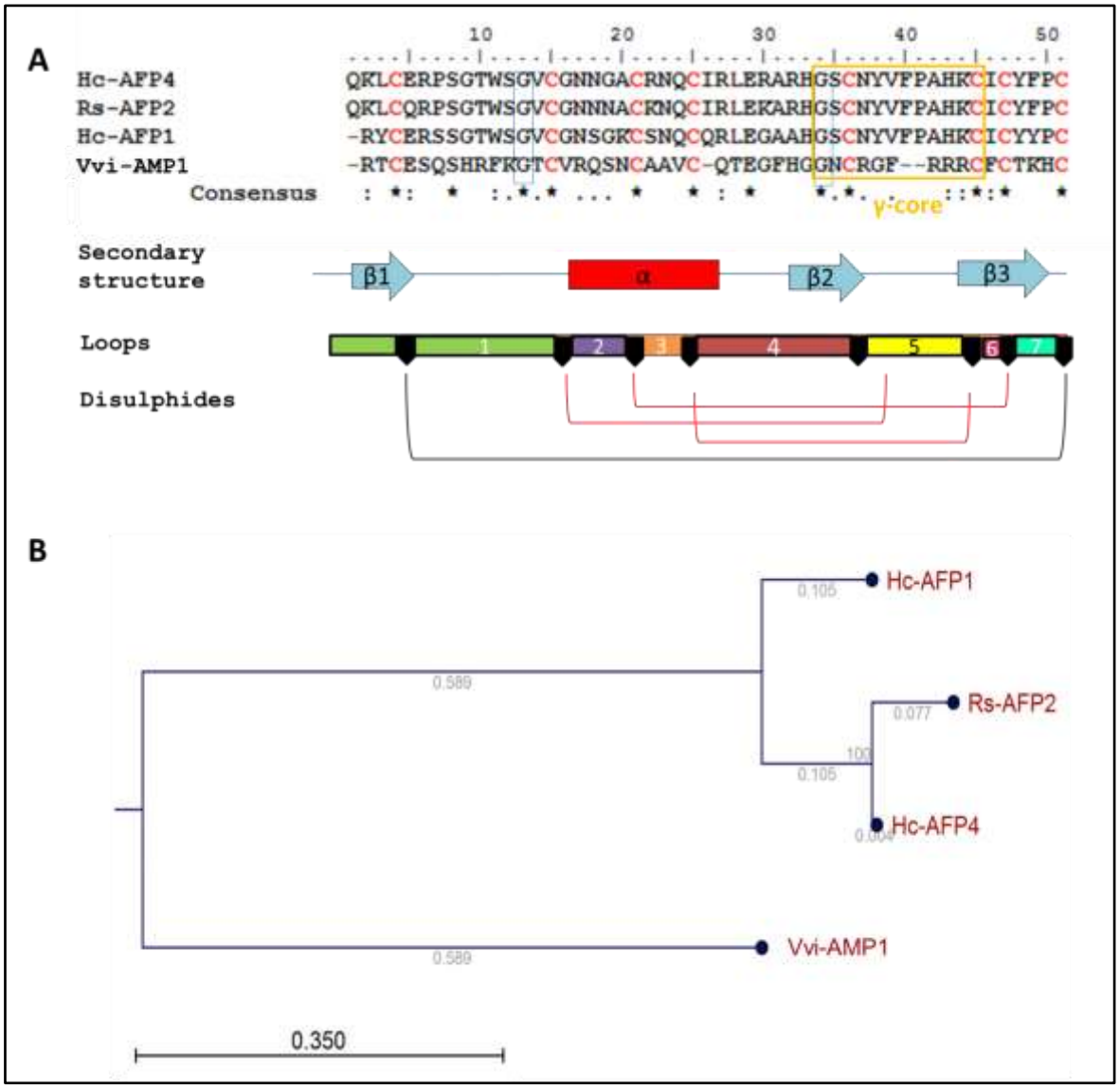

Figure 3.1. (A) Amino acid alignment of the mature regions of plant defensins *Heliophila coronopifolia* antifungal peptide 1 (Hc-AFP1) and *H. coronopifolia* antifungal peptide 4 (Hc-AFP4)*; Raphanus sativus* antifungal peptide 2 (Rs-AFP2), and *Vitis vinifera* antimicrobial peptide 1 (Vvi-AMP1). The eight conserved cysteine residues are indicated in red. Disulphide bridge formation of defensin sequences are indicated. The three disulphide bridges defining the conserved CSαβ motif are indicated in red. Sequences involved in the γ-core motif are boxed in yellow. The relative location of the secondary structures is indicated below the alignment followed by the location of each loop. Alignments were created with the MUSCLE algorithm (Edgar 2004) in CLC sequence viewer (CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark). (B) The phylogenetic relationship between plant defensins Hc-AFP1, Hc-AFP4, Rs-AFP2 and Vvi-AMP1. Phylogenetic trees were created using CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark).

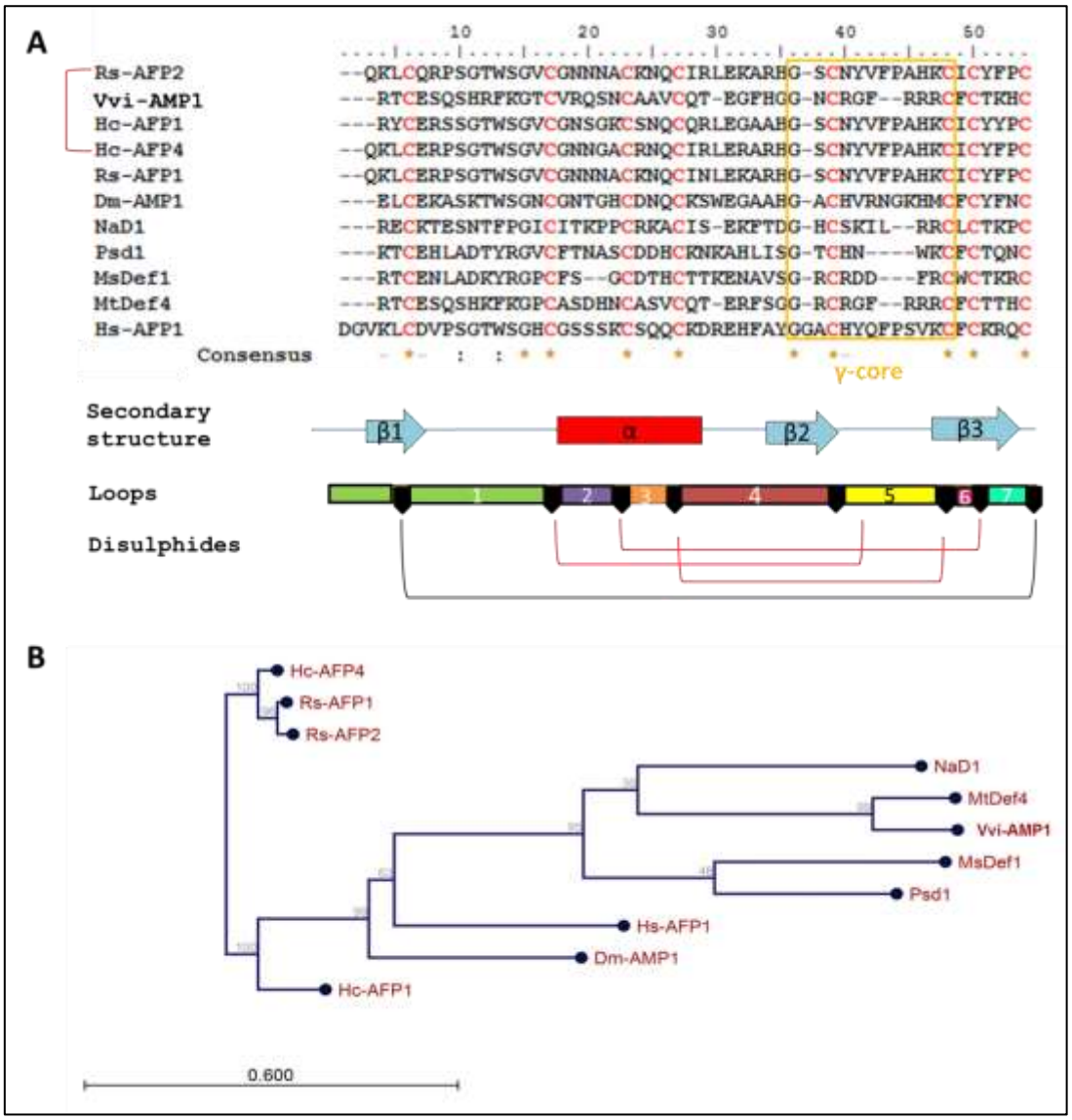

Figure 3.2. (A) Amino acid alignment of the mature regions of plant defensins Hc-AFP1, Hc-AFP2, Rs-AFP2 and Vvi-AMP1 with other members of the plant defensin super family that have been well characterized. The eight conserved cysteine residues are indicated in red. Disulphide bridge formation of defensin sequences is indicated. The three disulphide bridges defining the conserved CSαβ motif are indicated in red. Sequences involved in the γ-core motif are boxed in yellow. The relative location of the secondary structures is indicated below the alignment followed by the location of each loop. Alignments were created with the MUSCLE algorithm (Edgar 2004) in CLC sequence viewer (CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark). (B) The phylogenetic relationship between the plant defensins. Phylogenetic trees were created using CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark).

The sequence alignment and phylogenetic tree analysis were expanded to include the deduced amino acid sequences of the grapevine DEFL genes (refer to Table 3.1), as presented in Figure 3.3. The strong grouping of the *H. coronopifolia* peptides with Rs-AFP2 was maintained and the closest homology with this group was with the Vvi-AMP1, Vvi-AMP2 and Vvi-AMP3 defensins both with well supported nodes and bootstrap values of 100 and 86, respectively. This grouping then connected to the grapevine DEFL 59 peptide (previously renamed to Vvi-AMP4 by Giacomelli et al., (2012)), as closest neighbour in the tree with a weaker bootstrap value of 51. As expected, the *V. vinifera* predicted snakin peptides were the furthest removed from the defensin peptides in the phylogenetic tree.

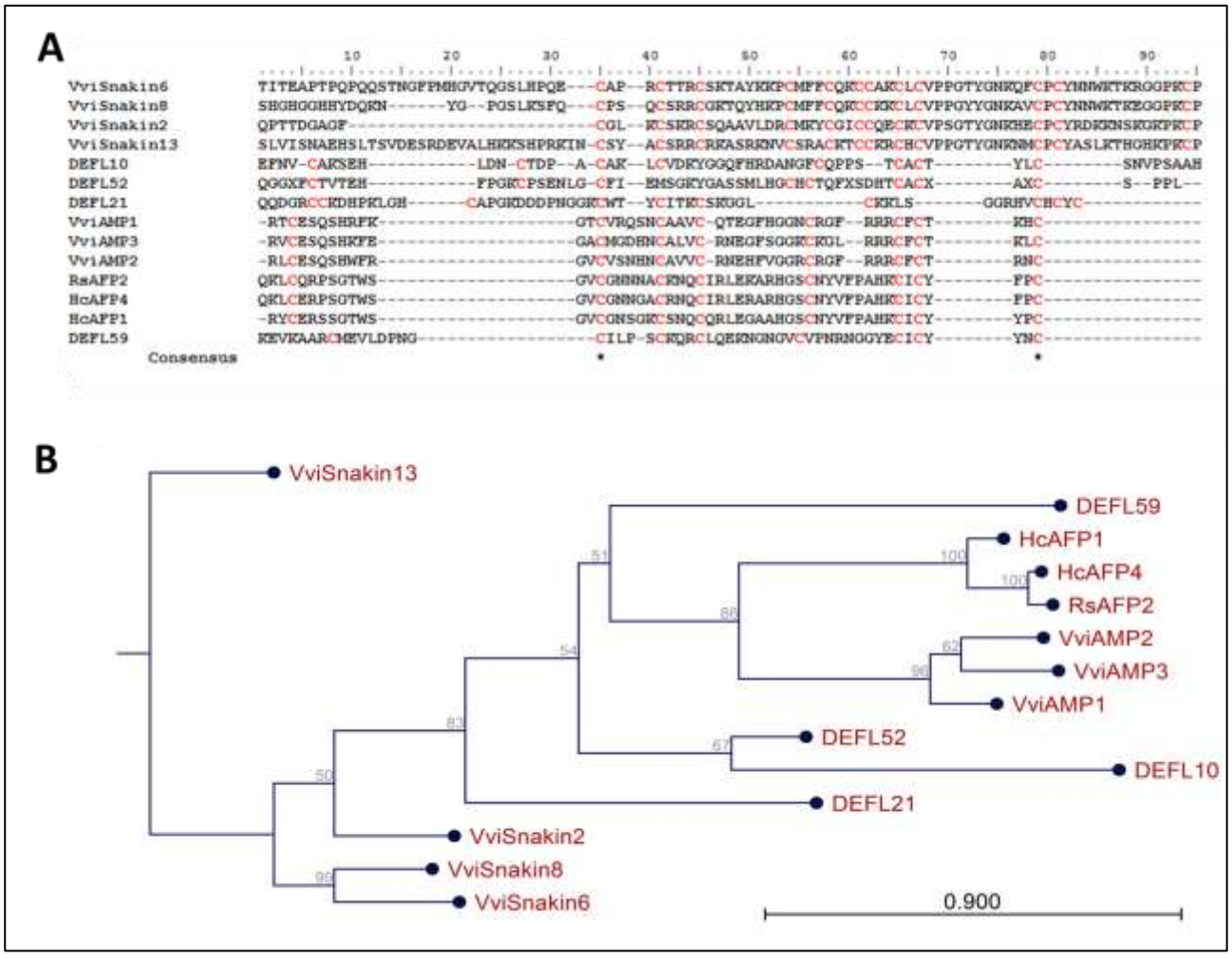

Figure 3.3. (A) Amino acid alignment of the mature regions of plant defensins Hc-AFP1, Hc-AFP2, Rs-AFP2 and Vvi-AMP1 with the deduced amino acid sequences of the grapevine DEFL genes. The eight conserved cysteine residues are indicated in red. Alignments were created with the MUSCLE algorithm (Edgar 2004) in CLC sequence viewer (CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark). (B) The phylogenetic relationship between plant defensins Hc-AFP1, Hc-AFP2, Rs-AFP2 and Vvi-AMP1 and the grapevine DEFL genes. Bootstrap values are indicated at the nodes of the tree. Phylogenetic trees were created using CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark).

3.3.2 Homology modelling of plant defensins Hc-AFP1, Hc-AFP4 and Rs-AFP2

Homology modelling of Hc-AFP1, Hc-AFP4 and Vvi-AMP1 was performed using the SWISS-MODEL online server form The Swiss Institute of Bioinformatics. The template results (refer to Table 3.4) of the homology modelling analysis coincided with the alignment analysis of the plant defensins. In order to test the template search function of SWISS-MODEL, *R. sativus* defensin, Rs-AFP2 was included in the search since the structure of this plant defensin peptide has been determined through NMR analysis (Vriens et al. 2016). The three best templates for the mature peptide sequence of Rs-AFP2 included Rs-AFP1, Rs-AFP2 and AhPDF1.1. However, NMR Rs-AFP2 template displayed the most favourable template and model characteristics and the template search function of SWISS-MODEL proved to be accurate.

AhPDF1.1 was chosen as the best template for the homology modelling of both Hc-AFP1 and Hc-AFP4 as it had the highest sequence identity, similarity and coverage and the models had the most favourable GMQE and QMEAN Z-score values. AhPDF1.1 was shown to play an role in zinc tolerance a function involved in its defence towards the necrotrophic bacteria, *Pectobacterium carotovorum* (Mirouze et al. 2006). The *M. truncatula* defensin 4 however showed the highest sequence identity, similarity and coverage and most favourable GMQE and QMEAN Z-score values for the predicted model as template for Vvi-AMP1. MtDef4 is a non-morphogenic defensin peptide isolated from the model legume plant, *M. truncatula* (El-mounadi et al. 2016).

Table 3.4. Template results of the three best matched to the target peptides and the quality scores of their predicted models. Models are ordered from the highest GMQE value. Templates chosen for homology modelling for each peptide is indicated in red. Secondary structure analysis and homology modelling of the mature peptide sequences were performed using the Swiss model server (https://swissmodel.expasy.org).

	Template								Models	
Defensin	**Template name**	**PDB ID**	**Sequence identity (%)**	**Oligo-state**	**Method**	**Sequence similarity**	**Range**	**Coverage**	**GMQE**	**QMEAN Z-score**
Hc-AFP1	Ah-PDF1.1	2m8b.1.A	80	Monomer	NMR	0,60	1-50	1,00	0,95	-0,92
	Rs-AFP2	2n2r.1.A	76	Monomer	NMR	0,59	1-50	1,00	0,92	-1,94
	Rs-AFP1	1ayj.1.A	76	Monomer	NMR	0,58	1-50	1,00	0,95	-3,71
Hc-AFP4	Rs-AFP1	1ayj.1.A	92	Monomer	NMR	0,65	2-51	0,98	0,99	-3,41
	Rs-AFP2	2n2r.1.A	92,16	Monomer	NMR	0,65	1-51	1,00	0,99	-2,91
	Ah-PDF1.1	2m8b.1.A	94	Monomer	NMR	0,66	2-51	0,98	0,98	-1,41
Vvi-AMP1	Mt-DEF4	2lr3.1.A	76,6	Monomer	NMR	0,58	1-47	1,00	0,82	-1,61
	NaD1	6b55.1.A	40,43	Homo-20-mer	X-ray	0,43	1-47	1,00	0,78	-0,63
	NaD1	6b55.1.J	40,43	Homo-20-mer	X-ray	0,43	1-47	1,00	0,78	-0,54
Rs-AFP2	Rs-AFP1	1ayj.1.A	0,96	Monomer	NMR	0,66	2-51	0,98	0,99	-3,37
	Rs-AFP2	2n2r.1.A	100	Monomer	NMR	0,67	1-51	1,00	0,99	-2,69
	Ah-PDF1.1	2m8b.1.A	90	Monomer	NMR	0,64	2-51	0,98	0,98	-0,92

In order to further test the SWISS model sever the predicted model of Rs-AFP2 was compared and aligned to the determined structure of Rs-AFP2 (Figure 3.4). The homology model of Rs-AFP2 had a very good fit to its template with the distribution of the conformational angles of residues all in the favourable and allowed regions of the Ramachandran plot (Figure 3.4D).

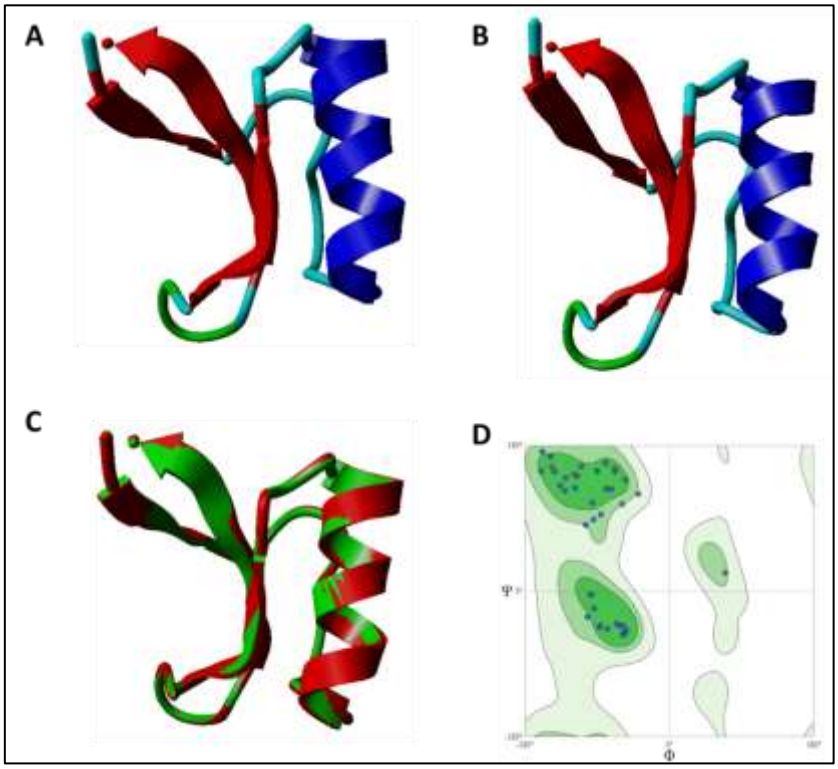

Figure 3.4. (A) Rs-AFP2 predicted model by Swiss model server (B) Rs-AFP2 structure determined with NMR (C) Structural alignment of Rs-AFP2 predicted model (red) and template, Rs-AFP2 structure (NMR) (green). (E) Ramachandran plot of the generated Rs-AFP2 model.

The sequence alignments and the models of the different peptides and their respective templates are shown in Figures 3.5 (Hc-AFP1 and template, AhPDF1.1), Figure 3.6 (Hc-AFP4 and template, AhPDF1.1) and Figure 3.7 (Vvi-AMP1 and template MtDef4). From the alignment analysis of the mature peptide region of Hc-AFP1 with template AhPDF1 it was clear that there were 10 amino acid differences, with the majority residing in the α-helix region. In spite of these differences, the homology model of Hc-AFP1 had a good fit to its template AhPDF1.1 with the distribution of conformational angles of resides all in favourable and allowed regions of the Ramachandran plot (Figure 3.5 E). Hc-AFP4, with the same template for homology modelling, only differed from the template with four amino acids. Most of the amino acid differences occurred in the β1 sheet and Loop 1 region and with one amino acid difference in the Loop 4 region (Figure 3.6). Overall this model, like that of Hc-AFP1, had a good fit to the template AhPDF1, as seen in the Ramachandran plot in figure 3.6 E, with the distribution of conformational angles of resides all in favourable and allowed regions.

Vvi-AMP1 was the peptide with the highest divergence from its template MtDef4, with 11 amino acid differences. The majority of these resided in the α-helix region (Figure 3.6 A-C), but the homology model still had a good fit to the template (figure 3.6E).

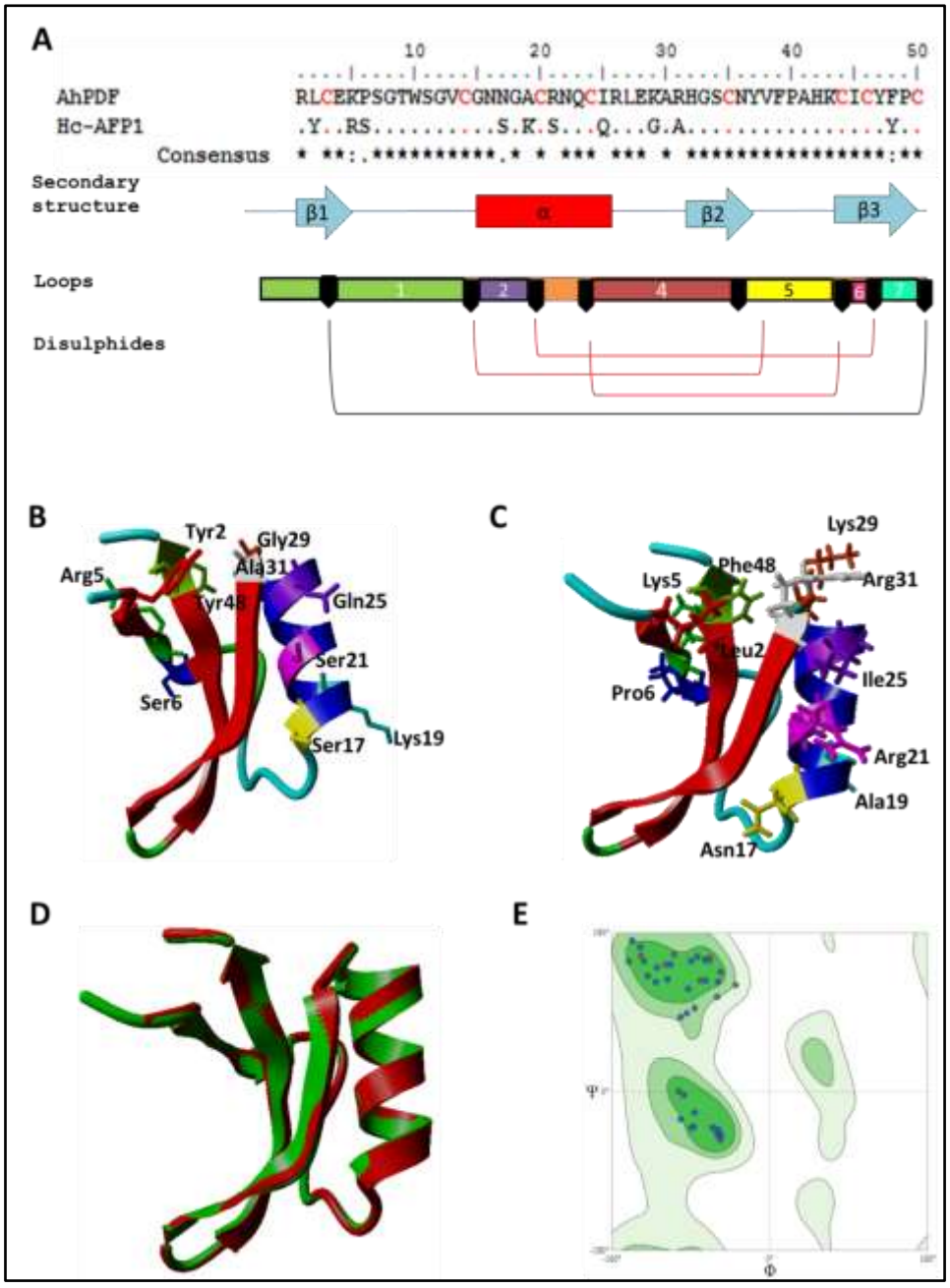

Figure 3.5. (A) Amino acid alignment of the mature regions of plant defensins Hc-AFP1 and its most suitable template AhPDF1.1. The eight conserved cysteine residues are indicated in red. Disulphide bridge formation of defensin sequences is indicated. The three disulphide bridges defining the conserved CSαβ motif are indicated in red. The relative location of the secondary structures is indicated below the alignment followed by the location of each loop. Alignments were created with the MUSCLE algorithm (Edgar 2004) in CLC sequence viewer (CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark). Structural models of (B) Hc-AFP1 and (C) AhPDF1.1 depicting the amino acid differences observed in the alignment. (D) Structural alignment of Hc-AFP1 (red) and template, AhPDF1 (green). (E) Ramachandran plot of the generated Hc-AFP1 model.

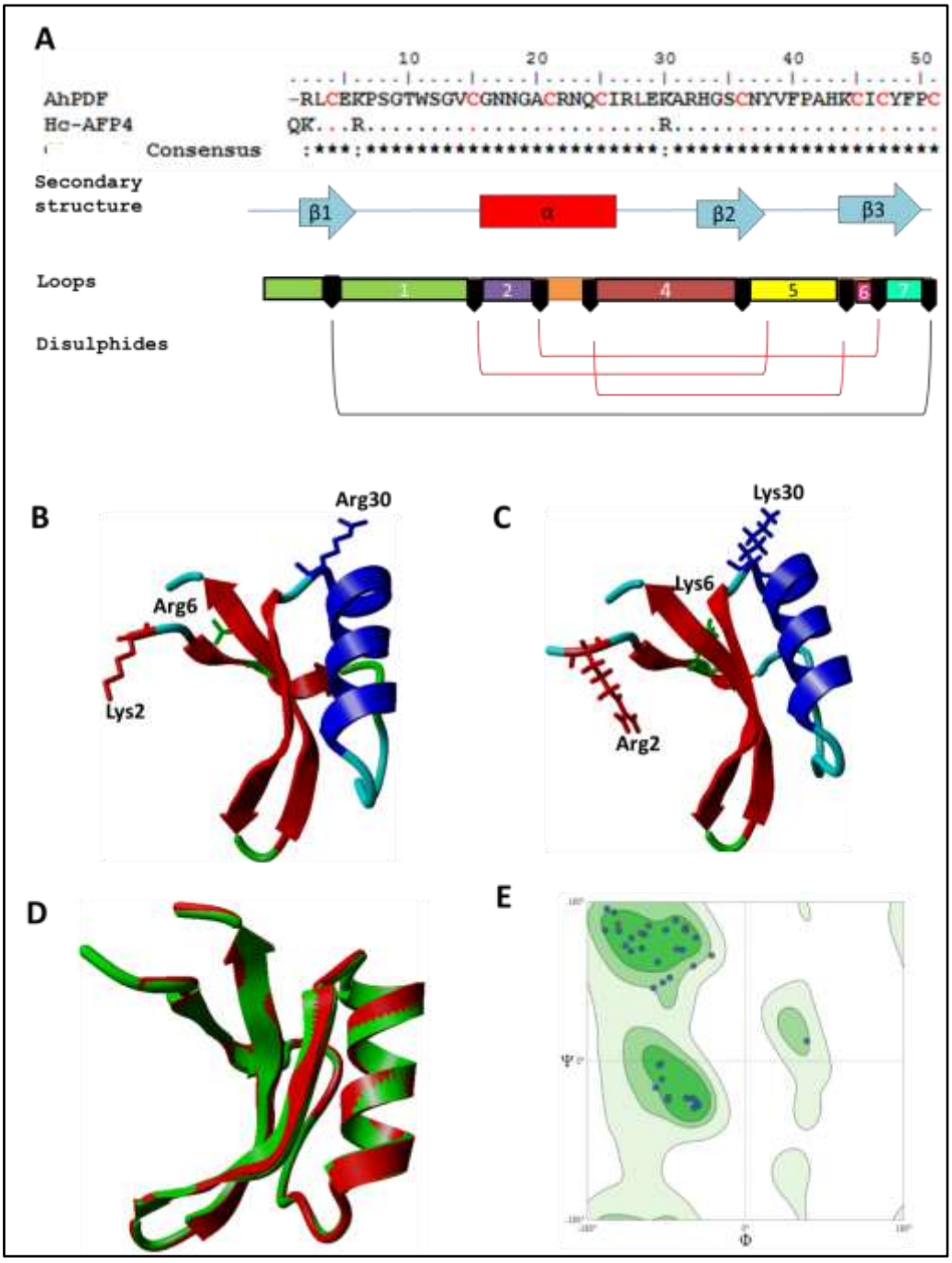

Figure 3.6. (A) Amino acid alignment of the mature regions of plant defensins Hc-AFP4 and most suitable template AhPDF1.1. The eight conserved cysteine residues are indicated in red. Disulphide bridge formation of defensin sequences is indicated. The three disulphide bridges defining the conserved CSαβ motif are indicated in red. The relative location of the secondary structures is indicated below the alignment followed by the location of each loop. Alignments were created with the MUSCLE algorithm (Edgar 2004) in CLC sequence viewer (CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark). Structural models of (B) Hc-AFP4 and (C) AhPDF1.1 depicting the amino acid differences observed in the alignment. (D) Structural alignment of Hc-AFP4 (red) and template, AhPDF1 (green). (E) Ramachandran plot of the generated Hc-AFP4 model.

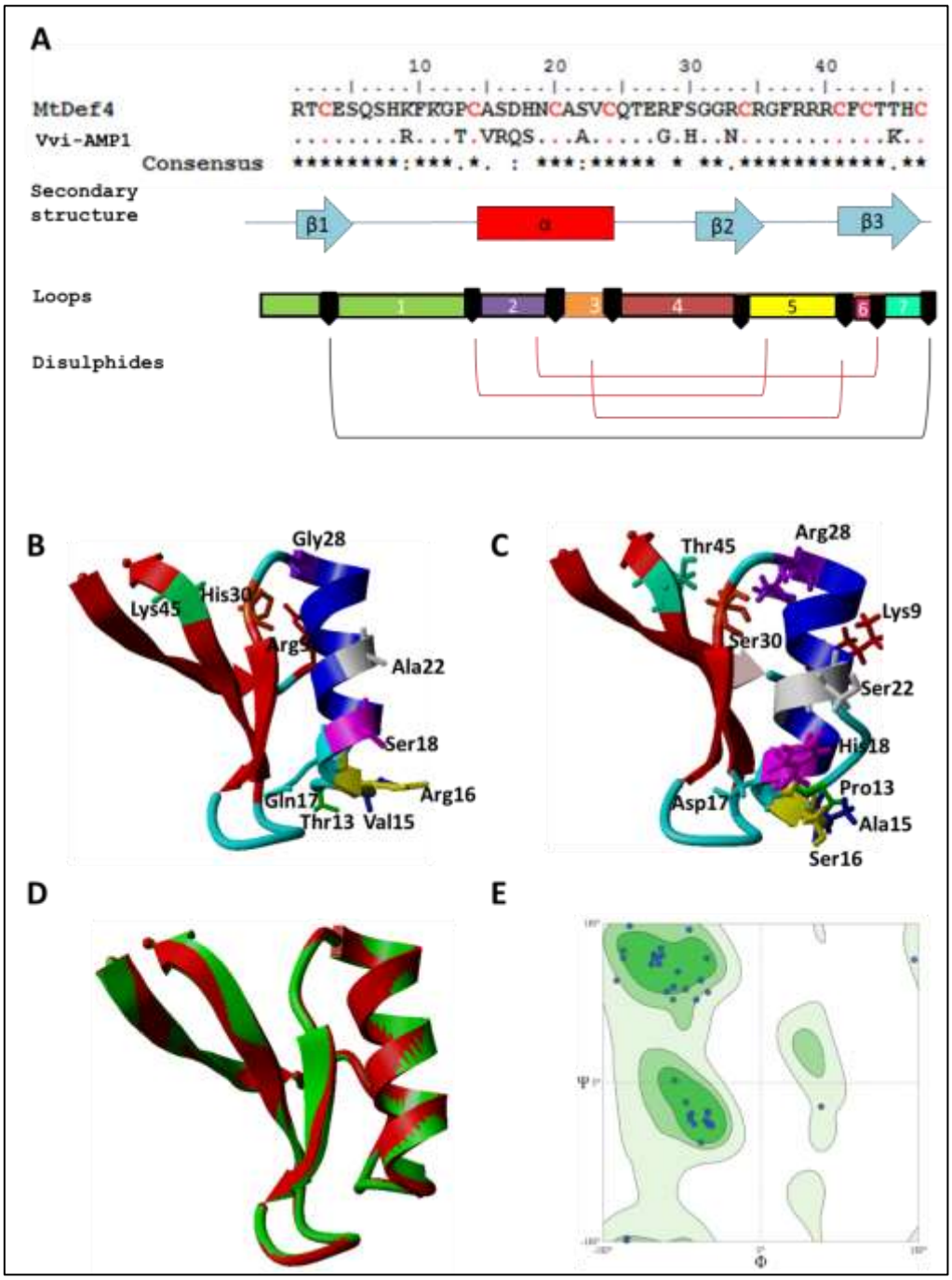

Figure 3.7. (A) Amino acid alignment of the mature regions of plant defensins Vvi-AMP1 and most suitable template MtDef4. The eight conserved cysteine residues are indicated in red. Disulphide bridge formation of defensin sequences is indicated. The three disulphide bridges defining the conserved CSαβ motif are indicated in red. The relative location of the secondary structures is indicated below the alignment followed by the location of each loop. Alignments were created with the MUSCLE algorithm (Edgar 2004) in CLC sequence viewer (CLC sequence viewer version 8.0 (QIAGEN Aarhus A/S, Kingdom of Denmark). Structural models of (B) Vvi-AMP1 and (C) MtDef4 depicting the amino acid differences observed in the alignment. (D) Structural alignment of Vvi-AMP1 (red) and template, MtDef4 (green). (E) Ramachandran plot of the generated Vvi-AMP1 model.

3.3.3. Backbone alignment of homology models Hc-AFP1, Hc-AFP4, Vvi-AMP1 and the NMR determined structure of Rs-AFP2

Although plant defensins share limited sequence homology, their three dimensional structure is highly conserved. Homology modelling revealed that all the defensins under study had the typical

defensin structure consisting of an α-helix with three helical turns and three antiparallel β-sheets, stabilized by four disulphide bridges (Bruix et al. 1993; Fant et al. 1998). The aligned backbones of the homology models of all the peptides are presented in figure 3.8A. This model again confirmed that the backbone structures of Hc-AFP1, Hc-AFP4 and Rs-AFP2 formed a more compact alignment with each other than that of Vvi-AMP1. Furthermore, it was clear from this structural alignment that there were significant differences in the loops on either side of the α-helix and within the α-helix region, with the greatest differences in the loop connecting the β2 and β3 sheets. This loop constitutes the γ-core motif of the peptides and as mentioned earlier this motif is responsible for determining the antifungal activity of plant defensin peptides. According to the global RMSD values of the alignments, Vvi-AMP1 and Rs-AFP2; and Vvi-AMP1 and Hc-AFP4 differed the most in structures from one another. In contrast, Rs-AFP2 and Hc-AFP4 had the most similar structures with the lowest RMSD value and is consistent with the sequence alignments of these four peptides.

Structure function analysis is a valuable tool in the prediction of peptide functions. From plant defensins we know that the antifungal activity of these peptides is mainly concentrated in the loop connecting the β2 and β3 sheets. Synthetic peptides synthesized from the loop connecting the β2 and β3 sheets of Rs-AFP2 were demonstrated to have similar activities as the Rs-APF2 peptide itself (Schaaper et al. 2001). Furthermore, the four *H. coronopifolia* defensin peptides showed 72% homology at amino acid level, but differed in their activities (De Beer and Vivier 2011). Homology modelling revealed that these peptides formed two structurally defined groups with Hc-AFP1 and Hc-AFP3 in one group and Hc-AFP2 and Hc-AFP4 in another group. This grouping on structural level was reflected in their biological activities as the two peptides within a group showed similar IC_{50} values towards the organisms tested (De Beer and Vivier 2011). Therefore, comparing plant defensin peptides on sequence and three-dimensional level can be very valuable in predicting their biological activity.

It would be interesting to see if the predicted structural variation in the different peptides will lead to phenotypic differences in the transgenic grapevine plants that overexpress them. From literature two plant defensins isolated from petunia, *Petunia hybrida* defensin 1 (PhD1) and, *P. hybrida* defensin 2 (PhD2) was overexpressed in banana. These peptides share 76% amino acid sequence identity, but had differential activity *in vitro.* This difference on sequence level and the differences in their *in vitro* activities were, however, not reflected in the transgenic banana plants as the transgenic plants overexpressing PhD1 and PhD2 presented the same level of resistance towards *Fusarium oxysporum* infection (Lay et al. 2003a; Ghag et al. 2012).

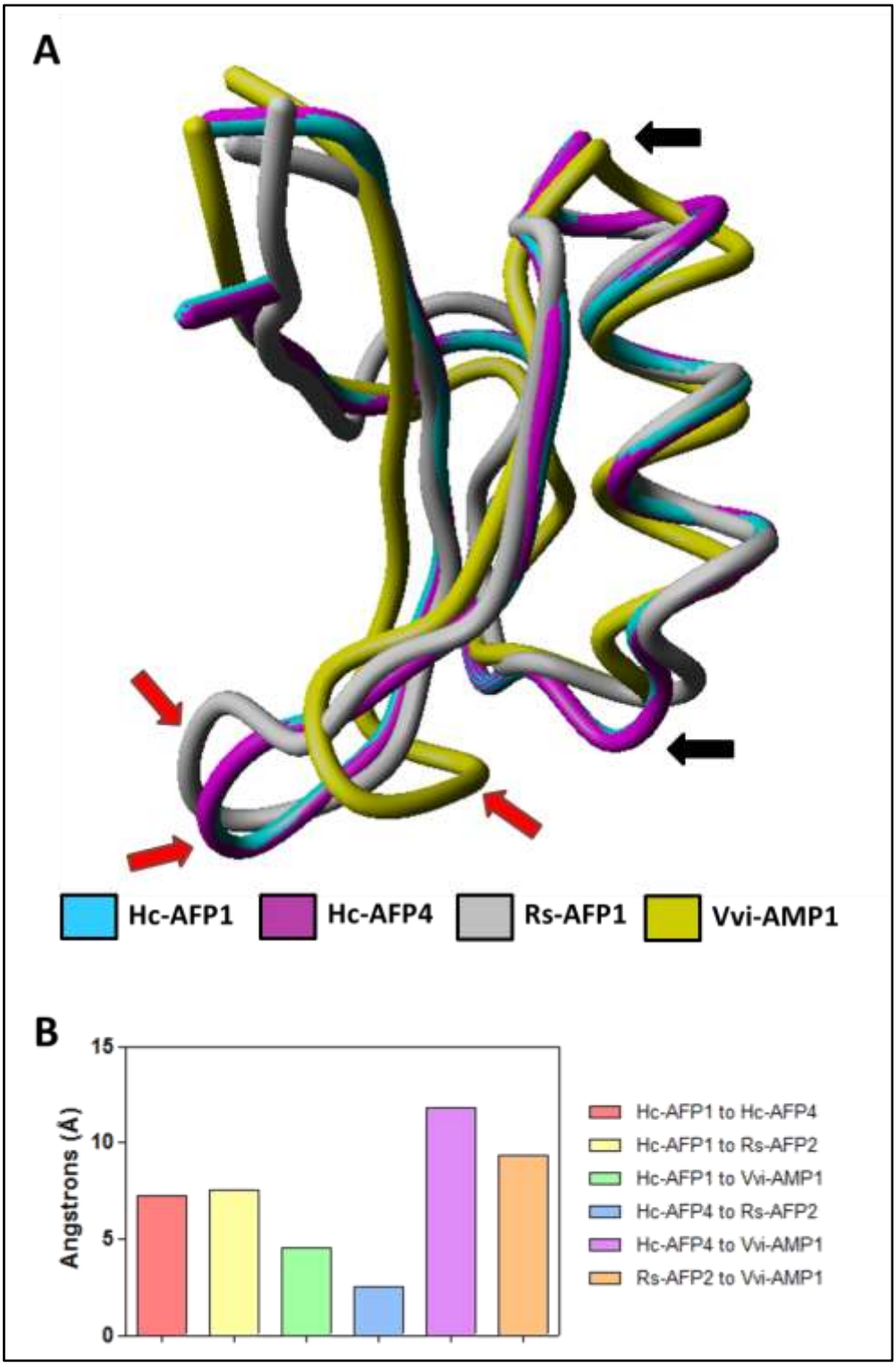

Figure 3.8. (A) Structural alignment of the backbones of Hc-AFP1, Hc-AFP4, Rs-AFP2 and Vvi-AMP1 with (B) the global root mean square deviation (RMSD) of the alignment analysis. Black arrows indicate positions of significant differences in structure, whereas the red arrows indicate the difference in presentation of the loop 5 region connecting β-sheets 2 and 3.

3.4 CONCLUSIONS

From the data presented in this chapter we conclude that the Hc-AFP1, 4 and Rs-AFP2 peptides were more related to one another on sequence and structural level compared to the Vvi-AMP1 peptide. The major differences between these peptides resided within the sequence and structure of the γ-core motif. This motif is known to determine the type of antifungal activity of plant defensins and this is reflected in the *in vitro* activity of these peptides with Hc-AFP1, 4 and Rs-AFP2 peptides being morphogenic peptides and Vvi-AMP1 being a non-morphogenic defensin. It remains to be established whether the few structural differences in the peptides and the different *in vitro* activities of these peptides will also be reflected in their *in vivo/ in planta* activities and roles.

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Addendum A to Chapter 3

This Addendum contains relevant and additional data not shown in Chapter 3.

Table A3.1. Summary of some of the characteristics of the antifungal activity of various defensin peptides.

Defensin	Origen	Expression in native hosts	*In vitro* fungal activiy	Morphogenic/ Non-morphogenic	Membrane permeabilization	Membrane target	References
Hc-AFP1	*H. coronopiflia*	Stem and Flowers	*Botrytis cinerea; Fusarium solani*	Morphogenic	Yes	ND	De Beer and Vivier et al 2011
Hc-AFP4	*H. coronopiflia*	Seeds	*Botrytis cinerea; Fusarium solani*	Morphogenic	Yes	ND	De Beer and Vivier et al 2011
Rs-AFP1	*R. sativus*	Seeds	*Botrytis cinerea; Fusarium culmorum; Alternaria brassicicola*	Morphogenic		ND	Terras et al 1995; Cools et al 2017
Rs-AFP2	*R. sativus*	Seeds	*Botrytis cinerea; Fusarium culmorum; Fusarium oxysporum; Alternaria brassicicola; Colletotrichum lindemuthianum; Ascochyta pisi; Nectria haematocca; Phoma betae; Pyricularia oryzae; Trichoderma haatum; Verticullium dahliae*	Morphogenic	Yes	GlcCer	Terras et al 1992; Osborn et al 1993; Cools et al 2017

Table A3.1. (cont.)

Defensin	Origen	Expression in native hosts	*In vitro* fungal activiy	Morphogenic/ Non-morphogenic	Membrane permeabilization	Membrane target	References
Vvi-AMP1	*V. vinifera*	Berries	*Verticulim dahliae; Fusarium oxysporim; Botrytis cinerea*	Non-morphogenic	Yes	ND	De Beer and Vivier et al 2008
Dm-AMP1	*Dahlia merkii*	Seeds	*Alternaria brassicicola, A. flavus, B. cinerea, C. albicans, C. glabrata, C. krusei, C. sphaerospermum, F. solani, F. culmorum, L. maculans, N. crassa, P. digitatum, S. cerevisiae, S. tritici, Trichoderma viride, V. albo-atrum*	Non-morphogenic	Yes	$M(IP)_2C$, ergosterol	Osborn et al 1995; Thevissen et al 2000; Thevissen et al 2007; Parisi et al 2018; Hayes et al 2013; Cools et al 2017; Aerts et al, 2006; Thevissen 1996; Thevissen et al 1999

Table A3.1. (cont.)

Defensin	Origen	Expression in native hosts	*In vitro* fungal activiy	Morphogenic/ Non-morphogenic	Membrane permeabilization	Membrane target	References
NaD1	*Nicotiana alata*	Flowers	*Aspergillus nidulans, B. cinerea, Candida albicans, Colletotrichum graminicola, Cryptococcus gattii, Cryptococcus neoformans, Fusarium graminearum, F. oxysporum, Puccinia coronate, P. sorghi, Saccharomyces cerevisiae, Thielaviopsis basicola, Verticillium dahliae*	Non-morphogenic	Yes	$PI(4,5)P_2$	Lay et al 2003; Hayes et al 2013; Van weerden et al 2010; Bleakley et al 2014; Parisi et al 2018; Dracatios et al 2014; Cools et al 2017; Lay et al 2003; Poon et al 2014; Payne et al 2016; Bleakly et al 2016; Van weerden et al 2008; Lay et al 2012
Psd1	*Pisum sativum*	Seeds	*Aspergillus niger, Avicularia. versicolor, Fusarium solani, F. moniliformae, F. oxysporum, Neurospora crassa, S. cerevisiae, T. mentagrophytes*	-	No	GlcCer, ergosterol	Almeida et al 2000; Parisi et al 2018;
MtDef4	*Medicago trucatula*	Seeds	*F. graminearum, N. crassa, Puccinia tritici*	Non-morphogenic	Yes	PA	Sagaram et al 2011; El-Mounandi et al 2016; Anderson et al 2016; Parisi et al 2016; Cools et al 2017

Table A3.1. (cont.)

Defensin	Origen	Expression in native hosts	*In vitro* fungal activiy	Morphogenic/ Non-morphogenic	Membrane permeabilization	Membrane target	References
MsDef1	*Medicago sativa*	Seeds	*F. graminearum, N. crassa V. dahlia*	Morphogenic	Yes	GlcCer, PI(3,5)P2	Sagaram et al 2011; Spelbrink et al 2004;Ramamoorthy et al 2007; Parisi et al 2018; Cools et al 2017
HsAFP1	*Heuchera sanguinea*	Seeds	*F. culumorum, Candia albicans, B. cinerea, Cladusporium sphaerospernum, Leptoshaera maculans, Pennicilium digitatum, Trichoderma viride, Streptoria tritici, Verticilium albo-atrum*	Morphogenic	Yes	ND	Osborn et al 1995; Vriens et al 2015; Aerts et al 2011

ND = Not determined

Glc: Glucosylceramide; M(IP)2C: Mannosyldiinositolphosphorylceramide; PA: Phosphatidic acid; PI(4,5)P2: Phosphatidylinositol (4,5) biphosphate

Chapter 4

Research results

Genetic, phenotypical and physiological characterization of seven transgenic grapevine populations ectopically expressing plant defensin peptides

The authors contributed as follows to the work presented: Mr Cobus Smit constructed the *in silico* pipeline used in this analysis. HB performed all analysis, data-interpretation and compiling of results under the guidance of MAV and MR; HB drafted and finalized the chapter with inputs MAV and MR

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RESEARCH RESULTS

Genetic, phenotypic and physiological characterization of seven transgenic grapevine populations ectopically expressing defensin peptides

4.1 INTRODUCTION

Plant defensin peptides have broad spectrum antifungal activity and are non-toxic towards eukaryotic cells. Furthermore, plant defensin peptides are single gene products ensuring the rapid delivery of these peptides without an excessive energy input form the plant (Osborn et al. 1995; Thomma et al. 2002). These peptides have been transformed into a wide range of plants with subsequent enhanced broad range fungal resistance (Stotz et al. 2013; Lacerda et al. 2014; Goyal and Mattoo 2014). The majority of these transgenic plant defensin studies have been focussed on confirming that the transgenically produced peptides are active, or whether the transgenic plants have increased defence against infections. The transgenic plants were rarely comprehensively phenotyped to provide an understanding of the *in planta* function of these peptides. Forward and reverse genetics and mutant analysis to aid in our understanding of functional impacts of defensins are also largely still lacking.

Only a few reports have been made on phenotypical changes and growth defects induced by the ectopic expression of plant defensin peptides. The overexpression of a tomato defensin, Defensin 2 (Def2) in tomato cv. Zhongshu 5 affected the regeneration ability and only a limited amount of transformed plantlets could be recovered. Moreover, the growth of these transgenic tomato plants was also affected by the ectopic expression of this peptide. The transgenic seedlings had smaller leaves, the maturation of the fruits was delayed and the tomato fruits generated by these transgenic plants showed defects in seed production. The flower development and maturation of the pollen grains were also negatively affected (Stotz et al. 2009). Another report of non-defence-related phenotypes induced by the ectopic expression of plant defensin peptides was of the ectopic expression of *Nicotiana alata* defensin 1 (NaD1) in cotton plants. The ectopic expression of this plant defensin induced toxic effects in the transgenic cotton plants that included growth defect and infertility. These toxic effects were correlated to the level of NaD1 expression in the transgenic plants (Anderson et al. 2009). Similarly, transgenic tobacco plants ectopically expressing a defensin-like gene (Spi1) isolated from the Norway spruce had low germination levels and were difficult to maintain (Elfstrand et al. 2001). In addition, the plant defensins Rs-AFP2, MsDef1 and MtDef2 from *Raphanus sativus, Medicago sativa and M. tracatula* respectively have demonstrated to inhibit the growth of roots and root hair of the well-established plant model *Arabidopsis*. (Allen et al. 2008; De Oliveira Carvalho and Gomes 2011; Parisi et al. 2018).

Plant defensin peptides are known to be constitutively expressed or induced in reaction to an external stimulus like pathogen infection, wounding or hormones (Broekaert et al. 1995; Thomma et al. 2002; De Beer and Vivier 2008). An *in silico* expression analysis conducted on grapevine defensin-like (DEFL) genes demonstrated that the putative co-expression patterns of these genes are driven by tissue and cultivar specificity and are developmentally regulated. The expression of *Vitis vinifera* snakin 8 (VviSnakin8) and *V. vinifera* antimicrobial peptide 3 (Vvi-AMP3) were found to be cultivar specific and was exclusively expressed in the aerial tissues of Cabernet Sauvignon. In contrast, the expression of *V. vinifera* snakin 6 (VviSnakin6) was also tissue specific with expression exclusively in aerial tissues, but in various cultivars (Du Plessis 2012). Furthermore, *V. vinifera* antimicrobial peptide 1 (Vvi-AMP1) was isolated from the cultivar Pinotage and the expression was demonstrated to be exclusively in the berry tissues from the onset of berry ripening (De Beer and Vivier 2008). However, Giacomelli et al. (2012) demonstrated that Vvi-AMP1 is expressed in other tissues than berries in the cultivar Pinot noir, further corroborating the cultivar specific expression of some of these genes.

Here we present an analysis of seven transgenic grapevine populations expressing plant defensins: Four populations in *V. vinifera* cultivar Sultana expressing Vvi-AMP1, Hc-AFP1, Hc-AFP4 and Rs-AFP2 encoding genes; and three populations in *V. vinifera* cv. Red Globe expressing Hc-AFP1, Hc-AFP4 and Rs-AFP2 encoding genes. The *Heliophila coronopifolia* antifungal peptide 1 (Hc-AFP1) is exclusively expressed in the stem and flower tissue of its native host, whereas *H. coronopifolia* antifungal peptide 4 (Hc-AFP4) and the radish defensin, *Raphanus sativus* antifungal peptide 2 (Rs-AFP2) are exclusively expressed in the seeds of their hosts.

The populations expressing Hc-AFP1, Hc-AFP4 and Rs-AFP2 had to be characterised in this study, whereas the Sultana population expressing Vvi-AMP1 was already previously geneticially characterised, as well as for defence phenotypes (Tredoux 2011; Du Plessis 2012) and was included here to expand on the previous characterisation. To contextualise the native grapevine defensin gene expression, the gene expression patterns of known grapevine DEFL genes in the grapevine gene atlas (Fasoli et al. 2012) is presented. The uncharacterised transgenic populations were analysed to confirm their transgenic status, analyse gene integration patterns, as well as defensin gene expression. Furthermore, in order to determine if the ectopic expression of these plant defensin peptides impacted on growth characteristics of the populations, the lines were subjected to phenotypical characterisation for growth parameters and a subset was analysed for photosynthetic capacity.

4.2 MATERIALS AND METHODS

4.2.1 Gene expression analysis: *In silico* pipeline construction, analysis and data mining

The *in silico* methodology described below was developed by Mr. Cobus Smit (IWBT, Stellenbosch University). Sixteen previously identified DEFL genes (Du Plessis 2012) were analysed on the NimbleGen 090918 *Vitis vinifera* exp HX12 array (NCBI GEO Acc. GPL13936). Firstly, a tBLASTn search against the CRIBI.v1 transcriptome was performed to identify putative DEFL-like genes matching the sixteen target genes. Expression patterns for the putative DEFL-like genes were extracted for specific experiments on the NimbleGen platform using a computational method written in Python. Expression patterns were visualised as cluster maps using z-score normalisation, average linkage and Euclidian distancing. The output form this *in silico* pipeline was used to give an overview of the expression patterns of the DEFL genes in the grapevine expression atlas (Fasoli et al. 2012). Significant up or down regulation of these DEFL-genes were extracted and further analysed. Line and bar graphs of the expression data (log_2 fold change) were constructed using GraphPad PRISM 5 for windows (©1992-2007 GraphPad Software, La Jolla California USA, www.graphpad.com).

4.2.2 Plant growth conditions

The characterised Vvi-AMP1 *V. vinifera* (cv. Sultana) and the uncharacterised, putative transgenic *V. vinifera* (cv. Sultana and cv. Red Globe) populations overexpressing Hc-AFP1, Hc-AFP4, Rs-AFP2 respectively were obtained from the grapevine transformation and regeneration platform of the IWBT. The vector construction and the plant transformation are described in Addendum A to Chapter 4. The transgenic populations were maintained *in vitro* on Murashige and Skoog basal salt mixture (Murashige and Skoog 1962) with 15 g/L sucrose. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C for 20 minutes. Commercially available expanded perlite was used as a substrate. The cultures were maintained in a tissue culture growth room at constant temperature of 24±2°C subjected to a 16 hour photoperiod with a light intensity of 50 µ $mol.s^{-1}.m^{-2}$ provided by cool-white fluorescent tubes. Sub-culturing was executed every 6 weeks. The transgenic plantlets from each line, as well as untransformed controls were multiplied *in vitro* to establish a working population for hardening off and subsequent experimentation. The transgenic Sultana) population ectopically expressing Vvi-AMP1 had previously been evaluated for their transgenic status and the data for the lines selected to work with in this analysis is shown in Table 4.1 (Tredoux 2011).

Vegetatively propagated plantlets from the selected transgenic lines, as well as several wild type *V. vinifera* (cv. Sultana) and *V. vinifera* (cv. Red Globe) individual plants were systematically hardened off in perlite and water. These plantlets were maintained at 25°C and moderate humidity in a greenhouse environment. The humidity was systematically reduced over time and the plantlets

were transferred to potting soil (Double Grow, Durbanville, South Africa) and maintained under the same conditions. These plants were watered twice a week and supplemented with Nitrosol natural organic plant food (Envirogreen Pty Limited, Fleuron, Braamfontein, South Africa) once every four weeks.

Table 4.1. Genotypical characterisation of four transgenic *V. vinifera* (cv. Sultana) lines ectopically expressing the Vvi-AMP1 plant defensin (obtained from Tredoux, 2011) and the experiments these lines were used in. Characterization included PCR screening. Southern and Northern Blot analysis. Number of integrations refers to the number of copies of Vvi-AMP1 transgene integrated into the genome. A "+" indicates a positive result whereas a "-" indicates a negative result.

Cultivar	Construct	Plant line	PCR	Southern Blot	Number of transgene integrations	Northern Blot	Experiments
Sultana	**Vvi-AMP1**	8	+	+	2	+	Phenotyping
		9	+	+	3	+	Phenotyping
		14	+	+	3	+	Phenotyping
		19	+	+	5	+	Leaf gas exchange measurements

4.2.3 PCR screening and Southern Blot analysis

Transgenic grapevine lines transformed with the respective *Hc-AFP1, Hc-AFP4* and *Rs-AFP2* genes were PCR screened and analysed by Southern Blot to confirm the successful integration of the transgene and determine the copy number of each line.

The initial PCR screening was performed by Dr K du Plessis (IWBT). Leaf discs were harvested form putative transgenic and WT *in vitro* plantlets using a standard paper punch. The Sigma REDExtract-N-Amp^{TM} Plant PCR Kit was used for DNA extraction and PCR screening according to the manufacturer's instructions to confirm transgene presence. Oligonucleotide primers were designed (Table 4.2) to amplify the coding region of the specific transgenes (De Beer 2008). The typical PCR reaction mixture consisted of 5 x PCR Ready Mix (containing Hot Start antibody for specific amplification of genomic DNA and an inert dye that acts as a tracking dye), 0.25 µM primers and 100 ng of template in 20 µl reactions. PCR program was as follows: Initial denaturation at 95°C for 5 min; followed by 40 amplification cycles consisting of denaturation at 95°C for 30 sec, elongation at 60°C for 30 sec; and a final 5 min elongation step at 72°C.

Table 4.2. Primers and their amplification products used in this study.

Primer name	Sequence	Primer partner	Tm (˚C)
Hc-AFP1 5'	CGCGAAGCTTAGGTACTGTGAGAGATCGAG	Hc-AFP1 3'	64
Hc-AFP1 3'	CGCGGGATCCTCAACATGGGTAGTAACAGA	Hc-AFP1 5'	64
Hc-AFP4 5'	CGCGAAGCTTCAGAAGTTGTGTGAGAGACC	Hc-AFP4 3'	64
Hc-AFP4 3'	CGGCGGATCCTTAACATGGGAAGTAACAGA	Hc-AFP4 5'	63

Table 4.2. (cont.).

Primer name	Sequence	Primer partner	Tm (˚C)
Rs-AFP2 5’	ATGGCTAAGTTTGCTTCTAT	Rs-AFP2 3’	46
Rs-AFP2 3’	TTAACAAGGGAAATAACAGA	Rs-AFP2 5’	44

Genomic DNA was extracted from ground leaf material obtained from *in vitro* plantlets for Southern Blot analysis according to the protocol of Reid et al. 2006. The genomic DNA was RNAse-treated prior to the restriction enzyme digestion. 30 µg Genomic DNA was restricted with *Eco*RV and separated on 0.8% (w/v) agarose TAE gels at 35V overnight at a constant current. *Eco*RV does not digest within the transgenes but restricts once outside the 5’ end of the gene, thus providing an approximation of transgene insertion events. The digested DNA was transferred to positively charged Hybond-N nylon membrane (Sambrook et al. 1989) and crosslinked to the membranes with UV. DIG Easy Hyb (Roche Diagnostics GmbH, Mannheim, Germany) was used for pre-hybridisation at 42°C 3 hours. Hybridization was performed by using the respective Hc-AFP1, Hc-AFP4 and Rs-AFP2 probes at the 42°C for 20 hours. Chemiluminescent detection was performed according to the DIG application manual for filter hybridization (Roche Diagnostics GmbH, Mannheim, Germany). Each hybridization signal represents a single copy of the transgenes respectively. Lambda DNA/HindIII, 2 (©Thermo Fisher Scientific Inc, USA) was used in this analysis.

4.2.4 RNA isolations and Northern Blot analysis of transgenic lines

RNA isolations were performed according to the protocol of Reid et al. 2006. Whole plant tissue (100 mg) was grounded to a fine powder in the presence of liquid nitrogen and extracted with 900 µl extraction buffer (2% [*w/v*] CTAB, 2% [*w/v*] PVP-40, 2% 2-mercaptoethanol, 100 mM Tris-HCL pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5 g L^{-1} spermidine). Five microgram total RNA was separated on a 1.2% (*w/v*) formaldehyde agarose gel and transferred to positively charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany) as described by Sambrook et al. (1989). Pre-hybridization was performed at 50°C for 3 hours. Hereafter these membranes were probed with the same DIG-labeled probes used for Southern Blotting. Hybridization was performed at 50°C for 20 hours. Chemiluminescent detection was performed according to the DIG application manual for filter hybridization (Roche Diagnostics GmbH, Mannheim, Germany).

4.2.5 Phenotyping for growth characteristics of *in vitro*-grown grapevine transgenic plantlets ectopically expressing Hc-AFP1, Hc-AFP4, Rs-AFP2 and Vvi-AMP1

Phenotyping for growth characteristics was performed according to the method adapted from Cui et al. (2016). In short, shoot segments (1.5 - 2.0 cm in length) containing two fully opened leaves and a growth point was excised from six week old *in vitro* wild type and transgenic plantlets,

respectively from the *V. vinifera* (cv. Sultana) and *V. vinifera* (cv. Red Globe) populations. These excised shoot segments were transferred to commercially available expanded perlite wetted with liquid Murashige and Skoog basal salt mixture (Murashige and Skoog 1962) media containing 15 g/L sucrose. These plantlets were cultured under the same conditions as previously described. The time required for axillary bud elongation was recorded. Vegetative growth including shoot length, average internode length, number of roots and length of the longest root were measured after six weeks. Each plant line was represented by five biological replicates and the whole experiment was repeated three times. All data was normalized to the wild type of each respective experiment. The results are presentative of three individual conducted experiments for each transgenic population. Graphs were constructed using GraphPad PRISM 5 for windows (©1992-2007 GraphPad Software, La Jolla California USA, www.graphpad.com). A T-test was performed with significant differences analysed at $P \leq 0.05$, using statistical software Statistica, Version 13 (Dell Inc., Tulsa, OK, USA).

4.2.6 Leaf gas exchange measurements

Uniquely transformed selected transgenic *V. vinifera* (cv. Sultana) transgenic lines (Table 4.3) were as well as several wild type *V. vinifera* (cv. Sultana) were systematically hardened off in perlite and water. These plantlets were maintained at 25°C and moderate humidity in a greenhouse environment. The humidity was systematically reduced over time and the plantlets were transferred to potting soil (Double Grow, Durbanville, South Africa) and maintained under the same conditions.

Table 4.3 The *V. vinifera* (cv. Sultana) plant lines selected for leaf gas exchange measurements

Construct	Plant lines	Repeats
Hc-AFP1	9	4
	11	4
	78	4
Rs-AFP2	3	4
	4	4
	5	4
Vvi-AMP1	19	4

Two days prior to photosynthesis measurements, plants were moved to a room with controlled low light, temperature and humidity conditions. Temperature was maintained at 22°C and relative humidity at 48% and light at 15.86 µmol $m^{-2}s^{-1}$ photosynthetic active radiations (PAR). Light measurement was done with TinyTag LiCor (Gemini data loggers Ltd, West Sussex, United Kingdom) sensor, measuring light between 400 nm and 700 nm. Photosynthesis measurements were executed using a Licor Li6400XT portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, Nebraska, United States). During measurements, the leaf chamber temperature block was maintained at 22°C which was within range of the air temperature during the measurement period. The molar air flow rate inside the chamber was set to 500 µmol mol^{-1} and the photosynthetic

photon flux was set to 1000 µmol m^{-2}. All measurements were taken at a reference CO_2 concentration of 400 µmol mol^{-1}. Four mature, healthy, fully expanded leaves were selected per plant for photosynthesis measurements. After placing the Li-Cor cuvette on the leaf for a minimum of two minutes, stability of multiple parameters was monitored and logged. The variables reported were photosynthetic CO_2 assimilation, stomatal conductance, transpiration and leaf temperature. Graphs were constructed using GraphPad PRISM 5 for windows (©1992-2007 GraphPad Software, La Jolla California USA, www.graphpad.com). Significant differences was determined with a One-way ANOVA followed by a Fisher LSD post hoc test with significant differences analysed at $P \leq 0.05$, using statistical software Statistica, version 13 (Dell Inc., Tulsa, OK, USA).

4.3 RESULTS

4.3.1 *In silico* analysis of grapevine defensin genes in the grapevine gene expression atlas

4.3.1.1 Mapping of the 16 previously identified grapevine DEFL genes to the V1 transcriptome

A previous *in silico* analysis identified 16 DEFL genes in grapevine. It originated form a total of 97 putative DEFL genes consisting out of 79 genes identified form a previous study by Giacomelli et al. (2012) and 18 genes from a separate study by Tredoux (2011). These genes were evaluated and compared to what is known for antimicrobial peptides in terms of their size, binding to the VitisAffy Gene chip (Gautier et al. 2004), unique ETS and evidence of gene expression. After this filtering was applied, 16 putative DEFL genes were retained (Du Plessis 2012) and mapped to the V1 transcriptome through a tBLASTn analysis in order to determine if these genes each map to a unique sequence in the V1 transcriptome. Not all of the 16 DEFL gene sequences resulted in a satisfying hit (Table 4.4) For DEFL26, DEFL70, DEFL29 and DEFL34 no significant hit could be obtained with the tBLASTn analysis and they were removed from the subsequent analysis. Some of the genes, namely Vvi-AMP1, Vvi-AMP2 and Vvi-AMP3 mapped to the same VIT ID. The same result was seen for the VviSnakin6 and VviSnakin8 genes. Moreover, other DEFL genes mapped to more than one VIT identity in the V1 transcriptome. These DEFL genes included DEFL10, DEFL21, VviSnakin2, VviSnakin6 and VviSnakin8. These genes were therefore renamed for the purpose of this *in silico* analysis and the final list of genes used for the *in silico* analysis, with their matching V1 and V0 identities are indicated in Table 4.4.

Table 4.4 Blast results of 16 DEFL protein sequences against the V1 transcriptome with their matching V1 and V0 identities and designate names used in this chapter.

DEFL Protein	BLAST Hits in PN40024	GPL17894	GPL13936	DEFL gene name referred to in chapter
		V1_ID	V0_ID	
DEFL1	NSH	-	-	-
DEFL10	2	VIT_02s0025g02280	CHR2_GSVIVT00019623001_T01	**DEFL10_1**
		VIT_02s0025g02300	CHR2_JGVV25_210_T01	**DEFL10_2**
DEFL21	3	VIT_00s0713g00010	CHRUN_JGVV713_1_T01	**DEFL21_1**
		VIT_00s0623g00010	CHRUN_PDVV623_1_T01	**DEFL21_2**
		VIT_00s0699g00010	CHRUN_JGVV699_1_T01	**DEFL21_3**
DEFL26	NSH	-	-	-
DEFL70	NSH	-	-	-
DEFL29	NSH	-	-	-
DEFL52	1	VIT_04s0023g00660	CHR4_GSVIVT00019080001_T01	**DEFL52**
DEFL59	2	VIT_18s0001g02910	CHR18_RANDOM_JGVV170_3_T01	**DEFL59_1**
		VIT_00s1256g00010	CHRUN_JGVV1256_1_T01	**DEFL59_2**
DEFL34	NSH	-	-	-
VviAMP1	2	VIT_01s0010g02030	CHR1_JGVV10_144_T01	**Vvi-AMPs_1**
		VIT_07s0130g00030	CHR7_JGVV130_3_T01	**Vvi-AMPs_2**
VviAMP2	2	VIT_01s0010g02030	CHR1_JGVV10_144_T01	**Vvi-AMPs_1**
		VIT_07s0130g00030	CHR7_JGVV130_3_T01	**Vvi-AMPs_2**
VviAMP3	2	VIT_01s0010g02030	CHR1_JGVV10_144_T01	**Vvi-AMPs_1**
		VIT_07s0130g00030	CHR7_JGVV130_3_T01	**Vvi-AMPs_2**
VviSnakin2	3	VIT_03s0091g00390	CHR3_JGVV91_81_T01	**VviSnakin2_1**
		VIT_18s0001g09460	CHR18_JGVV1_619_T01	**VviSnakin2_2**
		VIT_07s0129g00580	CHR7_JGVV129_56_T01	**VviSnakin2_3**
VviSnakin6	4	VIT_08s0007g05860	CHR8_JGVV7_297_T01	**VviSnakin6_1**
		VIT_00s0189g00070	CHRUN_JGVV189_5_T01	**VviSnakin6_2**
		VIT_14s0108g00740	CHR14_JGVV108_70_T01	**VviSnakin6,8_1**
		VIT_17s0000g06210	CHR17_JGVV0_371_T01	**VviSnakin6,8_2**
VviSnakin8	2	VIT_14s0108g00740	CHR14_JGVV108_70_T01	**VviSnakin6,8_1**
		VIT_17s0000g06210	CHR17_JGVV0_371_T01	**VviSnakin6,8_2**
VviSnakin13	1	VIT_18s0072g01110	CHR18_JGVV72_17_T01	**VviSnakin13**

NSH – No significant hits

4.3.1.2. Analysis of the tissue specific relative expression patterns of grapevine DEFL genes during different stages of development in the gene atlas

The *in silico* pipeline was used to obtain an overview of the baseline expression of the grapevine DEFL genes in the different grapevine organs and to determine during which specific developmental stages these genes were expressed in the respective grapevine organs. The developmental stages are described using the modified E-L system (Coombe 1995) It was clear that the DEFL genes targeted in the analysis were expressed in all grapevine plant organs in the Corvina cultivar that was used for the gene atlas. Although all genes displayed basal expression levels in all organs, there were developmental and tissue-specific differential expression of some of the genes in some organs and tissues (Addendum B to Chapter 4, Figure B4.1). The expression patterns during flowering and in the flower tissues (Figure 4.1); leaf and tendril development (Figure 4.2); seed and rachis (Figure 4.3); and stem and bud development (Figure 4.4) are

presented to show examples of this tissue-specific and developmental patterns for the nine DEFL genes included in the analysis. The red and black asterisks in these figures indicate the relative gene expressions of the Vvi-AMPs and DEFL59 (Vvi-AMP4, as the closest homolog) to the peptides that form part of this study (refer to Chapter 3 for the homolog characterisation). During flower development, several snakins, Vvi-AMPs and unknown DEFL defensins were expressed at higher levels and these increased levels were maintained throughout the early to later stages of inflorescence development (EL23) (Figure 4.1.B). Furthermore, several of the defensins (also the Vvi-AMPs and DEFL59) showed enriched expression in the tissues/sub-organs of the flowers, including strong expression in the pollen (Figure 4.1.C).

This differential tissue -specific enriched expression of some DEFL genes was also seen in the leaf tissue and during leaf development (Figure 4.2B), as well as in the tendril and during tendril development (Figure 4.2.C). The Vvi-AMPs_2 was not enriched in leaves, but expressed at elevated levels, together with VviSnakin6_1 and VviSnakin6_2, DEFL10_1, DEFL10_2, DEFL21_1, DEFL21_2, DEFL21_3 and DEFL52 during tendril development.

In the seed and during seed development, DEFL genes VvSnakin2_1, VvSnakin6_1 VviSnakin6_2, VviSnakin13, VviAMPs_1, DEFL59_2 DEFL10_1, DEFL10_2 and DEFL52 were predominantly expressed (Figure 4.3.B), with Vvi-AMPs1_1 showing the highest expression of these DEFL genes. In the rachis, the Snakin DEFL genes were the most predominantly expressed DEFL genes (Figure 4.3.C). Interestingly, similar to the tendril development, except for VviAMP1_3, only VviSnakin6,8_1, VviSnakin6,8_2, VviSnakin2_1 and Vvi-AMPs_2 were expressed in the stem with a downregulation from developmental stage EL14 to EL43 (Figure 4.4 B). In the bud and during bud development (Figure 4.4 C), the VviSnakin, Vvi-AMPs and DEFL10 were predominantly expressed.

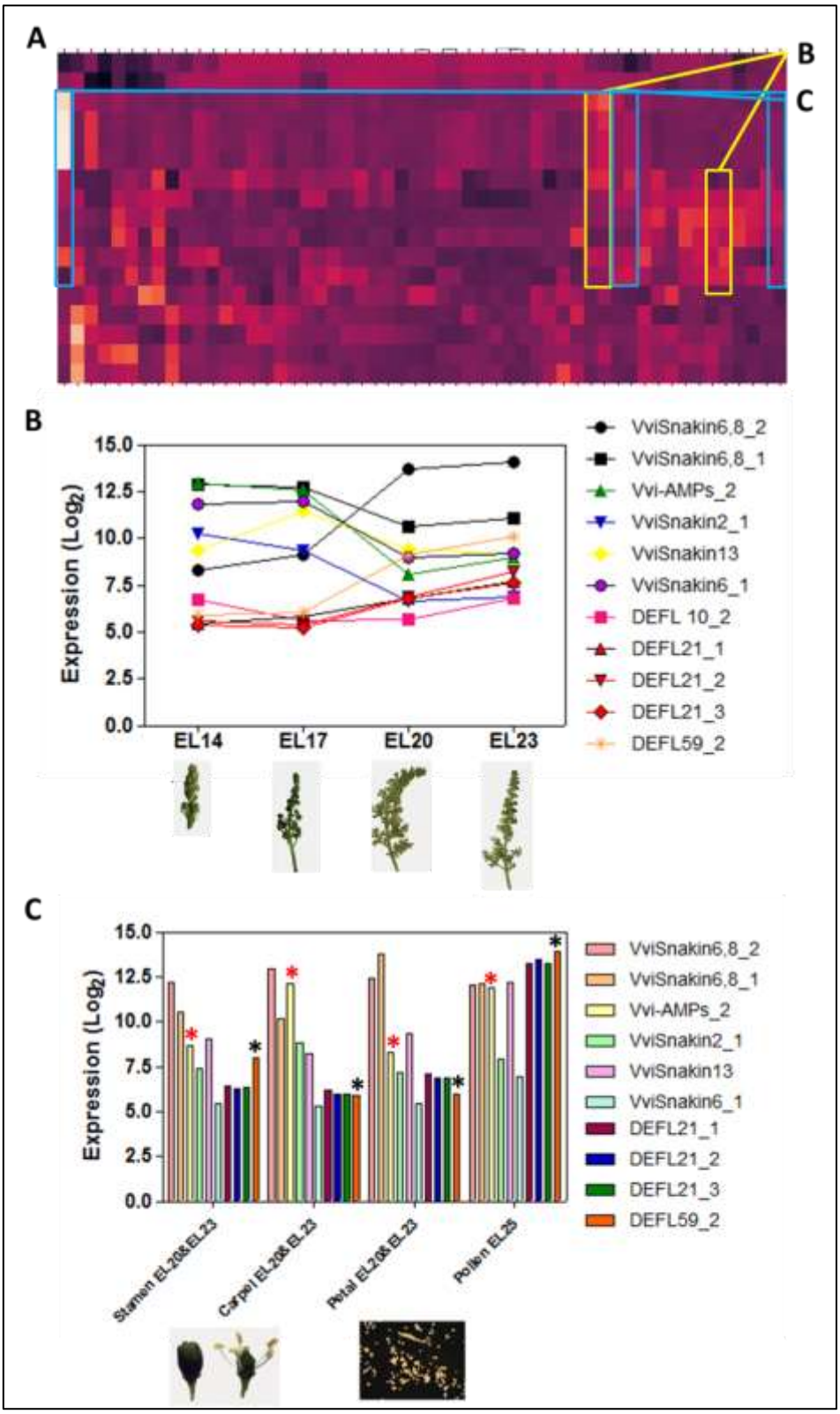

Figure 4.1. (A) Heat map depicting the tissue specific relative expression (Z-score normalized) of the DEFL genes during flowering and flower tissues in the Corvina gene expression atlas (Fasoli et al. 2012). (B) The relative expression (log_2, fold change) of some DEFL genes in the flower and in (C) stamen, carpel, petal and pollen per developmental stage (indicated by the modified E-L system) with the red and black asterisk indicating the expression of Vvi-AMP_2 and DEFL59_2 respectively.

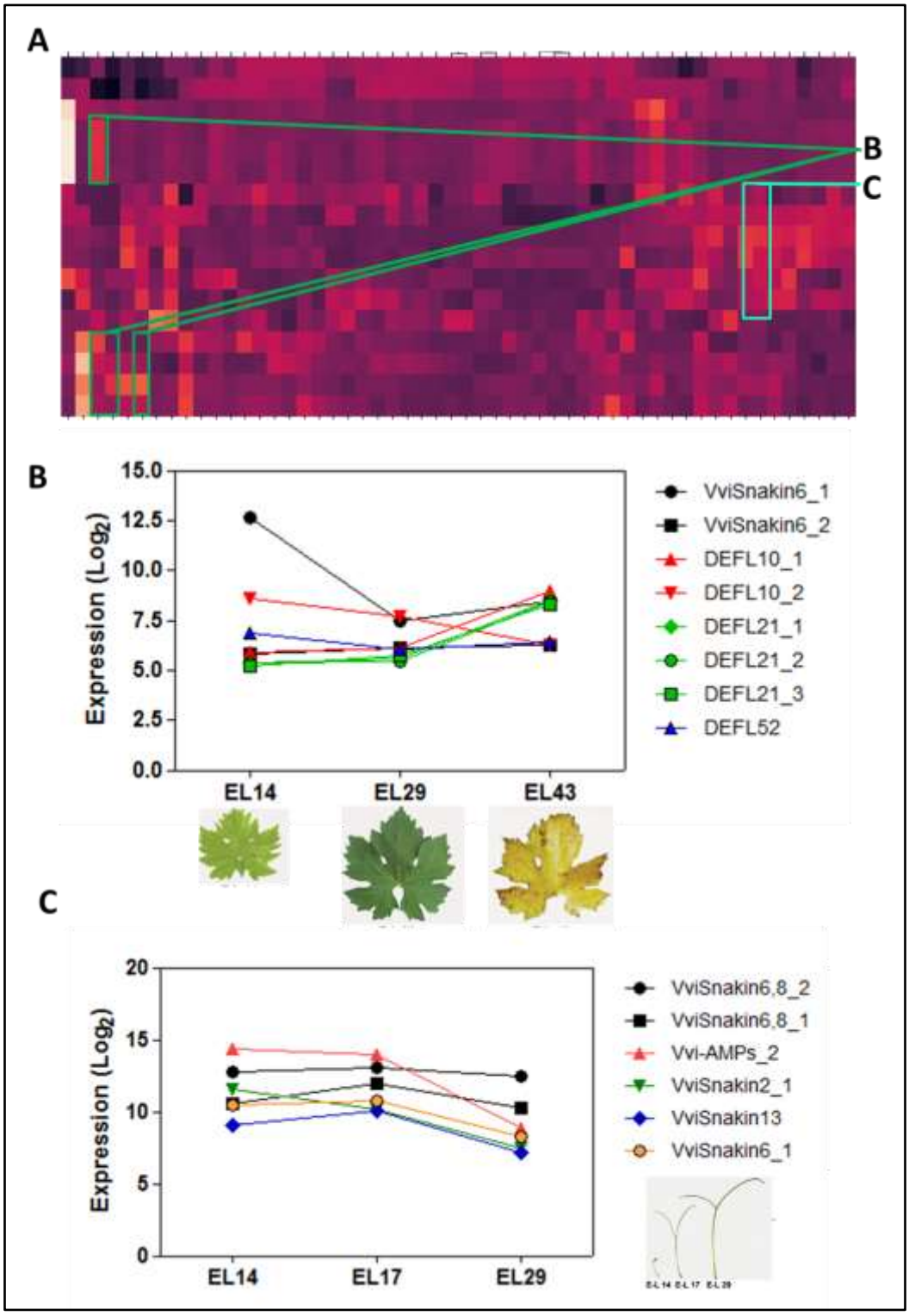

Figure 4.2. (A) Heat map depicting the tissue specific relative expression (Z-score normalized) of DEFL genes in leaf and tendril development in the Crovina gene expression atlas (Fasoli et al. 2012). (B) The relative expression (log_2, fold change) of some DEFL genes in the leaf and in (C) tendril per developmental stage (indicated by the modified E-L system).

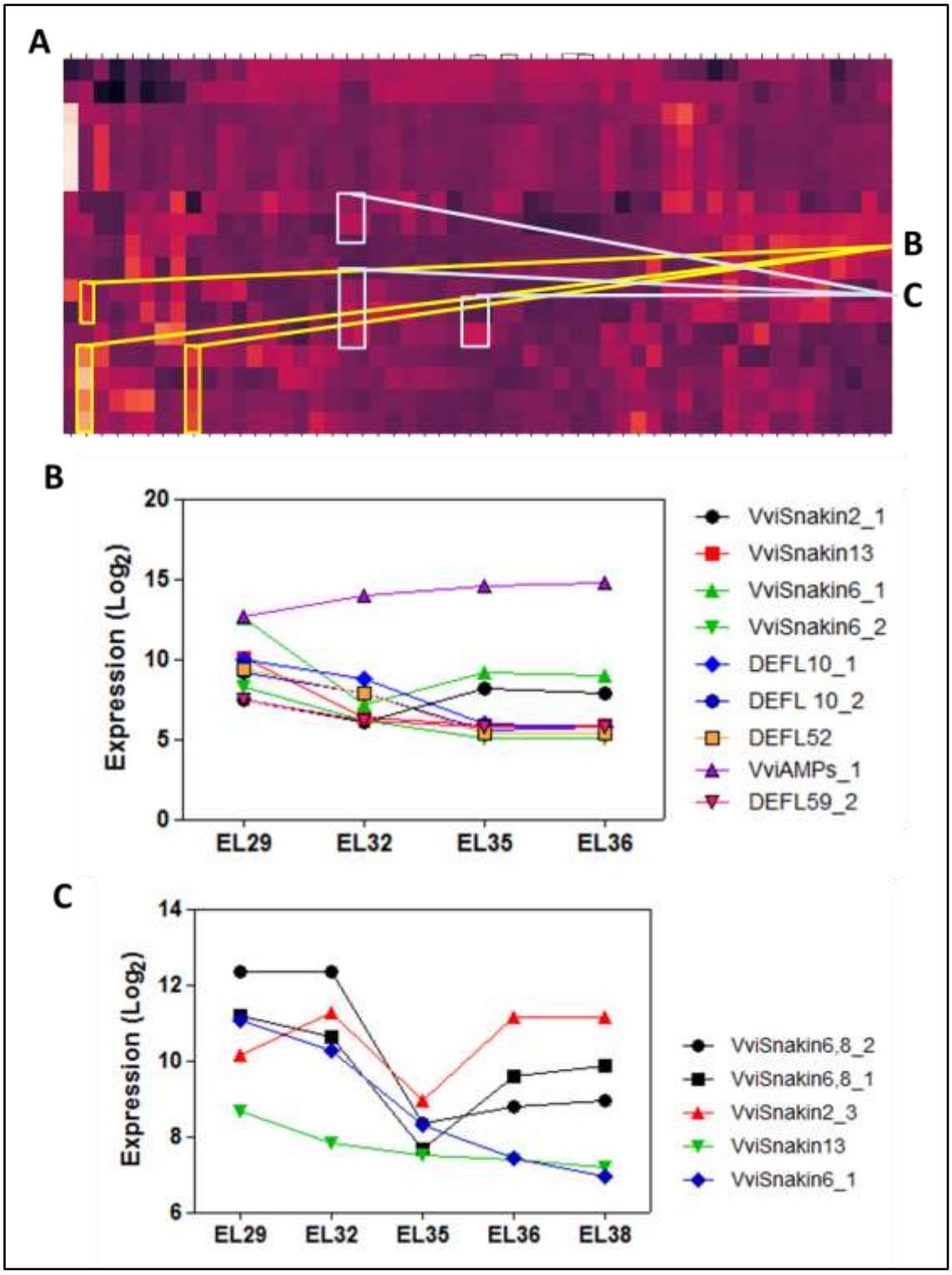

Figure 4.3. (A) Heat map depicting the tissue specific relative expression (Z-score normalized) of DEFL genes in seed and rachis developmental stages in the Corvina grapevine gene expression atlas (Fasoli et al. 2012). (B) The relative expression (log_2, fold change) of some DEFL genes in the seed and in (C) rachis per developmental stage (indicated by the modified E-L system).

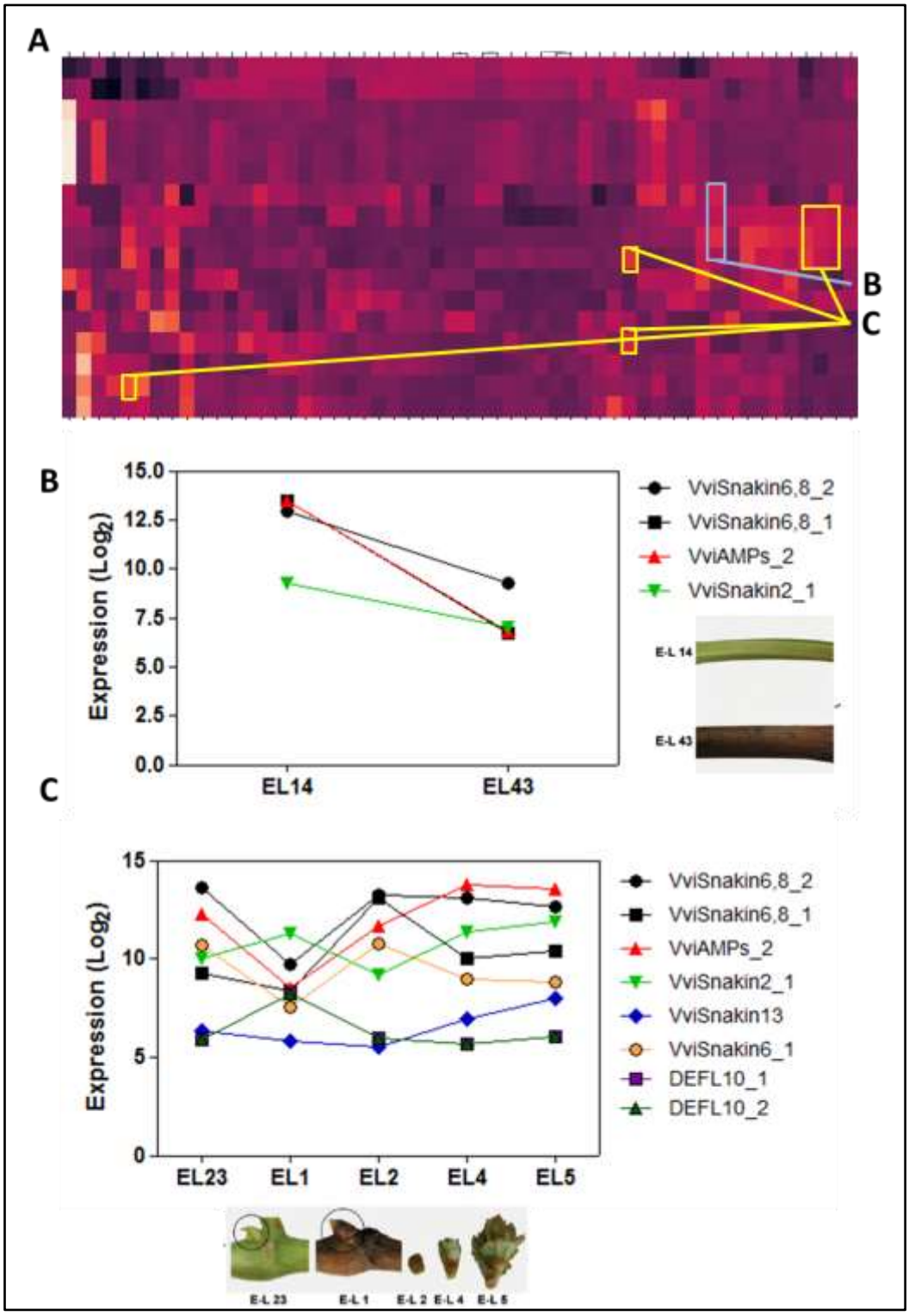

Figure 4.4. (A) Heat map depicting the tissue specific relative expression (Z-score normalized) of DEFL genes in stems and buds in the Corvina grapevine gene expression atlas (Fasoli et al. 2012). (B) The relative expression (log_2, fold change) of some DEFL genes in the stem and in (C) bud per developmental stage (indicated by the modified E-L system).

4.3.2 Characterising of grapevine populations ectopically expressing plant defensin peptides

4.3.2.1 Genetic analysis of the putative transgenic grapevine populations ectopically expressing Hc-AFP1, Hc-AFP4, and Rs-AFP2

PCR analysis confirmed that all the lines contained the respective transgenes, whereas the untransformed controls (Sultana and Red Globe) did not yield any amplicons (Table 4.5). Table 4.5 also lists all the unique transgenic lines, after clonal copies were identified through Southern Blot analysis (Addendum C to chapter 4, figures C4.1-C4.6) and the population renamed. The initial populations and genetic analysis before renaming are indicated in Addendum C to Chapter 4. The Hc-AFP1 constructs were stably integrated into the genomes of 10 independently transformed Sultana transgenic lines and eight expressed the transgene (Northern Blots presented in Addendum C to Chapter 4). Southern and northern Blot analyses of the *V. vinifera* (cv. Red Globe) Hc-AFP1 transgenic population revealed three individually transformed lines with two of these lines showing transgene expression. Furthermore, the *V. vinifera* (cv. Sultana) Hc-AFP4 transgenic population revealed nine unique integration patterns whereas the *V. vinifera* (cv. Red Globe) Hc-AFP4 transgenic population revealed that four unique integration patterns. Although all of these transgenic lines showed that they were successfully integrated, none of them expressed the Hc-AFP4 peptides (Addendum C to Chapter 4). The *V. vinifera* (cv. Sultana) Rs-AFP2 transgenic population comprised five uniquely transformed transgenic lines, but only four expressed the transgene (Red Globe Rs-AFP2 lines 1, 3, 4 and 5), whereas in the Red Globe background, four individually transformed lines were confirmed with three of them expressing the transgene. The individually transformed lines that showed expression of the transgenes were used to select lines for the phenotyping of growth phenotypes as well as preliminary physiology measurements as outlined in Table 4.5.

4.3.2.2 Phenotypical characterization of transgenic grapevine ectopically plant defensin peptides for their growth characteristics

The data presented for the phenotypical characterization discussed in this chapter is the combined data of three (n=3) individual experiments and in each experiment five copies per individual line was used, unless otherwise stated. The data for each individual experiment is provided in Addendum D to chapter 4.

In order to understand the baseline differences in growth characteristics between the two different cultivars used as transformation targets, the growth characteristics of the Sultana and Red Globe wild type plants were compared (Figure 4.5).

Table 4.5 Genetic analyses of the putative transgenic grapevine populations (Sultana and Red Globe) ectopically expressing Hc-AFP1, Hc-AFP4 and Rs-AFP2 and the experiments these transgenic lines were used in.

Cultivar	Construct	Plant line	PCR	Southern Blot	Number of transgene integrations	Northern Blot	Experiment
Sultana	**Hc-AFP1**	**Wild type**	-	-	0	-	Phenotyping; Leaf gas exchange
		6	+	+	1	+	-
		9	+	+	3	+	Leaf gas exchange
		11	+	+	1	+	Leaf gas exchange
		17	+	+	3	-	-
		28	+	+	3	+	Phenotyping
		55	+	+	1	-	-
		57	+	+	3	+	Phenotyping
		61	+	+	1	+	Phenotyping
		72	+	+	2	+	-
		78	+	+	1	+	Leaf gas exchange
Red Globe	**Hc-AFP1**	**Wild type**	-	-	0	-	Phenotyping
		9	+	+	2	-	-
		12	+	+	3	+	Phenotyping
		24	+	+	1	+	Phenotyping
Sultana	**Hc-AFP4**	1	+	+	4	-	-
		2	+	+	1	-	-
		8	+	+	1	-	-
		22	+	+	4	-	-
		23	+	+	2	-	-
		24	+	+	1	-	-
		28	+	+	5	-	-
		34	+	+	1	-	-
		40	+	+	1	-	-
Red Globe	**Hc-AFP4**	5	+	+	2	-	-
		16	+	+	1	-	-
		19	+	+	1	-	-
		24	+	+	3	-	-
Sultana	**Rs-AFP2**	1	+	+	1	+	Phenotyping
		2	+	+	2	-	Phenotyping
		3	+	+	1	+	Phenotyping; Leaf gas exchange
		4	+	+	2	+	Phenotyping; Leaf gas exchange
		5	+	+	3	+	Phenotyping; Leaf gas exchange
Red Globe	**Rs-AFP2**	1	+	+	1	+	Phenotyping
		2	+	+	2	+	Phenotyping
		3	+	+	1	+	Phenotyping
		4	+	+	8	-	-

The Red Globe wild type showed faster growth compared to the Sultana wild type, with less time required for the auxiliary bud elongation and more growth points developed compared to the control over six weeks (Figure 4.5 A and B). Furthermore, the Red Globe wild type also developed longer shoots and more roots compared to the Sultana wild type. There was no difference in the length of the longest root and internode length between the two different cultivars.

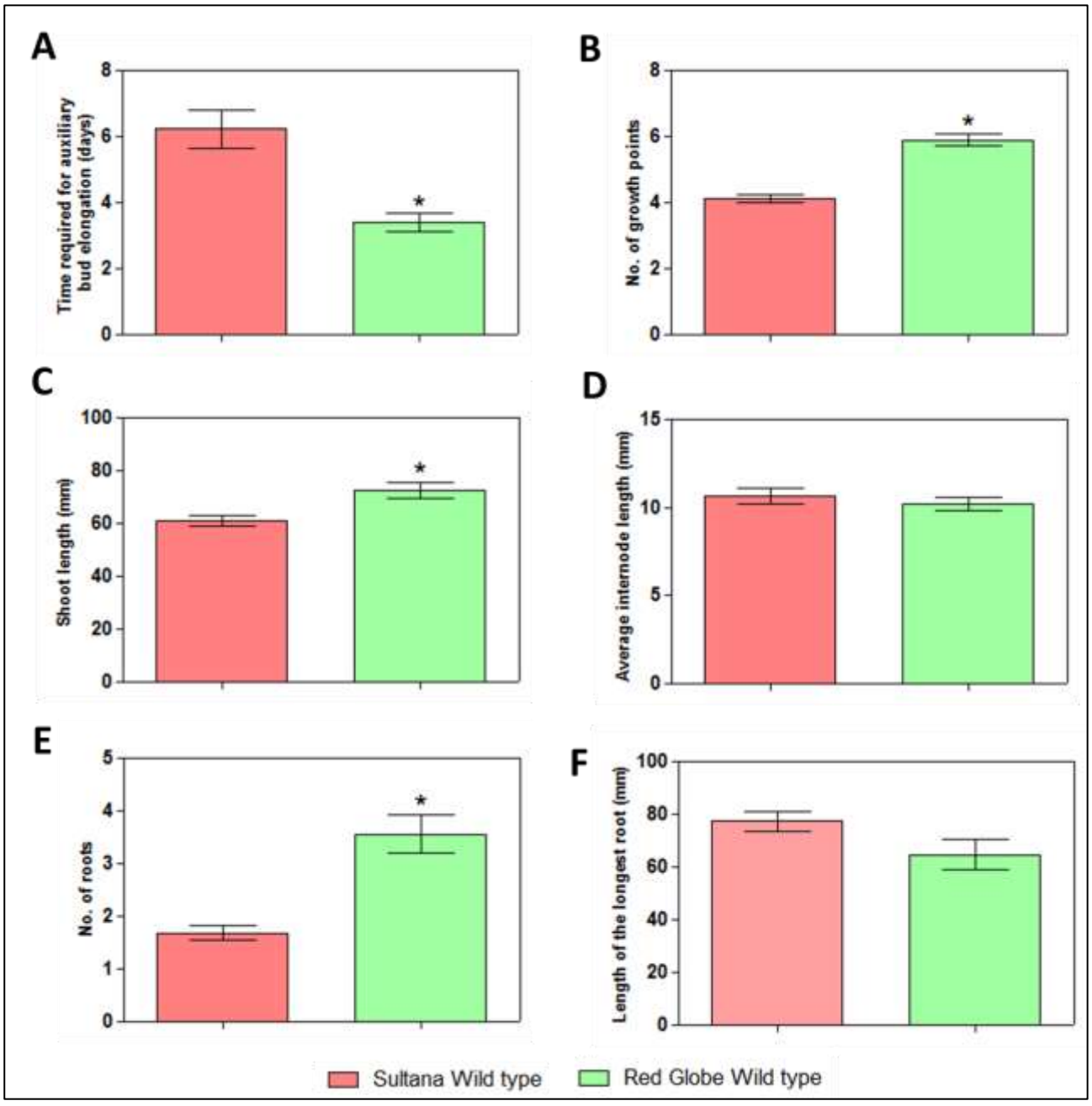

Figure 4.5. Phenotypic growth differences of the *V. vinifera* (cv. Sultana) and *V. vinifera* (cv. Red Globe) in terms of shoot growth and root formation. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is represented as means ± SEM and with asterisks indicating statistical differences between the two cultivars at $P \leq 0.05$.

It is clear from figure 4.6 that the ectopic expression of Vvi-AMP1 had little impact on the growth phenotypes of the transgenic *V. vinifera* (cv. Sultana) Vvi-AMP1 population (Figure 4.6); some lines had reduced or enhanced growth in some of the measured parameters, but overall very limited changes were observed.

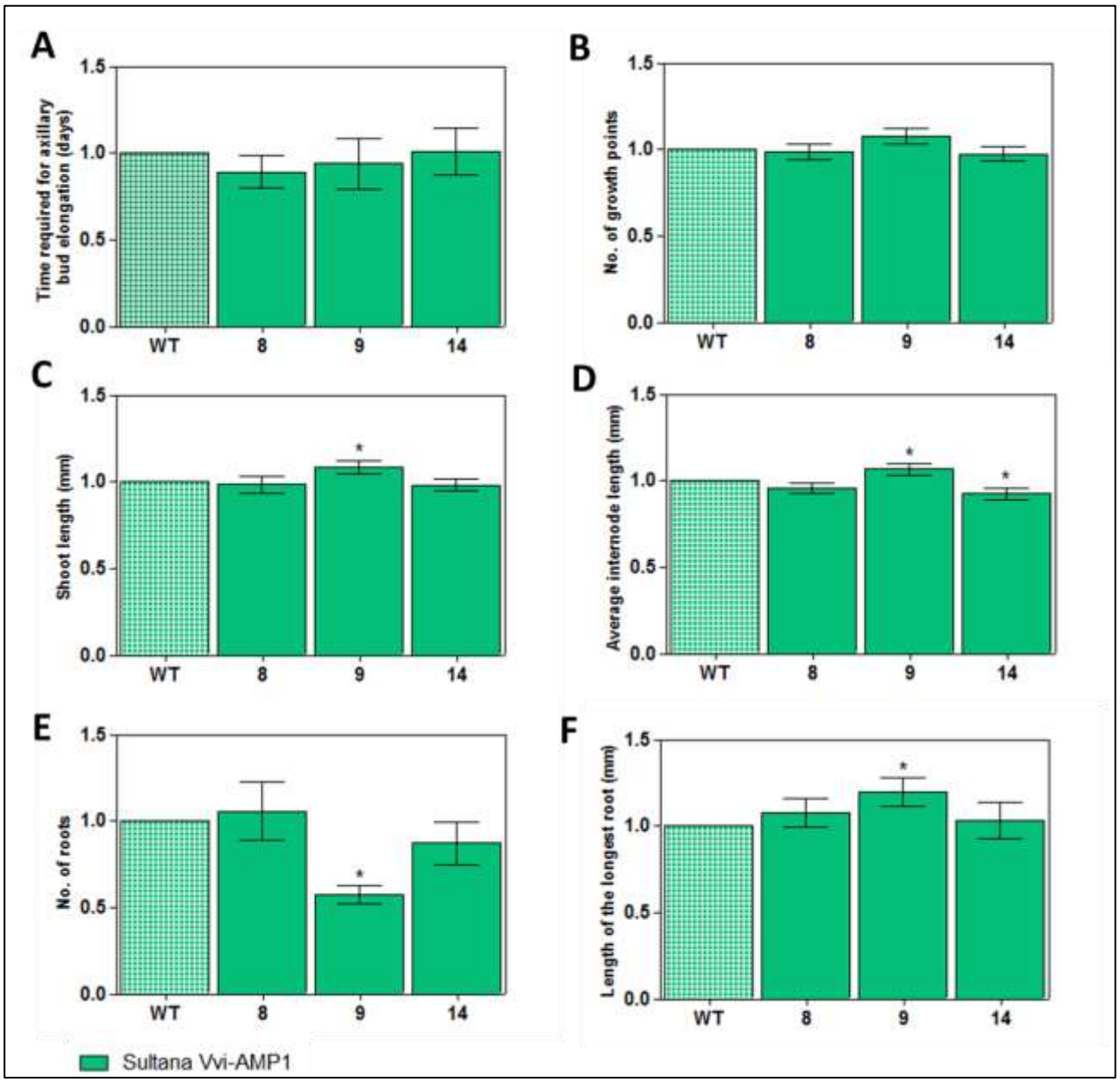

Figure 4.6. Effects of ectopically expressing Vvi-AMP1 on the phenotype of *in vitro* plantlets of *V. vinifera* (cv. Sultana) in terms of shoot growth and root formation. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is presented as means ± SEM and with asterisks indicating individual plant lines significantly different from the Wild type at $P \leq 0.05$ and asterisks with a caped line indicating the population significantly different from the Wild type at $P \leq 0.05$.

The phenotypical characterization of the transgenic *V. vinifera* (cv. Sultana and cv. Red Globe) Hc-AFP1 population showed that the ectopic expression of Hc-AFP1 affected the growth of both cultivars (Figure 4.7), but in different ways. It was clear that the Hc-AFP1 population in Red Globe was repressed in several categories of growth, whereas the same categories were not affected, or slightly improved in Sultana.

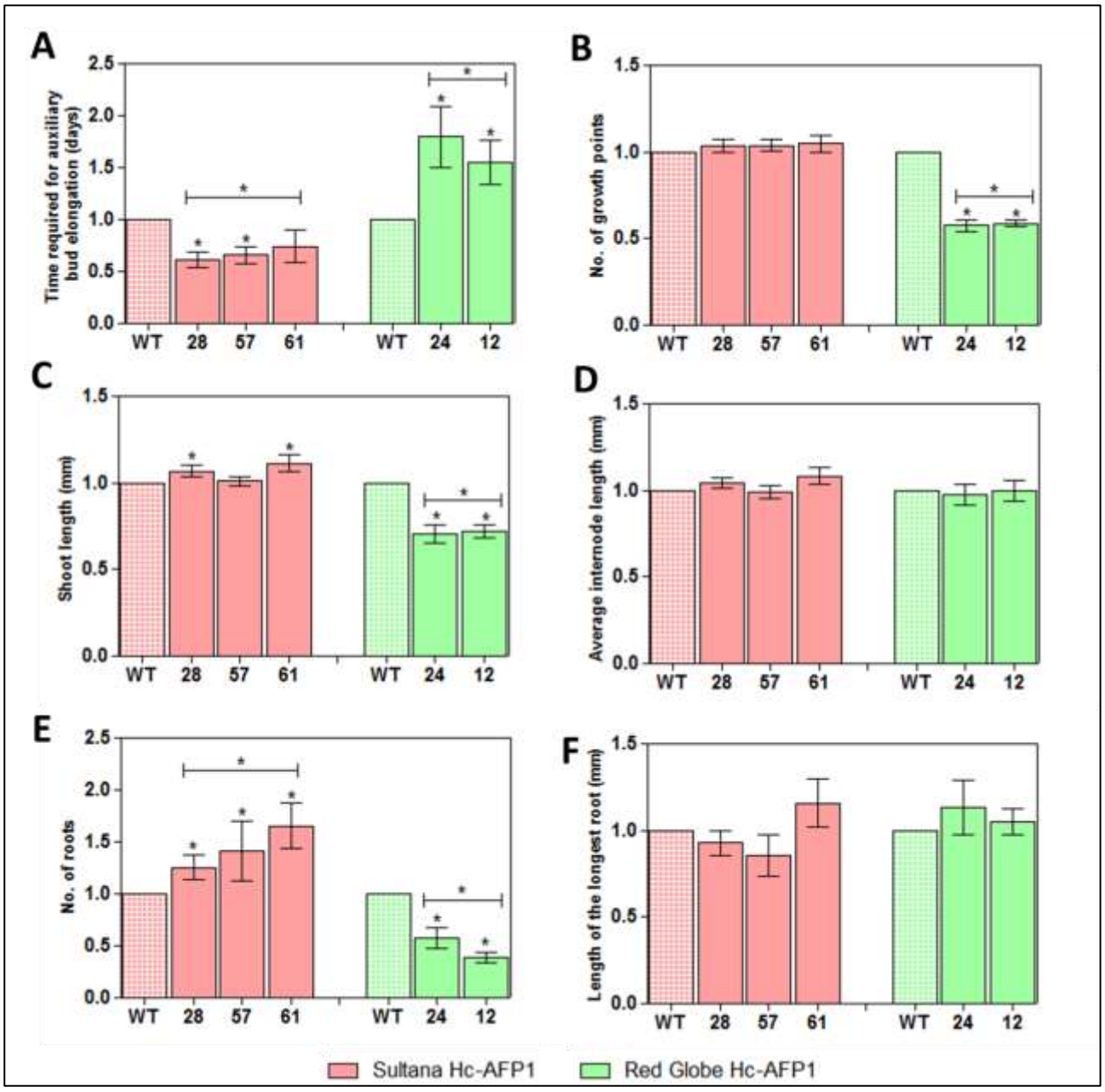

Figure 4.7. Effects of ectopically expressing Hc-AFP1 on the phenotype of in vitro plantlets of *V. vinifera* (cv. Sultana and cv. Red Globe) in terms of shoot growth and root formation. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is represented as means ± SEM and with asterisks indicating individual plant lines significantly different from the Wild type (WT) at $P \leq 0.05$ and asterisks with a caped line indicating the Hc-AFP1 population significantly different from the Wild type at $P \leq 0.05$.

The phenotypical characterization of the transgenic *V. vinifera* (cv. Sultana and cv. Red Globe) Rs-AFP2 populations again showed that the growth of the Red Globe population was more negatively affected by the ectopic expression of Rs-AFP2 compared to the Sultana population, although not all the lines behaved similarly (Figure 4.8).

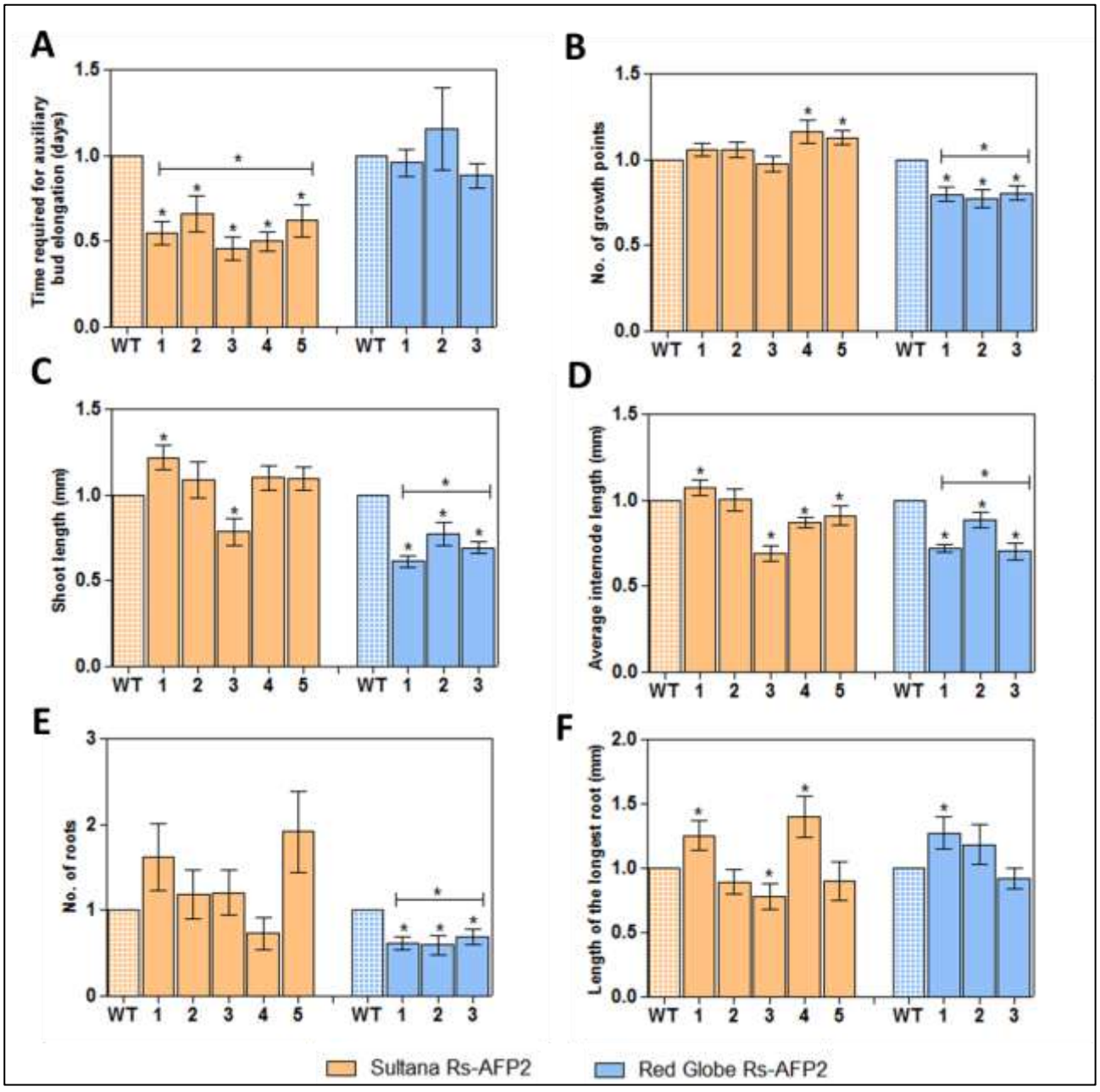

Figure 4.8. Effects of ectopically expressing Rs-AFP2 on the phenotype of in vitro plantlets of *V. vinifera* (cv. Sultana and cv. Red Globe) in terms of shoot growth and root formation. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is represented as means ± SEM and with asterisks indicating individual plant lines significantly different from the Wild type (WT) at $P \leq 0.05$ and asterisks with a caped line indicating the Rs-AFP2 population significantly different from the Wild type at $P \leq 0.05$.

In summary, it was clear from the phenotypical characterization that the phenotypes of the transgenic *V. vinifera* (cv. Red Globe*)* populations were more strongly affected than the phenotype of *t*ransgenic *V. vinifera* (cv. Sultana*)* populations, regardless of the defensin peptide it expressed.

Moreover, the growth of both cultivars were affected but in some cases in opposite ways. Based on this *in vitro* growth analysis, a selection of lines to be hardened-off was made for further characterisation, as presented in Table 4.6. Two non-expressing lines (Red Globe Hc-AFP1 Line 9 and Red Globe Rs-AFP2 line 4) were included as well, although they were not subjected to *in vitro* growth assays. Furthermore, the Sultana Vvi-AMP1 line 19 was also not subjected to *in vitro* growth assays.

Table 4.6 The plant lines of the different transgenic populations selected to harden off.

Cultivar	Construct	Plant line	PCR	Southern Blot	Number of transgene integrations	Northern Blot
Sultana	**Vvi-AMP1**	19	+	+	5	+
Sultana	**Hc-AFP1**	9	+	+	3	+
		11	+	+	1	+
		78	+	+	1	+
Red Globe	**Hc-AFP1**	9	+	+	1	-
		12	+	+	3	+
		24	+	+	1	+
Sultana	**Rs-AFP2**	3	+	+	1	+
		4	+	+	2	+
		5	+	+	3	+
Red Globe	**Rs-AFP2**	1	+	+	1	+
		2	+	+	2	+
		4	+	+	8	-

4.3.3 Physiological characterization of a subset of the transgenic grapevine lines ectopically expressing plant defensin peptides

A preliminary physiological characterization of hardened off plant lines of one of the genetic backgrounds, namely the *V. vinifera* (cv. Sultana) transgenic populations was performed to obtain an indication of potential physiological impacts of the transformation/transgene expressions (Figure 4.10). From the gas exchange measurements it was clear that the rate of photosynthesis of all the transgenic lines tested, except one, was reduced compared to that of the untransformed control. The stomatal conductance and transpiration data was more variable per construct, but the Vvi-AMP1 line and Rs-AFP2 populations reported reduced values on average. Except for Vvi-AMP1 line 19, none of the transgenic lines had a significant difference in leaf temperature when compared to the untransformed control. Vvi-AMP1 line 19 displayed a higher temperature when compared to the untransformed control.

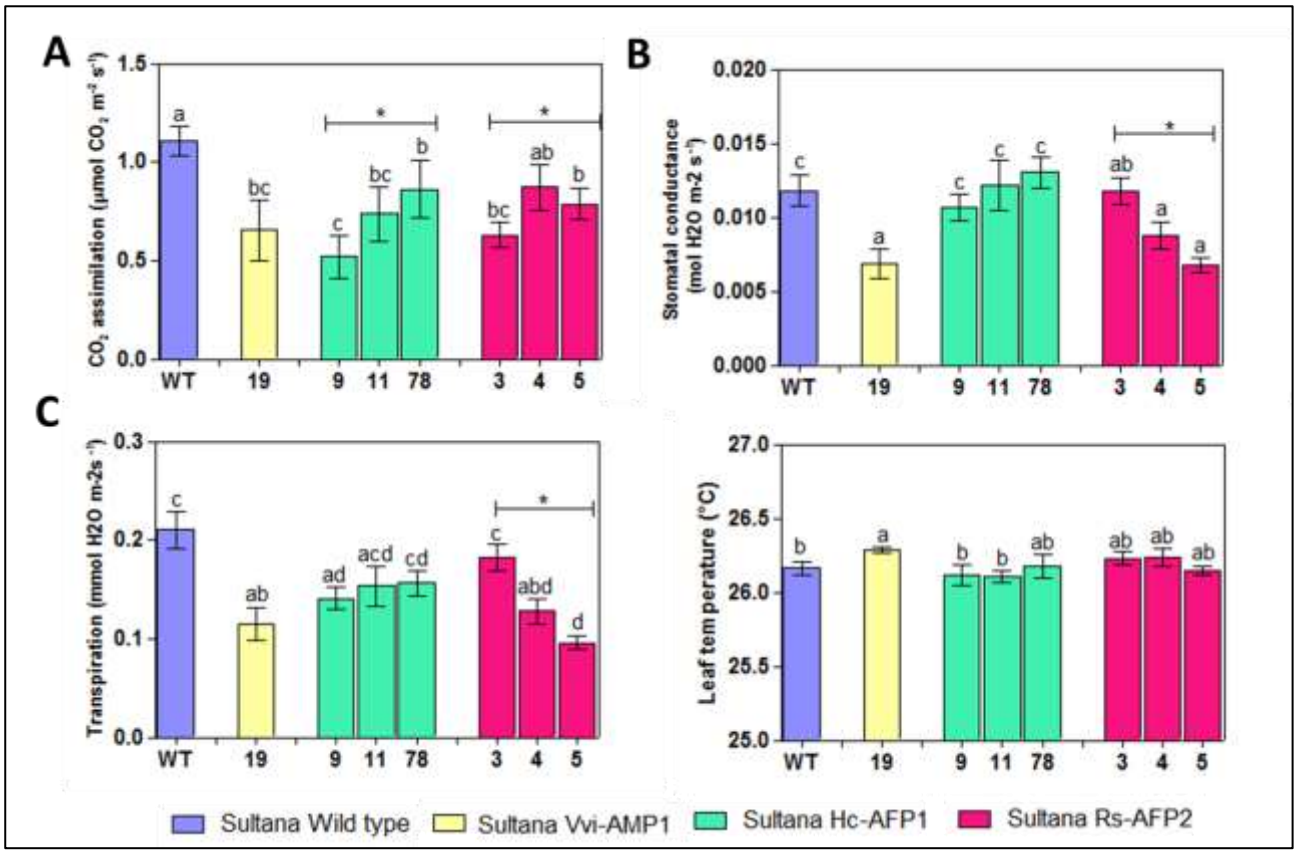

Figure 4.9. Physiological characterization of *V. vinifera (*cv. Sultana*)* wild type plants and transgenic plants through leaf gas exchange measurements. (A) Photosynthesis. (B) Stomatal conductance. (C) Transpiration. (D) Leaf temperature. Data is represented as means ± SEM and with different letters within the same parameter are significantly different at P ≤ 0.05. An asterisk with a caped line indicates the population is significantly different from the Wild type at P ≤ 0.05.

To summarise the growth and physiological analysis of the different analysis conducted, a summary figure (Figure 4.10) was prepared, to summarise the differences between the two genetic backgrounds (comparing the WT Sultana and Red Globe, as well as a key to summarise the findings for the transgenic populations.

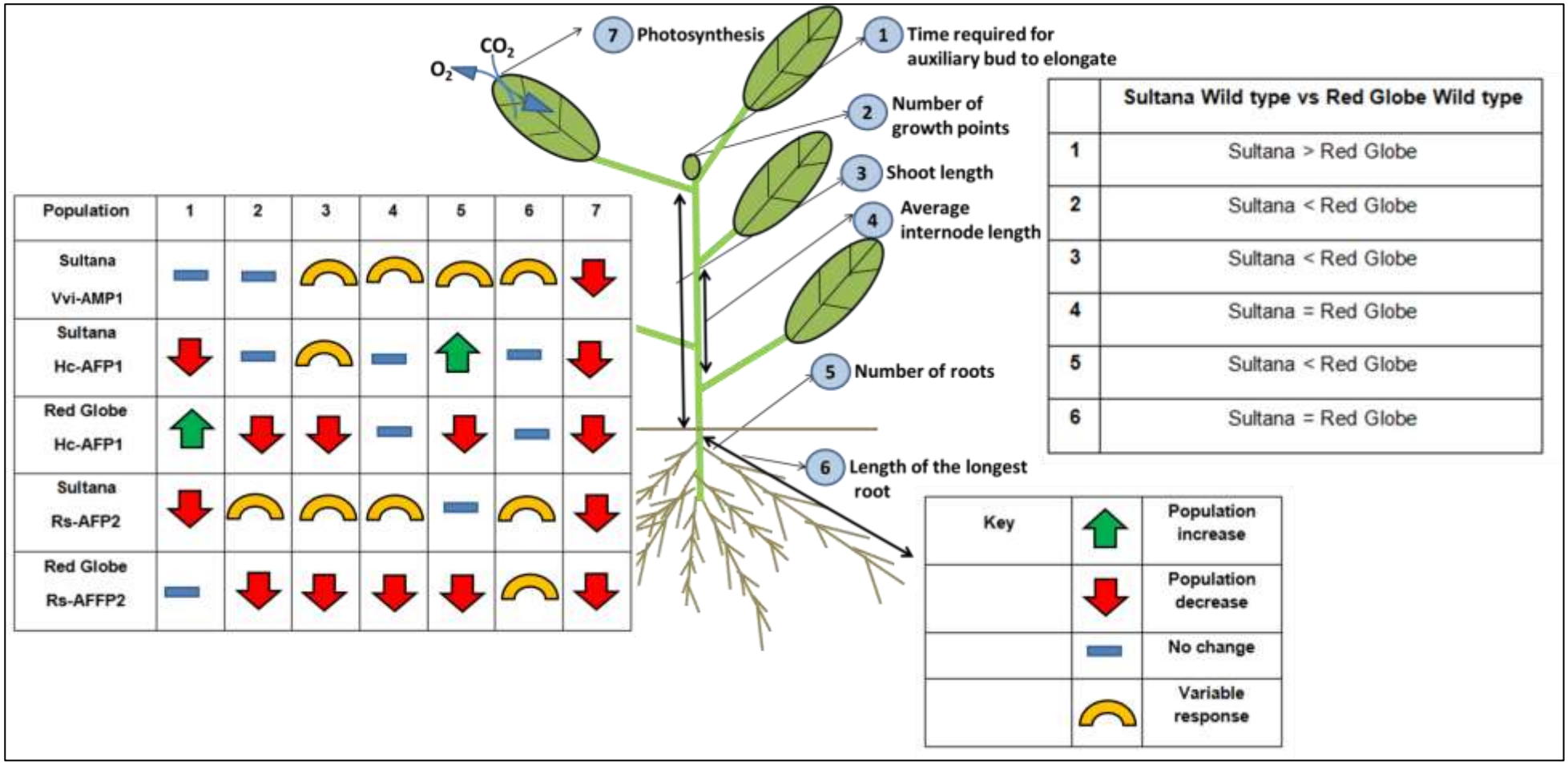

Figure 4.10 Summary of the phenotypic growth characterization results of the Sultana and transgenic populations expressing Hc-AFP1, Rs-AFP2 and Vvi-AMP1 peptides under non-stressed conditions and in comparison with the respective untransformed controls.

4.4 DISCUSSION

A great diversity of crop plants has been transformed with plant defensin peptides with successful resistance enhancement toward the economically important pathogens of each specific crop (Goyal and Mattoo 2014). Little is however reported on the broader potential impacts and non-defence related *in planta* function of these peptides within their transgenic host plants. Here several transgenic grapevine populations transformed with four different defensins were characterised. Three of the peptides (Hc-AFP1 and 4, and Rs-AFP2) were isolated from *Brassicaceae* species and therefore constitute heterologous expression in grapevine, whereas a previously characterised transgenic grapevine population, overexpressing a grapevine defensin, Vvi-AMP1 (De Beer and Vivier 2008; Tredoux 2011; Du Plessis 2012) was also included to expand the prior characterisation that only focused on defence phenotypes. PCR, Southern Blot and Northern Blot analyses were used to identify transgenic lines that expressed the transgenes and had unique integration patterns (of the Hc-AFP1 and 4, and Rs-AFP2 populations). No lines in either the Sultana or the Red Globe background could be identified that expressed the Hc-AFP4 peptide, despite all the lines being confirmed to be transgenic. It was concluded that these lines were silenced for the transgenes and no further work was conducted on them. The exact mechanism of the silencing observed in the Hc-AFP4 transgenic populations is not fully understood and will require further analysis. The integration patterns showed that between 1-5 copies of the genes occurred in the lines without expressing, and the silencing is therefore not necessarily related to numbers of transgene copies integrated. The Hc-AFP4 peptide is naturally expressed in the seeds of its native hosts and is very similar in sequence to Hc-AFP1 and Rs-AFP2, with a very similar predicted structure to Rs-AFP2 (Figure 3.1 and 3.7 in Chapter 3), which both yielded expressing transgenic population in the two genetic backgrounds.

The confirmed ectopic expression of the Hc-AFP1, Hc-AFP4, Rs-AFP2 and Vvi-AMP1 peptides in the seven transgenic grapevine populations theoretically should increase the level of peptides within/on the surface of the different plant organs. Given the fact that the grapevine genome contains several predicted defensin gene sequences, an *in silico* analysis was conducted to evaluate relative expression patterns of a subset of the DEFL genes as a framework for native expression in the host (of the transgenic populations heterologously or homologously expressing defensins in this study). It was found that the grapevine defensin-like genes are expressed in all organs, but complex differential expression patters were observed for individual predicted genes (Figures 4.1-4.4). Plant defensin peptides are known to be constitutively expressed and can be induced upon external stimulus (Broekaert et al. 1995). The Corvina gene atlas expression analysis revealed that the DEFL genes were indeed expressed in all grapevine tissues at various developmental stages, correlating with what is known about the expression patterns of plant defensin peptides. This analysis further revealed that the expression of the putative grapevine

Snakin and DEFL genes were not restricted by tissue, being expressed in all grapevine tissues tested in the atlas. These peptides have also been reported to be constitutively expressed in other plant hosts (Stotz et al. 2013). Snakin peptides are antimicrobial peptides with a very broad range of antimicrobial activity towards Gram positive and negative bacteria, as well as multiple fungal pathogens (De Souza Cândido et al. 2014). Snakin peptides were given their name due to their sequence homology to hemotoxic disintegrin-like snake venoms (Stotz et al. 2013). The first identified member of the snakin family, the tomato Gibberellic Acid-Stimulated Transcript 1 gene (GAST1) was shown to be expressed and present in all shoot organs (leaf, stem, petiole and flower) (Shi et al. 1992). Furthermore, the potato snakin peptide snakin-2 (StSN2) is developmentally expressed in tubers, stems, flowers, shoot apex, and leaves. This potato snakin is up-regulated by wounding and by abscisic acid treatment (Berrocal-Lobo et al. 2002). The grapevine snakin gene family remains uncharacterised and deserves attention given their ubiquitous expression patterns and interesting functions.

The microarray used in these experiments was the NimbleGen microarray © 2011 Roche (NimbleGen, Inc, Madison, WI, USA). This array is based on the V1 gene prediction by CRIBI of the 12x grapevine genome assembly by the French-Italian consortium (Jaillon et al. 2007). In order to obtain the nucleotide sequences of these DEFL genes and to determine if they map to a unique sequence in the V1 transcriptome, a tBLASTn analysis was performed of the DEFL protein sequences against the V1 transcriptome (V1 annotation on 12x V0 assembly). Not all of the DEFL genes however delivered satisfying hit in the V1 transcriptome. All genome sequences contain some sequence that is repetitive or very close to repetitive on the length scale of reads. Due to these repetitive sequences and the fact that plant defensins have short gene sequences, the chances are very high that these DEFL genes will map equally well to multiple positions (Li et al. 2008). The fact that some of these DEFL genes did not map to a unique sequence within the V1 transcriptome or mapped to a sequence at all, was a limitation of this *in silico* analysis. This was especially true for the *V. vinifera* defensin peptides.

The two VIT identities encoding the three Vvi-AMP peptide gene sequences was shown to be expressed in various grapevine organs in the gene atlas expression dataset (Fasoli et al. 2012). Since all three Vvi-AMP peptide sequences mapped to the same two VIT identities, it was impossible to distinguish the three from one another. Vvi-AMP peptides were shown to be expressed in the berry and throughout berry development. Vvi-AMP1 was exclusively expressed in berry tissue from the onset of ripening and throughout the ripening process (De Beer and Vivier 2008). This suggested a developmental role of Vvi-AMP1 in the berry ripening process. Furthermore, it also suggests a protective role in the berry as berries are very most vulnerable to pathogen attack during ripening. Moreover, according to the gene atlas data, Vvi-AMP peptides were also seen to be expressed in flower development and in the stamen, carpel, pollen and petal. This is in accordance with literature as Vvi-AMP2 is a grapevine flower specific plant defensin peptide that is specifically expressed in the pollen grains and specific areas of the ovary

parenchyma. Due to this specific expression pattern, this peptide has been suggested to play a role in the fertilization of grapevine (Nanni et al. 2014). The VIT identities coding for Vvi-AMP defensin peptides also revealed to be expressed in the stem, bud, tendrils and seeds. Vvi-AMP3 has been reported to be expressed in seed at the pre-veraison stage (Giacomelli et al. 2012). Furthermore, VIT identities coding for Vvi-AMP defensin peptides also showed the expression of these peptides in stem, bud, tendril, seeds and bud tissue. Except for the mapping ambiguities, this divergence in expression patterns can be attributed to the different cultivar as the expression of specifically Vvi-AMP1 has been shown to be different depending on the cultivar. In 'Pinotage' the Vvi-AMP1 peptide was exclusively expressed in berry tissue upon ripening and throughout the ripening process (De Beer and Vivier 2008). In 'Pinot-Noir', Vvi-AMP1 was expressed in the berry flesh (mesocarp) but also in leaves, roots, and flowers (Da Silva et al. 2005; Giacomelli et al. 2012). The gene expression atlas was conducted on *V. vinifera* cv. Corvina and might explain the differential expression seen with the Vvi-AMP defensin peptides (Fasoli et al. 2012).

4.4.1. The transgenic populations were altered in some growth and physiological parameters, indicating possible fitness cost of the overexpression

Transgenic *in vitro* cultures of *V. vinifera* (cv. Sultana) and *V. vinifera* (cv. Red Globe) were subjected to phenotypical characterization in order to determine if the integration of the expression cassette and/or the expression of the respective defensin peptide have an influence on the growth phenotype of the transgenic grapevine (Figure 4.10). This method proved to be a successful and effective method for high throughput phenotyping of grapevine plants as it is less time consuming than conventional phenotyping methods since this method is performed on tissue culture plants. Furthermore, the same alterations in the phenotype, for example "internode length" observed and classified in the tissue culture population was also observed in the hardened off transgenic population highlighting the functionality of this phenotyping method (results not shown). The *V. vinifera* (cv. Sultana) Vvi-AMP1 transgenic population was not significantly different from the untransformed control in terms of growth speed and plant size. This is consistent with the results of a previous phenotypical characterization (conducted on hardened off plants) for this population (Du Plessis 2012).

For the transgenic *V .vinifera* (cv. Sultana) and *V. vinifera* (cv. Red Globe) Hc-AFP1 and Rs-AFP2 populations it was clear that the integration of the expression cassette and/or the expression of the defensin peptides lead to mildly altered phenotypes of these transgenic grapevine plants in terms of growth, in some cases the speed of axillary bud growth was enhanced, also causing more growth points, whereas root formation was also often affected. When comparing just the Sultana and Red Globe cultivars in terms of the measured parameters, it was clear that the Red Globe cultivar showed faster growth compared to that of the Sultana cultivar with enhanced speed of auxiliary bud outgrowth, higher number of buds developed; longer shoot length and more developed roots. Moreover, the same genetic constructs lead to divergent effects on the growth

parameters in the different genetic backgrounds. The Sultana Hc-AFP1 population showed enhanced auxiliary bud outgrowth, whereas the Red Globe Hc-AFP1 population showed a reduction in the same assay and also developed a reduced amount of buds. The Sultana Hc-AFP1 population showed variable responses in shoot length; however the Red Globe population developed shorter shoots. Furthermore the Sultana populations typically developed more roots and the Red Globe population less (Figure 4.10). Overall it seemed as if the overexpression of the peptides in the Red Globe background lead to more of a reduction of growth indicators on a population level, whereas fewer parameters were affected in the Sultana populations, irrespective of the construct, or the outcomes were quite variable between the different transgenic lines of the population (Figure 4.10). It is clear that the genetic background was a major driver in the phenotypic diversity of these transgenic populations. This difference in transgenic cultivars transformed with the same defensin peptide was also observed in transgenic tomato plants expressing the tomato defensin Def2 (Stotz et al. 2009).

Considering the observed changes to the growth characteristics of the transgenic lines compared to their controls under non-stressed conditions, it is possible that the overexpression could perhaps lead to a higher metabolic load on the plants. The lower photosynthetic rates recorded for the transgenic Sultana population (under non-stressed conditions) would support this idea. It is well known that many resistance genes show a fitness cost of resistance. There is a substantial trade-off between growth performance and defence and genes that increase resistance is in most cases costly for a plant in the absence of pathogens (Zeller et al. 2013). The overexpression of an *Arabidopsis* resistance gene, Resistance to Powdery mildew 8.1 (RPW8.1) in rice conferred enhanced resistance to the transgenic rice plant, however this overexpression resulted in substantial fitness penalties of these transgenic rice plants, with a substantial reduction in yield component traits (Li et al. 2018). Altered effects on the growth of transgenic lines were also reported for the transformation of the tomato defensin DEF2 into the tomato plant. These transgenic tomato plants displayed a reduction in growth and produced smaller leaves and fruits (Stotz et al. 2009). Moreover, cotton plants transformed with the flower defensin, NaD1 revealed an altered growth phenotype with low yields of recovered seedlings with small leaves and short internodes (Anderson et al. 2009), similar to what was found for Sultana Vvi-AMP1 plants, as reported by Du Plessis (2012).

The Rs-AFP2 peptide has been linked to reductions in root growth before, similar to what we observed here as well, particularly in the red Globe population. It is suspected that these plant defensins abrogate the Ca^{2+} gradient in the root tip since plant root growth is dependent on Ca^{2+} channels. This is the same mechanism plant defensins deploy to inhibit the growth of fungal hyphae that are also dependent on Ca^{2+} gradient for hyphal growth. Although the Rs-AFP2 peptide does not hold a Ca^{2+} ion channel blocking activity, we know the mechanism of antifungal action involves membrane depolarization through Ca^{2+} influx and K+ efflux and can possibly explain this

observation (Jackson and Heath 1993; Allen et al. 2008; De Oliveira Carvalho and Gomes 2011; Parisi et al. 2018).

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Addendum A to Chapter 4

This Addendum contains relevant and additional data not shown in Chapter 4.

A4.1 Vector construction and plant transformation

The primers used in the vector construction and plant transformation are listed in Table A4.1. The vector construction and plant transformation was performed by Dr A de Beer at the Institute for Wine Biotechnology and is described in his PhD thesis (De Beer 2008). In short, the nucleotide sequences encoding for the mature peptide regions (mCDS) of the defensin peptides were isolated by PCR and cloned in to pGEM-T-Easy vector The fragment was cloned into pGEM-T-Easy (Promega Corporation, Madison, USA) and confirmed with sequencing. The Hc-AFPs were excised from pGEM(Hc-AFPs) to yield pGEM-mature Hc-AFPs with XhoI and SpeI and cloned into the XhoI and XbaI sites of pART7. The expression cassette with Hc-AFPs was subcloned form pART7 (Hc-AFPs) into the NotI sites of pART27 to give pART27 (Hc-AFPs) and placing the gene under the control of the 35S CaMV 35S promoter and nopaline synthase (NOS) terminator. The construct (Figure A4.1) was mobilised into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by electroporation as described by (Mattanovich et al. 1989). *V. vinifera* (cv. Sultana) and V. vinifera (cv. Red Globe) were transformed with pART27-Hc-AFP2 and pART27-Hc-AFP4 via Agrobacterium transformation of embryogenic callus (done by Dr Krishnan Vasant at the Institute for Wine Biotechnology). Transgenic embryos were generated under kanamycin selection (100 µg/mL) and the transgenic grapevine plantlets regenerated on MS medium without hormones at 25 °C under a 16 h/8h light cycle until enough material could be collected for clonal propagation of each transgenic line.

Table A4.1. Primer sets used in the construction of the expression vectors.

Primer name	Sequence	Primer partner	Restriction enzyme
Hc-AFP1 5'	CGCGAAGCTTAGGTACTGTGAGAGATCGAG	Hc-AFP1 3'	*XhoI*
Hc-AFP4 5'	CGCGAAGCTTCAGAAGTTGTGTGAGAGACC	Hc-AFP4 3'	*XhoI*
Hc-AFP1 3'	CGCGGGATCCTCAACATGGGTAGTAACAGA	Hc-AFP1 5'	*SpeI*
Hc-AFP4 3'	CGGCGGATCCTTAACATGGGAAGTAACAGA	Hc-AFP4 5'	*SpeI*
Rs-AFP2 5’	ATGGCTAAGTTTGCTTCTAT	Rs-AFP2 3’	-
Rs-AFP2 3’	TTAACAAGGGAAATAACAGA	Rs-AFP2 5’	-

Figure A4.1. The plant expression cassette used for grapevine transformation under the native signal peptide with antifungal peptides from *H. coronopifolia (*Hc-AFPs). The abbreviations represent: **RB,** T-DNA right border; **LB**, T-DNA left border; **CaM35S,** promotor of 35S RNA of cauliflower mosaic virus with duplicated enhancer region; **Tnos,** terminator of T-DNA nopaline synthase gene; **NativeSP,** signal peptide encoding domain of native *Hc-AFP* genes; **Mature Peptide**, mature protein encoding domain of plant defensin genes.

Addendum B to Chapter 4

This Addendum contains relevant and additional data not shown in Chapter 4.

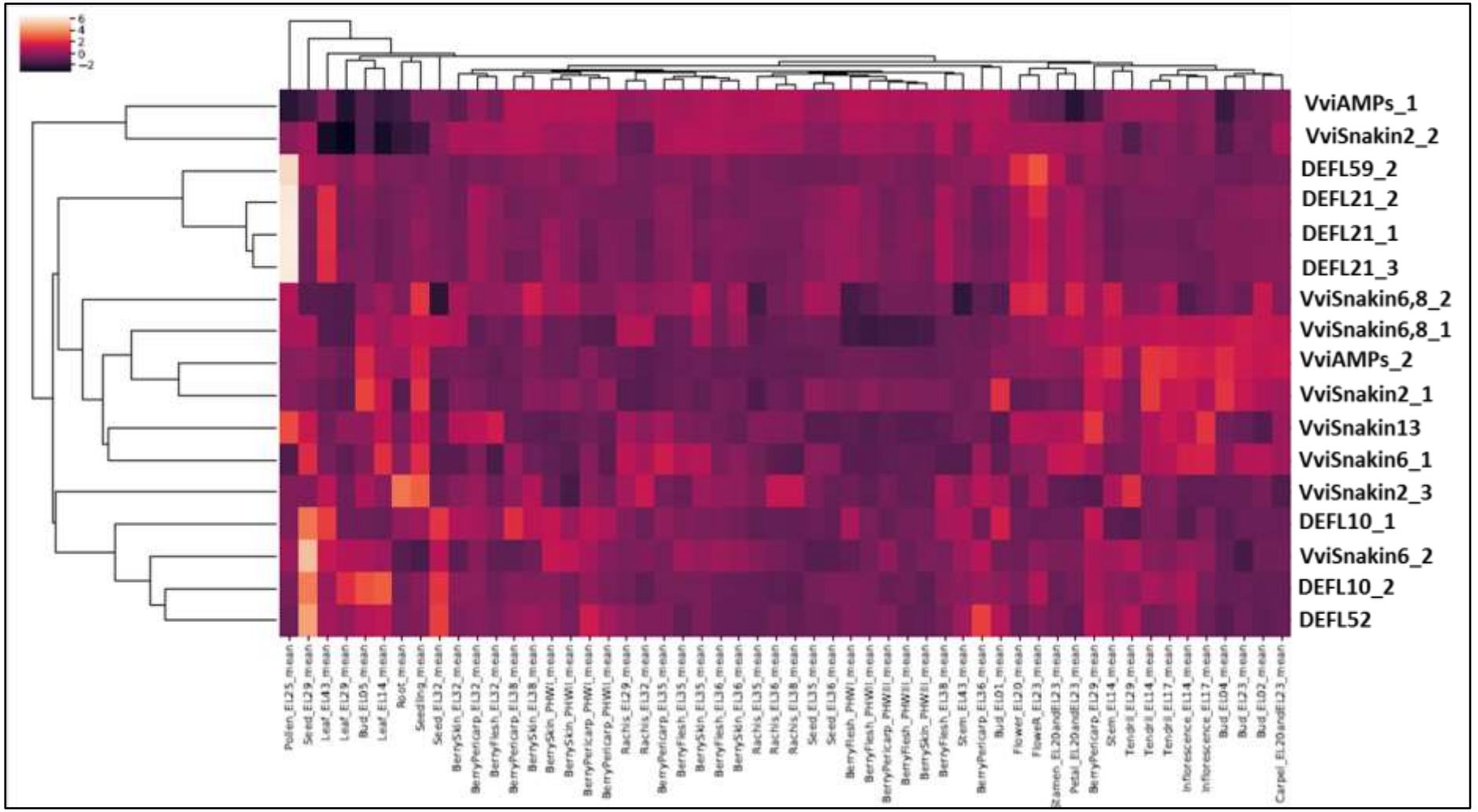

Figure B4.1. Heat map depicting the tissue specific relative expression (log_2, scaled and mean centred) of the 11 DEFL genes in various grapevine tissues during specific developmental stages by the grapevine gene expression atlas (Fasoli et al. 2012).

Addendum C to Chapter 4

This Addendum contains relevant and additional data not shown in Chapter 4.

C4.1 Genetic analysis of transgenic *V. vinifera* (cv. Sultana) ectopically expressing Hc-AFP1

Table C4.1: Summary of the genetic analysis of putative transgenic grapevine populations ectopically expressing the Hc-AFP1, Hc-AFP4 and Rs-AFP2. These analyses included PCR screening, Southern Blot analysis and Northern Blot analysis. Number of integrations refers to the number of transgene copies integrated in the genome. A "+" indicates a positive result whereas a "-" denotes a negative result. The untransformed *V. vinifera* (cv. Sultana and cv. Red Globe) lines were used as control. Transgenic plant lines were renamed according to their Southern Blot analysis results.

Cultivar	Construct	Plant line	PCR	Southern Blot	Number of transgene integrations	Northern Blot	Renamed
Sultana	**Wild type**	**Wild type**	-	-	0	-	-
	Hc-AFP1	6	+	+	1	+	6
		8	+	+	1	+	11
		9	+	+	3	+	8
		11	+	+	1	+	11
		17	+	+	3	-	17
		28	+	+	3	+	28
		55	+	+	1	-	55
		57	+	+	3	+	57
		61	+	+	1	+	61
		72	+	+	2	+	72
		78	+	+	1	+	78
Red Globe	**Hc-AFP1**	**Wild type**	-	-	0	-	-
		6	+	+	1	+	24
		9	+	+	2	-	9
		12	+	+	4	+	12
		17	+	+	4	+	12
		21	+	+	4	+	12
		24	+	+	1	+	24
Sultana	**Hc-AFP4**	1	+	+	4	-	1
		2	+	+	1	-	2
		8	+	+	1	-	8
		18	+	+	1	-	2
		22	+	+	4	-	22
		23	+	+	2	-	23
		24	+	+	1	-	24
		28	+	+	5	-	28
		32	+	+	4	-	11
		34	+	+	1	-	34
		36	+	+	4	-	22
		40	+	+	1	-	40

Table C4.1 (cont.)

Cultivar	Construct	Plant line	PCR	Southern Blot	Number of transgene integrations	Northern Blot	Renamed
Red Globe	**Hc-AFP4**	5	+	+	2	-	5
		16	+	+	1	-	16
		19	+	+	1	-	19
		24	+	+	3	-	24
Sultana	**Rs-AFP2**	3	+	+	3	+	5
		4	+	+	1	+	1
		5	+	+	1	+	3
		7	+	+	2	+	4
		9	+	+	2	-	2
		10	+	+	3	+	5
		11	+	+	3	+	5
		12	+	+	3	+	5
		16	+	+	3	+	5
		17	+	+	3	+	5
Red Globe	**Rs-AFP2**	1	+	+	1	+	3
		5	+	+	1	+	1
		6	+	+	2	+	2
		7	+	+	2	+	2
		9	+	+	1	+	1
		15	+	+	8	-	4

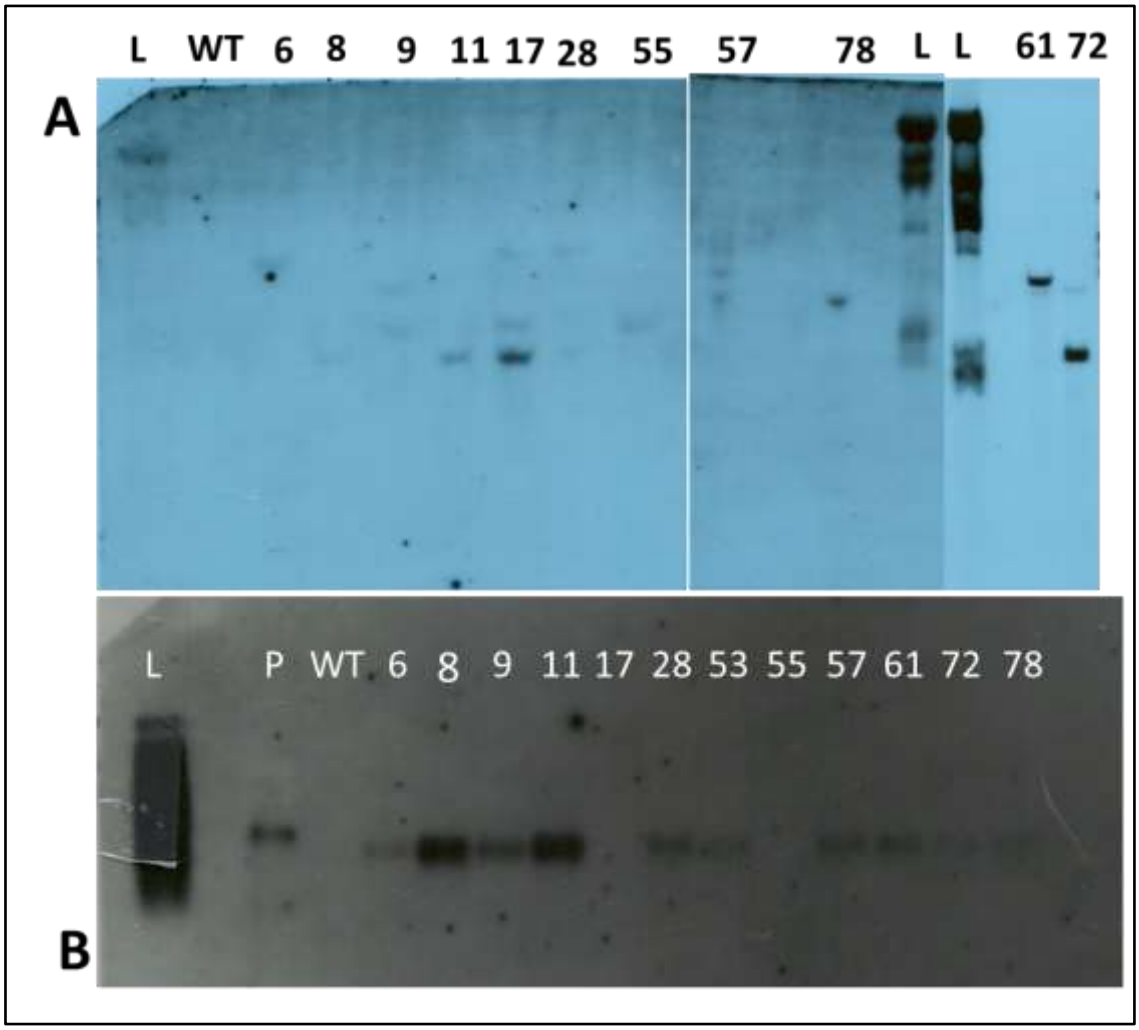

Figure C4.1. (A) The Southern blot analysis of 11 transgenic Hc-AFP1 *V. vinifera* (cv. Sultana) lines. The marker lane (L) contains the Lamda DNA/HindIII Marker, 2. The "WT" indicates the non-transformed *V. vinifera* (cv. Sultana) lines used as control and the numbers represent the names of the 11 transgenic Hc-AFP1 *V. vinifera* (cv. Sultana) lines. (B) The Northern blot analysis of 11 transgenic Hc-AFP1 *V. vinifera* (cv. Sultana) lines, performed by Dr. K du Plessis. The marker lane (L) contains the RiborulerTM High Range RNA Ladder (in bp). The "P" indicates the positive control, "WT" indicates the non-transformed *V. vinifera* (cv. Sultana) lines used as control and the numbers represent the names of the 11 transgenic Hc-AFP1 *V. vinifera* (cv. Sultana) lines.

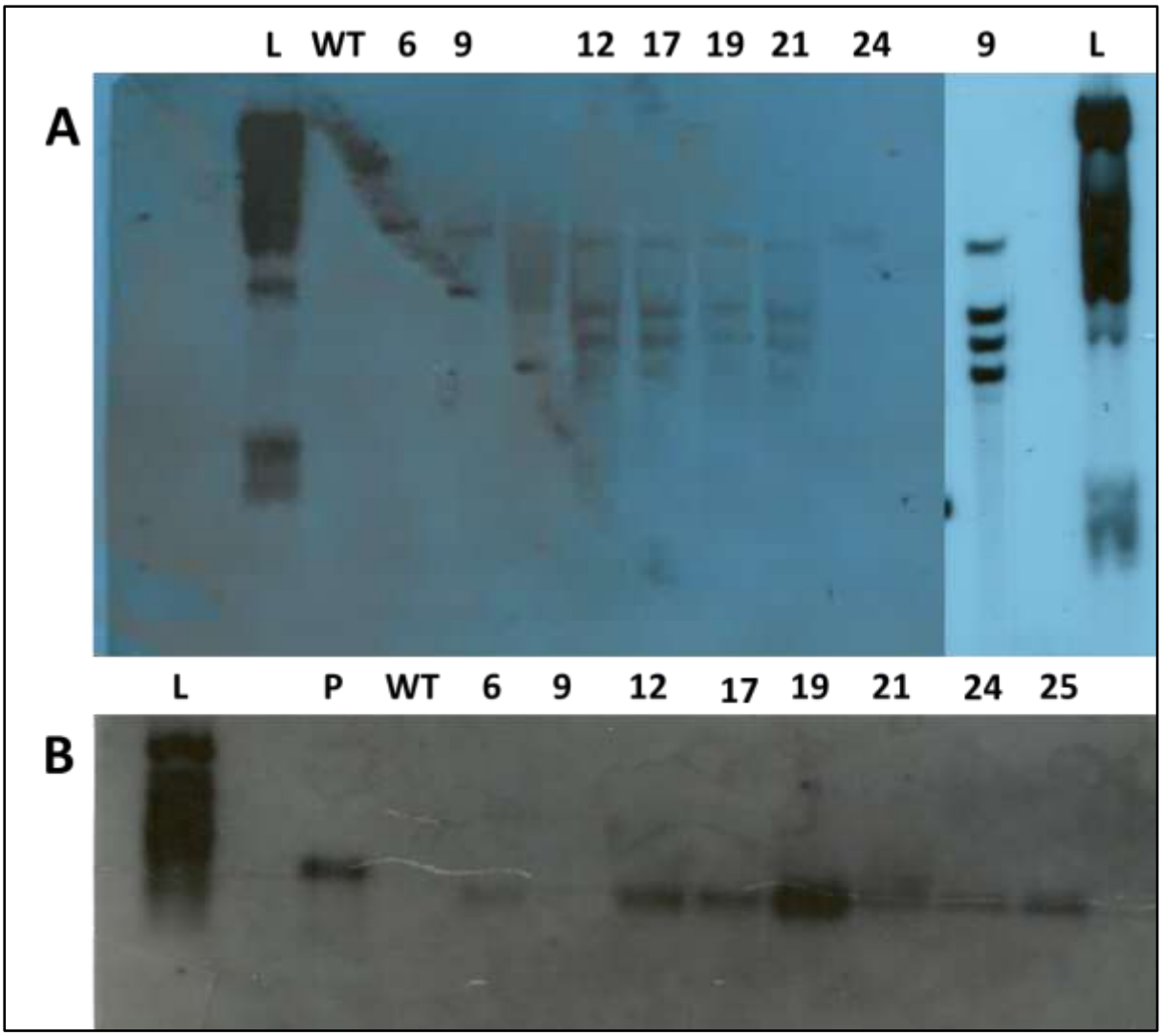

Figure C4.2: (A) The Southern blot analysis of 8 transgenic Hc-AFP1 *V. vinifera* (cv. Red Globe) lines. The marker lane (L) contains the Lamda DNA/HindIII Marker, 2. The "WT" indicates the non-transformed *V. vinifera* (cv. Red Globe) lines used as control and the numbers represent the names of the 11 transgenic Hc-AFP1 *V. vinifera* (cv. Red Globe) lines. (B) The Northern blot analysis of 11 transgenic Hc-AFP1 *V. vinifera* (cv. Sultana) lines, performed by Dr. K du Plessis. The marker lane (L) contains the RiborulerTM High Range RNA Ladder (in bp). The "P" indicates the positive control, "WT" indicates the non-transformed *V. vinifera* (cv. Red Globe) lines used as control and the numbers represent the names of the 11 transgenic Hc-AFP1 *V. vinifera* (cv. Red Globe) lines.

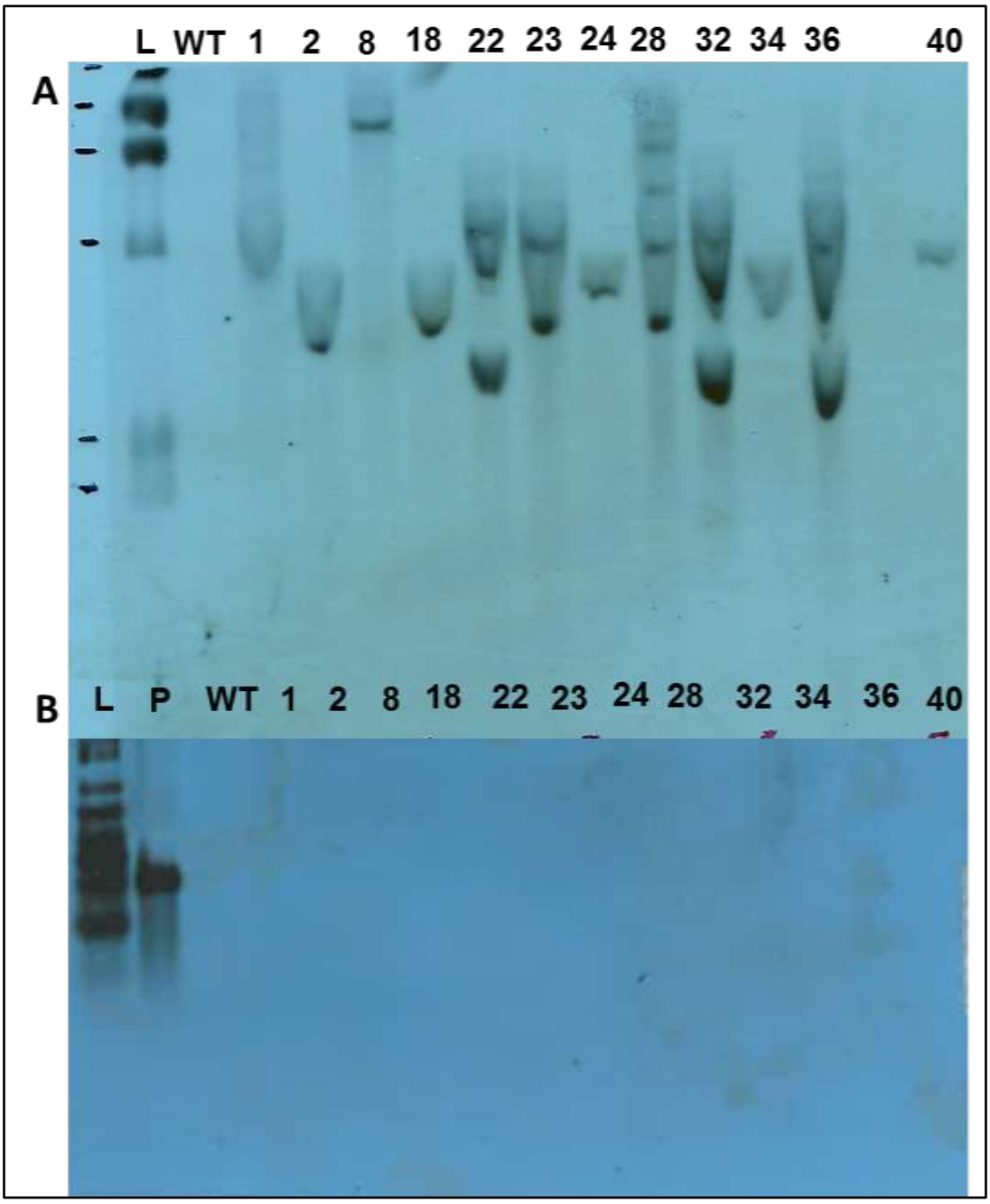

Figure C4.3: (A) The Southern blot analysis of 12 transgenic Hc-AFP4 *V. vinifera* (cv. Sultana) lines. The marker lane (L) contains the Lamda DNA/HindIII Marker, 2. The "WT" indicates the non-transformed *V. vinifera* (cv. Sultana) lines used as control and the numbers represent the names of the 11 transgenic Hc-AFP4 *V. vinifera* (cv. Sultana) lines. (B) The Northern blot analysis of 11 transgenic Hc-AFP4 *V. vinifera* (cv. Sultana) lines. The marker lane (L) contains the RiborulerTM High Range RNA Ladder (in bp). The "P" indicates the positive control, "WT" indicates the non-transformed *V. vinifera* (cv. Sultana) lines used as control and the numbers represent the names of the 12 transgenic Hc-AFP4 *V. vinifera* (cv. Sultana) lines.

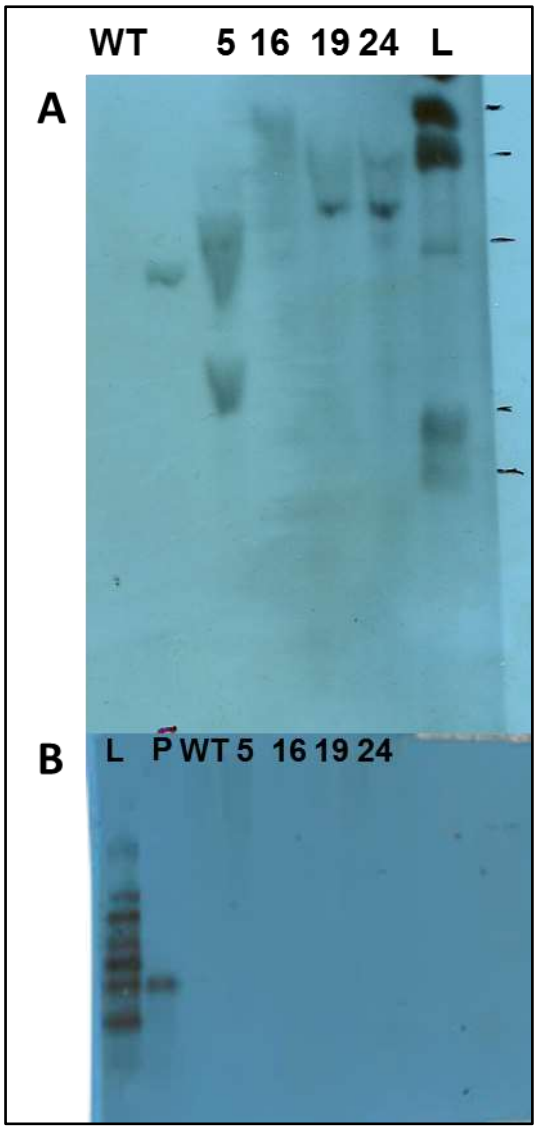

Figure C4.4. (A) The Southern blot analysis of 12 transgenic Hc-AFP4 *V. vinifera* (cv. Red Globe) lines. The marker lane (L) contains the Lamda DNA/HindIII Marker, 2. The "WT" indicates the non-transformed *V. vinifera* (cv. Sultana) lines used as control and the numbers represent the names of the 11 transgenic Hc-AFP4 *V. vinifera* (cv. Sultana) lines. (B) The Northern blot analysis of 11 transgenic Hc-AFP4 *V. vinifera* (cv. Red Globe) lines. The marker lane (L) contains the RiborulerTM High Range RNA Ladder (in bp). The "P" indicates the positive control, "WT" indicates the non-transformed *V. vinifera* (cv. Red Globe) lines used as control and the numbers represent the names of the 12 transgenic Hc-AFP4 *V. vinifera* (cv. Red Globe) lines.

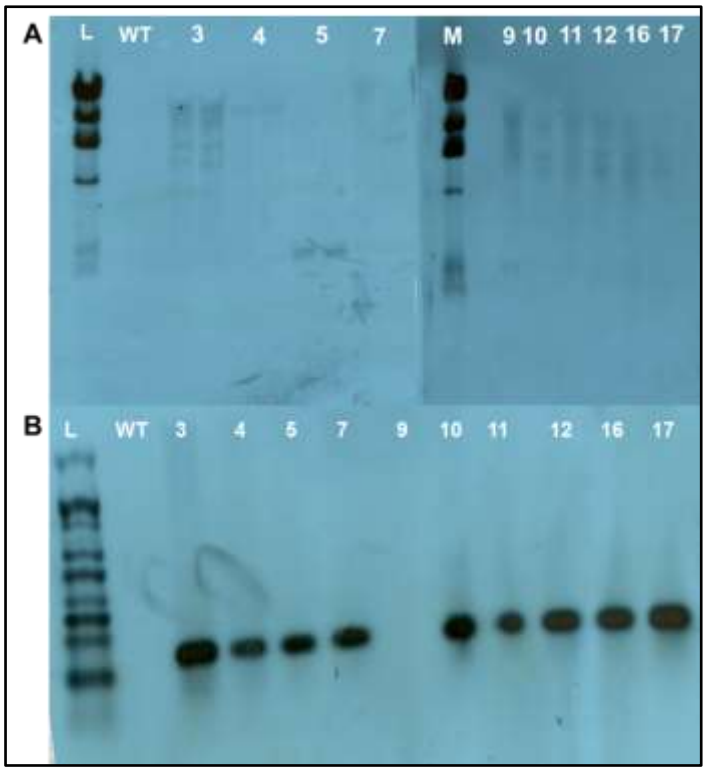

Figure C4.5. (A) The Southern blot analysis of 10 transgenic Rs-AFP2 *V. vinifera* (cv. Sultana) lines. The marker lane (L) contains the Lamda DNA/HindIII Marker, 2. The "WT" indicates the non-transformed *V. vinifera* (cv. Sultana) lines used as control and the numbers represent the names of the 11 transgenic Rs-AFP2 *V. vinifera* (cv. Sultana) lines. (B) The Northern blot analysis of 11 transgenic Rs-AFP2 *V. vinifera* (cv. Sultana) lines. The marker lane (L) contains the RiborulerTM High Range RNA Ladder (in bp). The "P" indicates the positive control, "WT" indicates the non-transformed *V. vinifera* (cv. Sultana) lines used as control and the numbers represent the names of the 12 transgenic Rs-AFP2 *V. vinifera* (cv. Sultana) lines.

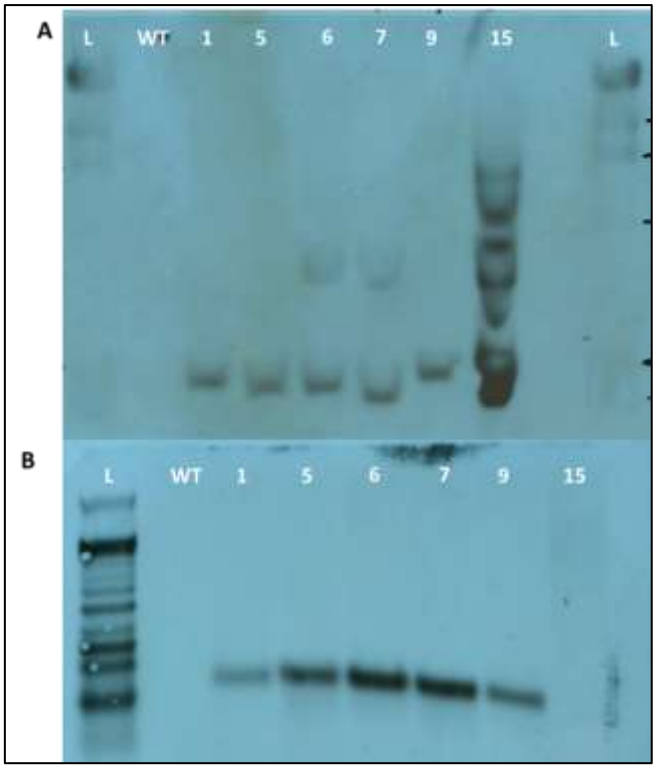

Figure C4.6. (A) The Southern blot analysis of six transgenic Rs-AFP2 *V. vinifera* (cv. Red Globe) lines. The marker lane (L) contains the Lamda DNA/HindIII Marker, 2. The "WT" indicates the non-transformed *V. vinifera* (cv. Sultana) lines used as control and the numbers represent the names of the 11 transgenic Rs-AFP2 *V. vinifera* (cv. Sultana) lines. (B) The Northern blot analysis of 11 transgenic Rs-AFP2 *V. vinifera* (cv. Red Globe) lines. The marker lane (L) contains the RiborulerTM High Range RNA Ladder (in bp). The "P" indicates the positive control, "WT" indicates the non-transformed *V. vinifera* (cv. Red Globe) lines used as control and the numbers represent the names of the 12 transgenic Rs-AFP2 *V. vinifera* (cv. Red Globe) lines.

Addendum D to Chapter 4

This Addendum contains relevant and additional data not shown in Chapter 4.

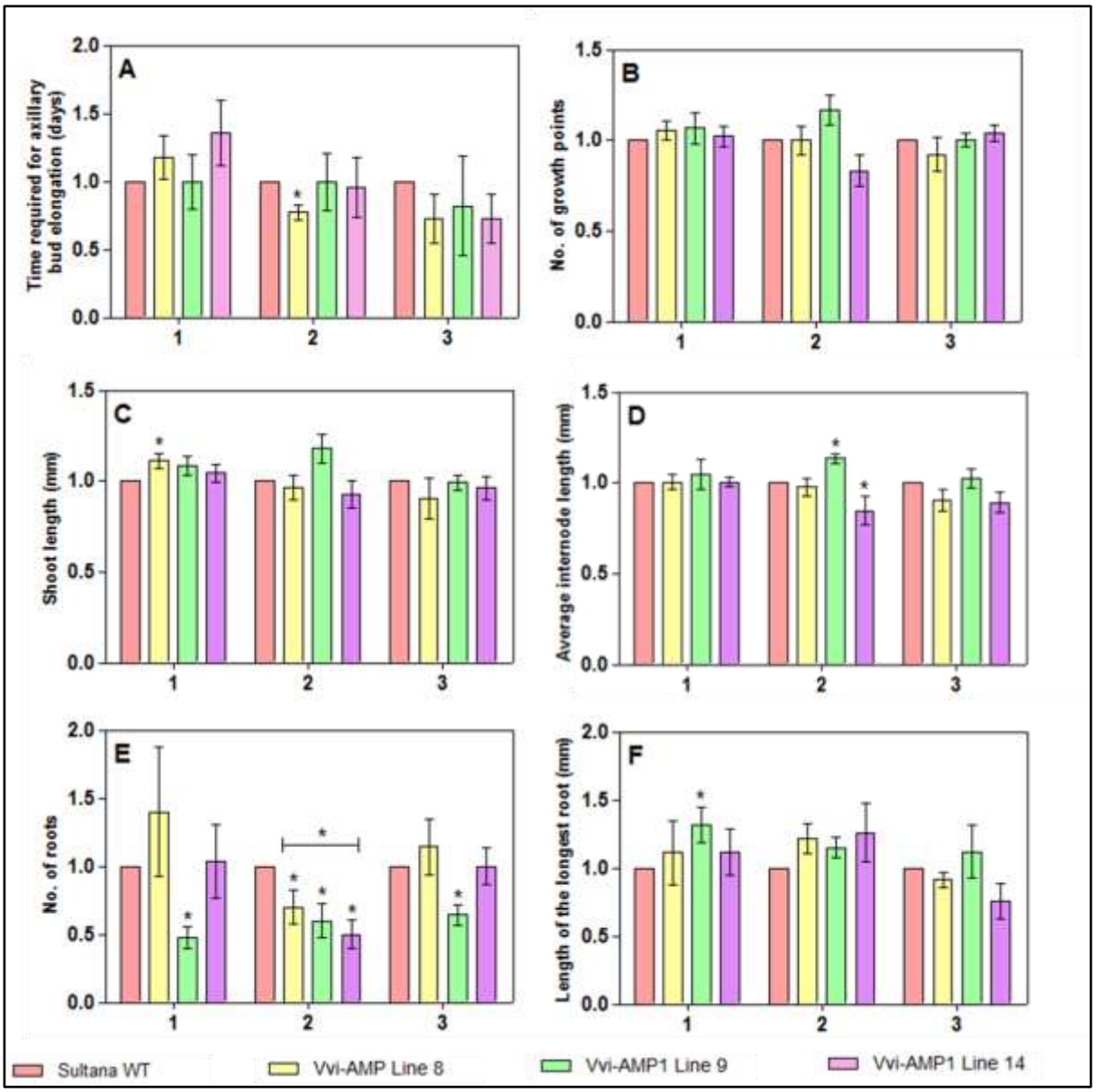

Figure D4.1 Effects of ectopically expressing Vvi-AMP1 on the phenotype of in vitro plantlets of grapevine cultivar Sultana in terms of shoot growth and root formation in three individual conducted experiments. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is represented as means ± SEM and with asterisks indicating individual plant lines significantly different from the Wild type at $P \leq 0.05$ and asterisks with a caped line indicating the Rs-AFP2 population significantly different from the Wild type at $P \leq 0.05$.

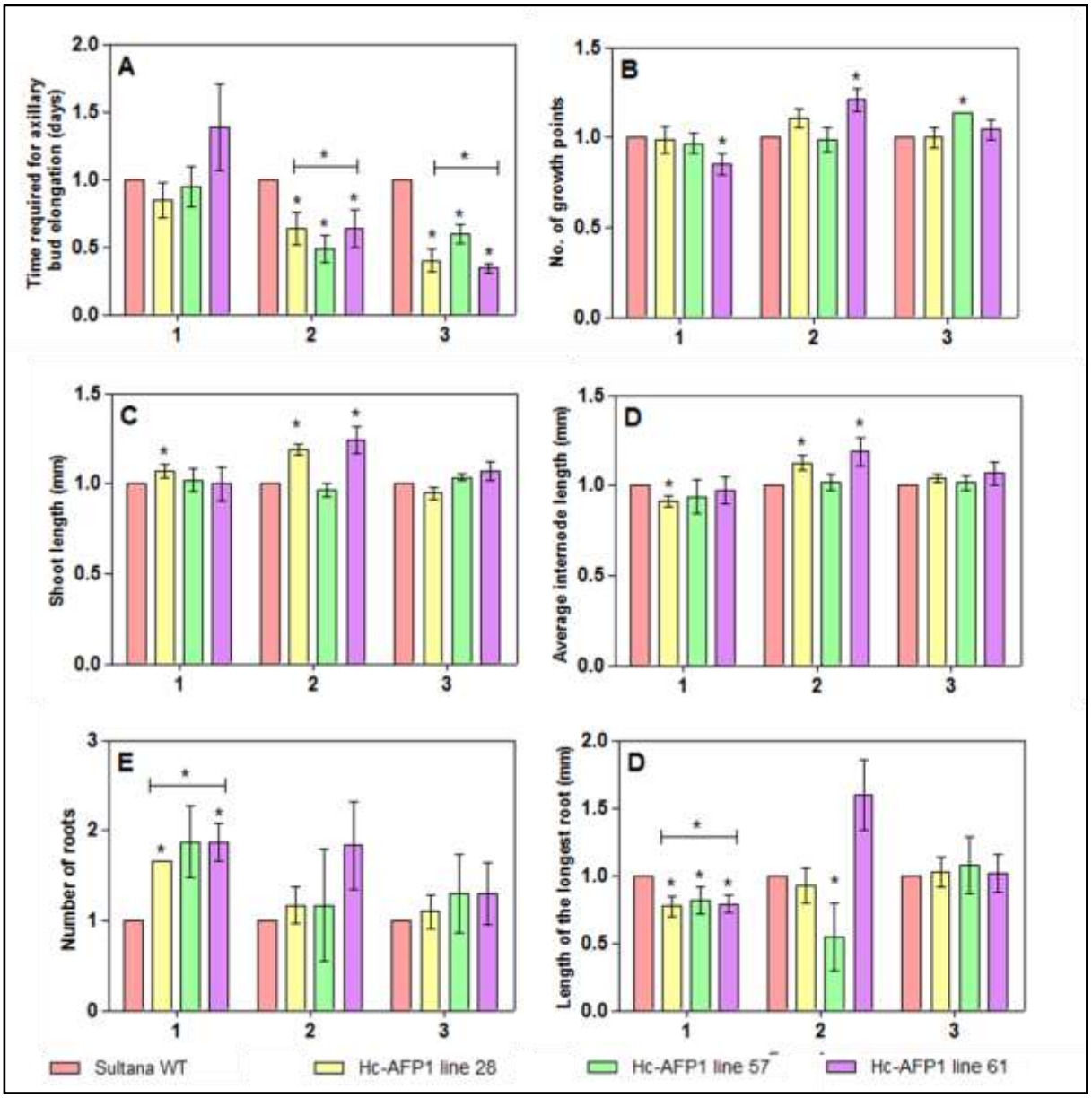

Figure D4.2 Effects of ectopically expressing Hc-AFP1 on the phenotype of in vitro plantlets of grapevine cultivar Sultana in terms of shoot growth and root formation in three individual conducted experiments. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is represented as means ± SEM and with asterisks indicating individual plant lines significantly different from the Wild type at $P \leq 0.05$ and asterisks with a caped line indicating the Rs-AFP2 population significantly different from the Wild type at $P \leq 0.05$.

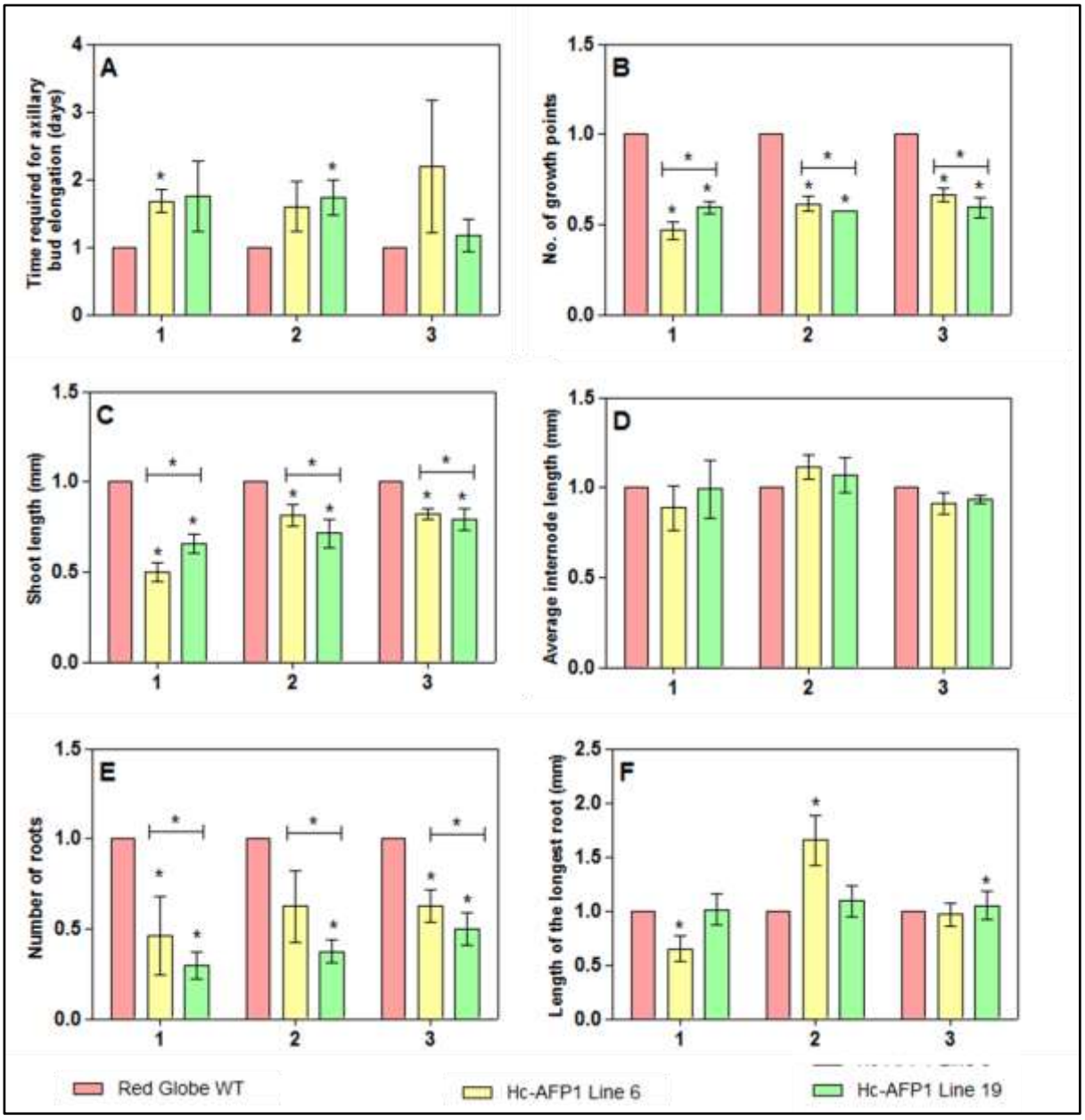

Figure D4.3 Effects of ectopically expressing Hc-AFP1 on the phenotype of in vitro plantlets of grapevine cultivar Red Globe in terms of shoot growth and root formation in three individual conducted experiments. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is represented as means ± SEM and with asterisks indicating individual plant lines significantly different from the Wild type at $P \leq 0.05$ and asterisks with a caped line indicating the Rs-AFP2 population significantly different from the Wild type at $P \leq 0.05$.

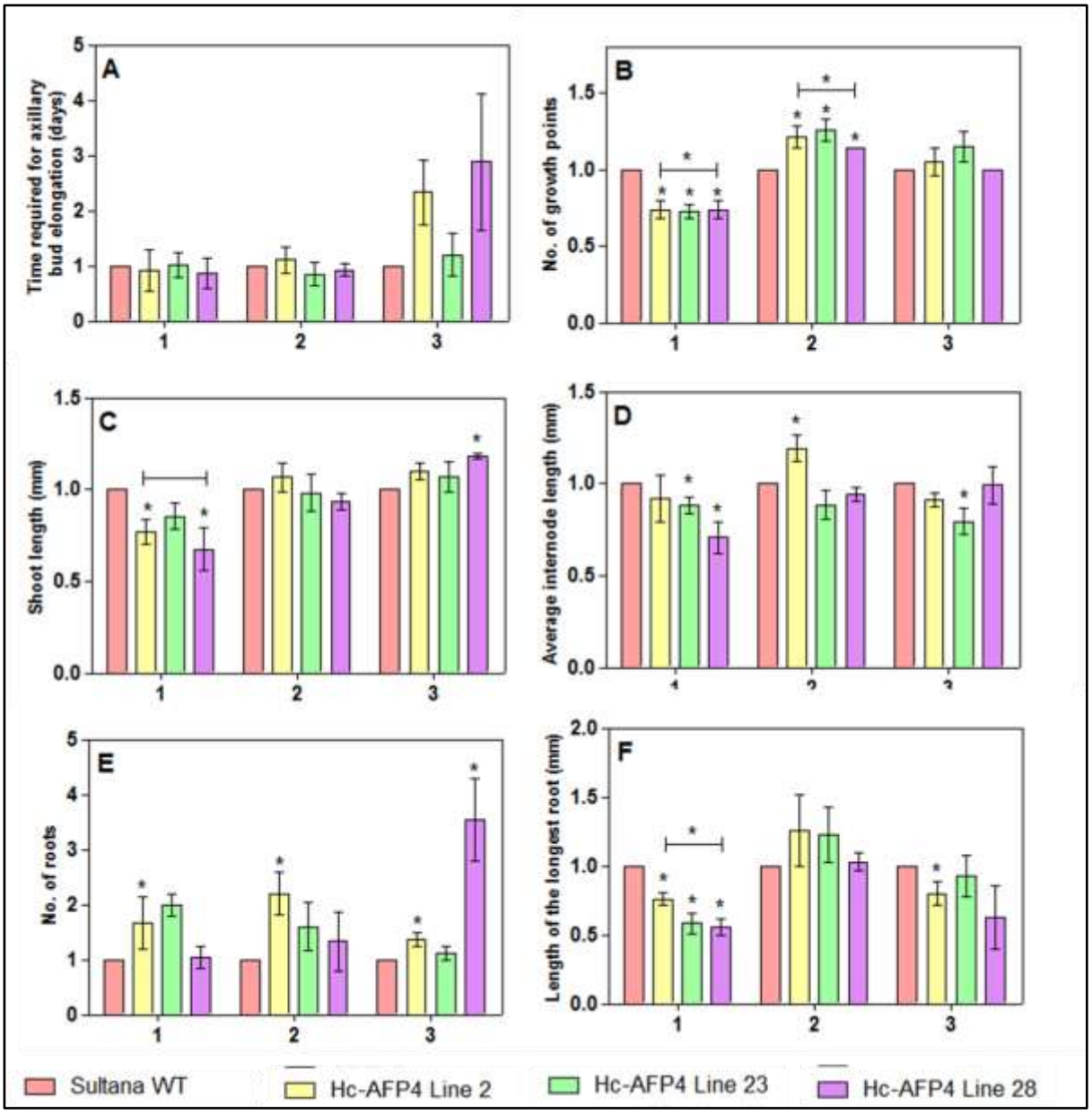

Figure D4.4 Effects of ectopically expressing Hc-AFP4 on the phenotype of in vitro plantlets of grapevine cultivar Sultana in terms of shoot growth and root formation in three individual conducted experiments. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is represented as means ± SEM and with asterisks indicating individual plant lines significantly different from the Wild type at $P \leq 0.05$ and asterisks with a caped line indicating the Rs-AFP2 population significantly different from the Wild type at $P \leq 0.05$.

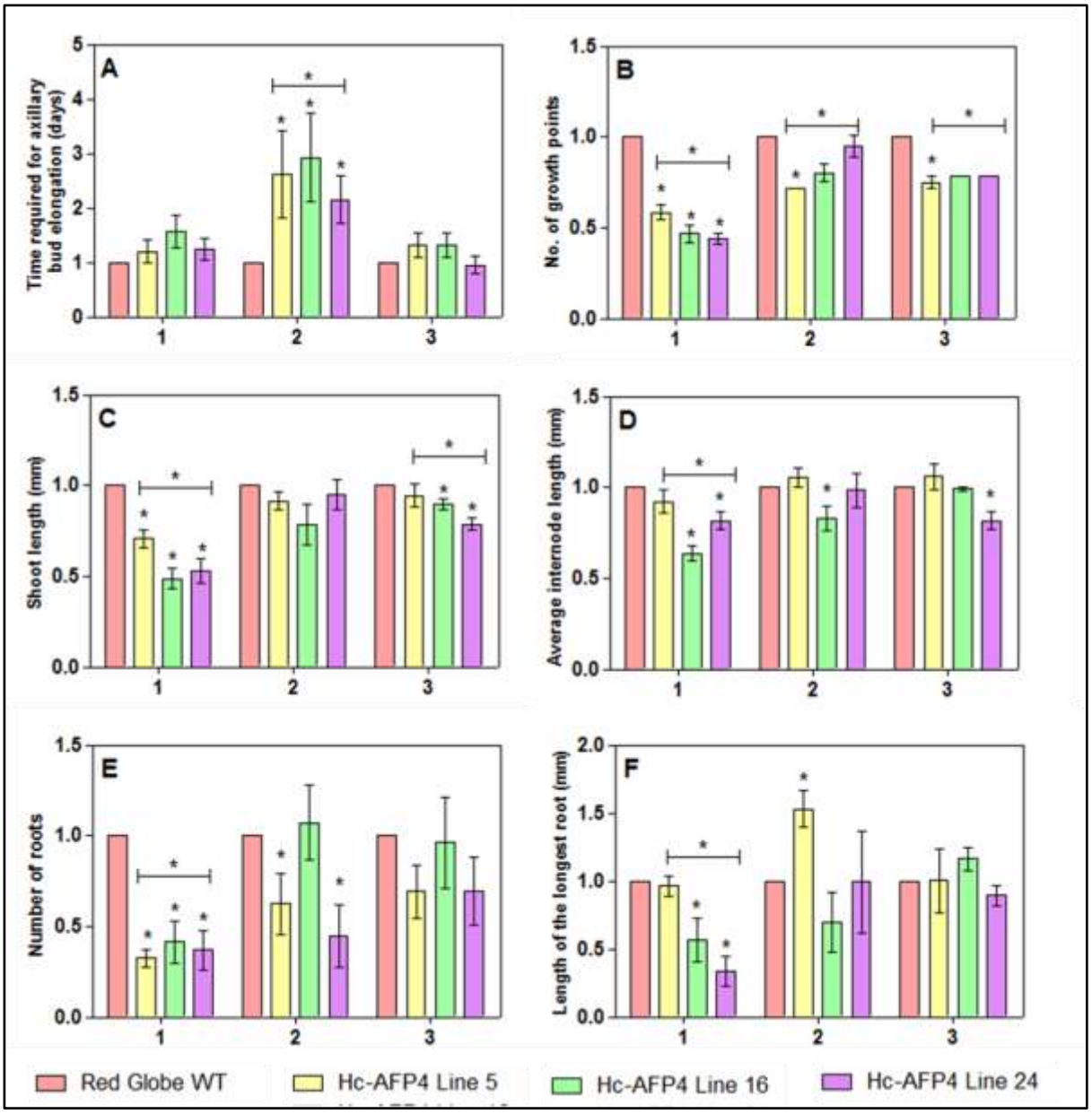

Figure D4.5 Effects of ectopically expressing Hc-AFP4 on the phenotype of in vitro plantlets of grapevine cultivar Red Globe in terms of shoot growth and root formation in three individual conducted experiments. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is represented as means ± SEM and with asterisks indicating individual plant lines significantly different from the Wild type at $P \leq 0.05$ and asterisks with a caped line indicating the Rs-AFP2 population significantly different from the Wild type at $P \leq 0.05$.

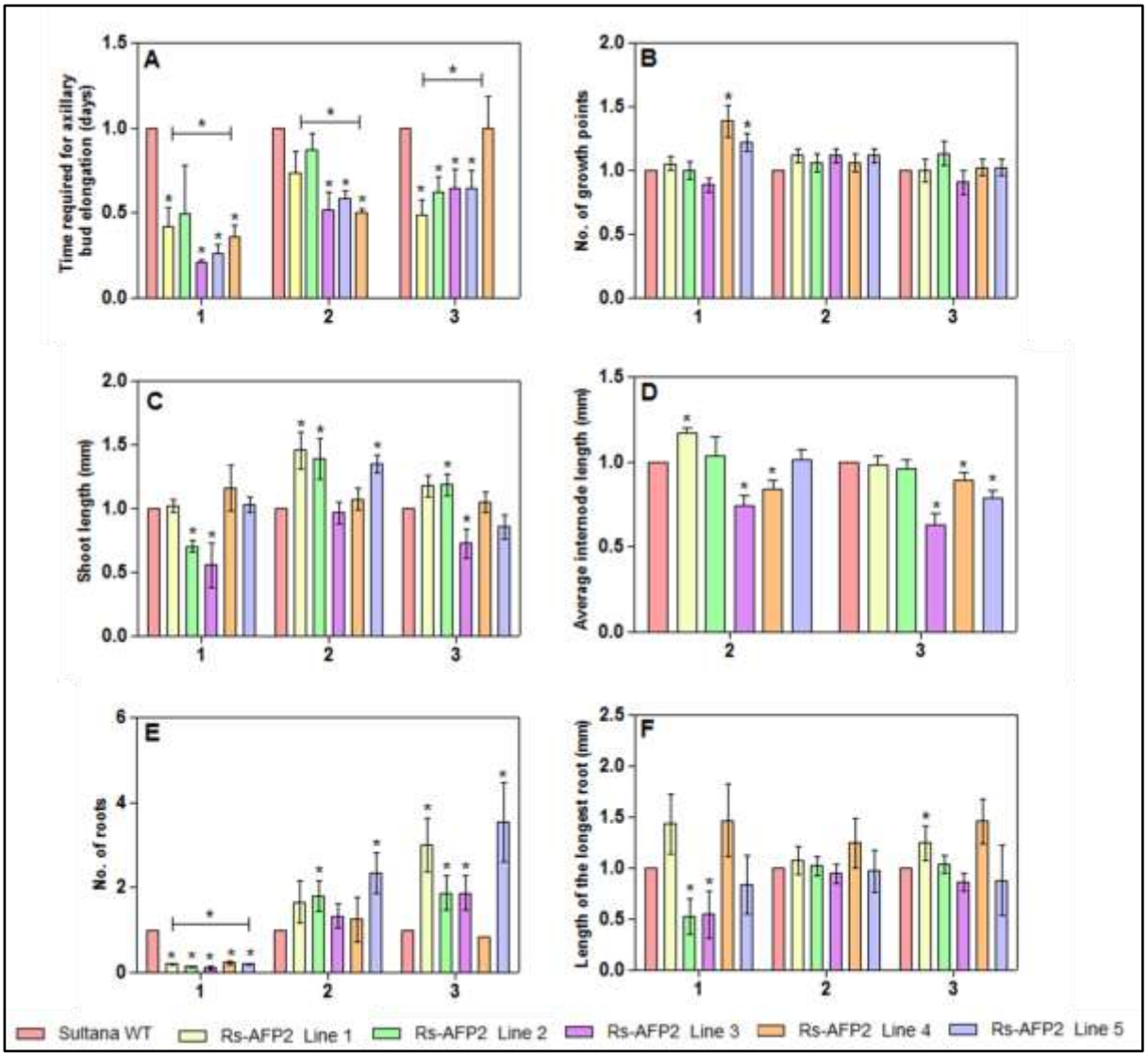

Figure D4.6 Effects of ectopically expressing Rs-AFP2 on the phenotype of in vitro plantlets of grapevine cultivar Sultana in terms of shoot growth and root formation in three individual conducted experiments. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is represented as means ± SEM and with asterisks indicating individual plant lines significantly different from the Wild type at $P \leq 0.05$ and asterisks with a caped line indicating the Rs-AFP2 population significantly different from the Wild type at $P \leq 0.05$.

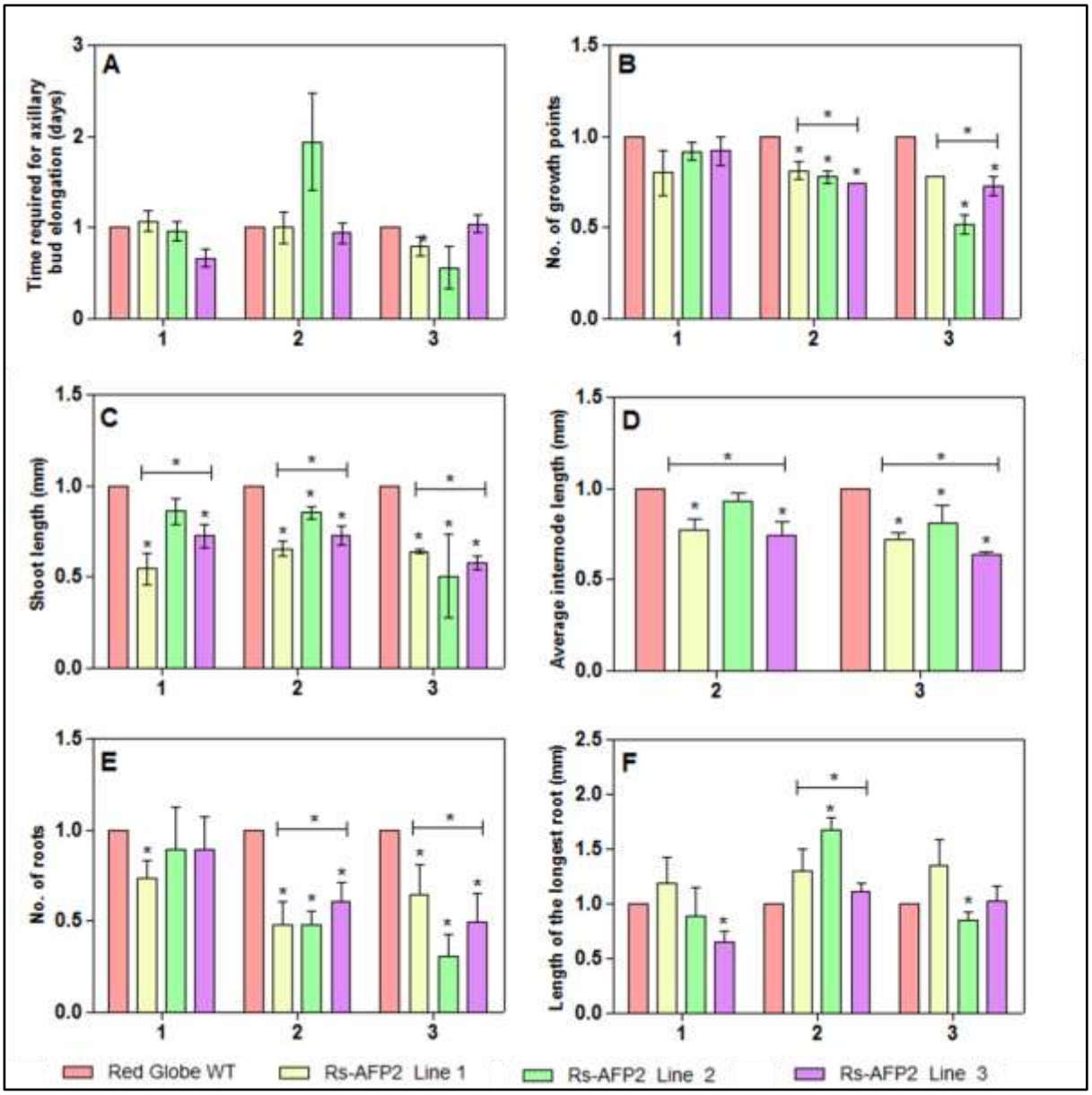

Figure D4.7 Effects of ectopically expressing Rs-AFP2 on the phenotype of in vitro plantlets of grapevine cultivar Red Globe in terms of shoot growth and root formation. (A) Time required for auxiliary bud elongation. (B) Number of auxiliary buds developed in six weeks. (C) Shoot length. (D) Average internode length. (E) Number of roots developed in six weeks. (F) Length of the longest root. Data is represented as means ± SEM and with asterisks indicating individual plant lines significantly different from the Wild type at $P \leq 0.05$ and asterisks with a caped line indicating the Rs-AFP2 population significantly different from the Wild type at $P \leq 0.05$.

Chapter 5

Research results

Challenging defensin expressing transgenic grapevine populations and their controls with *Botrytis cinerea*, *Erysiphe necator* and *Planococcus ficus* to describe and characterise potential defense phenotypes

The authors contributed as follows to the work presented: HB performed all experiments, data-interpretation and compiling of results under the guidance of MAV; HB drafted and finalized the chapter with inputs from MAV.

RESEARCH RESULTS

Challenging of defensin expressing transgenic grapevine populations and their controls with *Botrytis cinerea*, *Erysiphe necator* and *Planococcus ficus* to describe and characterize defense phenotypes

5.1 INTRODUCTION

Grapevine is one of the most important food crops worldwide. Most of the viticulture and wine production industries rely on the cultivars of the European grape species, *Vitis vinifera*. This species however lack genetic resistance against some prominent pathogens and pests and the cultivated varieties have become susceptible to various pathogens and pests due to the long history of domestication, vegetative propagation and human selection for perceived grape quality characteristics, rather than resistance traits (Qiu et al. 2015; Hu et al. 2018). Some of the most important pathogens of grapevine include *Botrytis cinerea, Erysiphe necator, Plasmopara viticola, Agrobacterium vitis, Xylella fastidiosa* and Grapevine Leaf Roll associated viruses; causing grey mold, powdery mildew, downy mildew, crown gall disease, Pierce's disease and leafroll diseases, respectively (Armijo et al. 2016). The latter two diseases also have insect vectors involved and in South Africa, mealybug control is an important aspect in the effort to limit the spread of leafroll infections. Disease management against several pathogens and pests depends on the frequent use of preventative chemical spaying programs (Qiu et al. 2015). This, however, leads to a rapid escalation in fungicide and pesticide resistance and produces a negative ecological footprint. Several strategies have been implemented in order to limit the use of chemicals, including conventional breeding programs and biological control (Compant et al. 2013; Qiu et al. 2015; Wang et al. 2017).

The antifungal activity of plant defensins has been extensively studied since their discovery, isolation and characterisation (Osborn et al. 1995; Terras et al. 1995; Broekaert et al. 1995; Thevissen et al. 1996, 2012; Thevissen 1997). Although the exact mechanism and all underlying cellular mechanisms involved are not completely understood, major advancements have been made towards the elucidation of the mode of action of defensin peptides. The mechanisms employed to achieve fungal cell death differ between plant defensins and include production of reactive oxygen species, apoptosis, membrane permeabilization, impairment of mitochondrial function, interference with divalent cation homeostasis, calcium channel blocking, cell cycle arrest, cell wall stress and septum mislocation (Cools et al. 2017; Parisi et al. 2018). Despite growing numbers of defensins being reported, isolated (Terras et al. 1992b; Osborn et al. 1995; Broekaert et al. 1995; Lay et al. 2003; De Beer 2008; Games et al. 2008) and characterised in terms of their structure-activity features (Fant et al. 1998; Lay et al. 2003, 2012; Lin et al. 2007; Sagaram et al. 2011; Poon et al. 2014; Baxter et al. 2015; Khairutdinov et al. 2017), as well as being tested

against pathogens for in vitro activities (Mendez et al. 1990; Terras et al. 1992b; Osborn et al. 1995; Almeida et al. 2000; Carvalho and Gomes 2009; Kvansakul et al. 2016), it is fair to state that the functional *in planta* roles of the defensin peptides in their native or target hosts have received less attention as of yet.

In this study, we ask two questions: (1) Are grapevine defensin encoding genes transcriptionally regulated in response to pathogens and pests and (2) do transgenic grapevine plants overexpressing defensins with known antifungal activities display resistance against important fungal pathogens and/or a grapevine insect pest? Towards the first question, the expression patterns of grapevine defensin-like (DEFL) genes were evaluated in a meta-analysis from published studies where grapevines were infected with *B. cinerea*, *E. necator* or *P. ficus*. These results prompted experiments where transgenic grapevine plants (Sultana and Red Globe) that overexpress Vvi-AMP1, Hc-AFP1, Rs-AFP2 were challenged with two strains of the grey mould pathogen and a powdery mildew strain to characterise the populations for their potential fungal resistances and defence phenotypes. A selection of transgenic and control plants was also used as hosts for the pest *P. ficus* to evaluate the impact of the defensin overexpression on insect mortality.

5.2 MATERIALS AND METHODS

5.2.1. *In silico* analysis and data mining

The *in silico* pipeline described in Chapter 4 was used to investigate the expression patters of DEFL genes in response to biotic stress. The transcriptomic data used for this study was obtained from a collection of publically available microarray expression studies (Table 5.1).

Table 5.1. Summary of the selected accessible *V. vinifera* microarray experiments form platform GPL13936 included in the analysis of the DEFL gene expression from NCBI.

Platform	Experiment accession	Description	Tissue
GPL13936	GSE32343	Grapevine response to *Planococcus ficus* feeding	Leaf
GPL13936	GSE65969	Analysis of the molecular dialogue between grey mould (*Botrytis cinerea*) and grapevine (*Vitis vinifera*) reveals a clear shift in defence mechanisms during berry ripening	Grape berries
GPL17894	GSE52586	Transcript and metabolite analysis of *Vitis vinifera* cv. Trincadeira berries infected with *Botrytis cinerea* reveals an activation of a non-sustained plant defence response	Grape berries

The resulting heat maps were thoroughly investigated for significant up or down regulation of the DEFL-genes. Line and bar graphs of the expression data (log_2 fold change) were constructed

using GraphPad PRISM 5 for windows (©1992-2007 GraphPad Software, La Jolla California USA, www.graphpad.com).

5.2.2. Infection assays

The transgenic *V. vinifera* (cv. Sultana) and *V. vinifera* (cv. Red Globe) ectopically expressing Hc-AFP1, Rs-AFP2 and Vvi-AMP1 respectively; as well as their untransformed controls were subjected to infection assays with biotrophic and necrotrophic fungal pathogens and with infestation assays with the soft scale insect, *Planococcus ficus* (mealybug), according to the schedule outlined in Table 5.2.

Table 5.2 Summary of the plant lines selected for pathogen and pest challenge experiments. For the whole plant infection assays (*Botrytis* and *Planococcus* challenges) the numbers represent the number of individual plants per transgenic line used, whereas for the detached leaf (*E. necator*) infection assays the numbers represent the number of detached leaves used per line.

Plants			**Challenging Organism**			
			Necrotroph: *B. cinerea*		Biotroph: *E. necator*	Soft scale insect: *P. ficus*
Cultivar	Construct	Line	**Details of infection/survival assay**			
			Whole plant infection assay: *B. cinerea* "Grape strain"	Whole plant infection assay: *B. cinerea* BO510	Detached leaf: dry inoculation	Whole plant survival assay: *P. ficus*
Sultana	Wild type		4	4	4	2
	Rs-AFP1	3	4	4	4	2
		4	4	-	4	2
		5	4	-	4	2
	Hc-AFP1	9	4	-	4	2
		11	4	4	4	2
		78	4	-	4	1
	Vvi-AMP1	19	4	4	4	2
Red Globe	Wild type		6	4	4	-
	Rs-AFP1	1	4	4	4	-
		2	4	-	4	-
		4*	4	-	4	-
	Hc-AFP1	9*	4	-	4	-
		12	4	4	4	-
		24	4	-	4	-

*Non expressing plant lines

5.2.2.1 *B. cinerea* isolates and culturing

Two different *B. cinerea* strains were used in this study, a hyper-virulent *B. cinerea* strain isolated from a vineyard in Stellenbosch (designated the "grape strain") (Joubert et al. 2006) and the *B. cinerea* B0510 strain (Amselem et al. 2011; Staats and van Kan 2012; Blanco-Ulate et al. 2014).

The grape strain was cultivated on sterile apricot halves (Rhodes, Rhodes Food Group Holdings, South Africa) at 23°C until sporulation, wheras *B. cinerea* B0510 was cultivated on 5% Malt Extract Agar (Biolab, Merck, South Africa) and 1% Yeast Extract (Biolab, Merck, South Africa). They were maintained in the dark at 20-21°C for 4-5 days. To induce sporulation, B0510 cultures were placed under constant artificial light conditions for 4 consecutive days.

B. cinerea spores were harvested in 5 ml sterile water containing 0.001% Tween 20. The spore suspension was filtered through sterile glass wool to remove mycelium fragments. Prior to an infection assay, the viability and germination potential of the harvested spores were determined growing the spores on 2% (w/v) water agar overnight at 23°C. Prior to an infection experiment, spores were hydrated overnight at 4°C and spore concentrations determined with a haemocytometer. A previously optimised pathosystem for grapevine, using whole plant infections (Moyo 2011) was followed by using 1000 *B. cinerea* spores per infection spot (see next section). Spores were diluted in 50% sterile red grape juice to a final concentration of 1000 spores per 5 µl infection spot.

5.2.2.2 Whole plant infection assay with *B. cinerea*

Hardened off plants selected for this infection assay were healthy and of a similar size, with numerous mature, fully expanded leaves. Four days prior to the infection assay, leaves of plants selected for the assay were gently washed with water to remove any residual components from routine greenhouse fumigation and application of contact fungicides and allowed to dry for two days. One day prior to the infection assay, plants were placed in Perspex high humidity infection chambers to allow for sufficient acclimatization. Room temperature and a light/dark cycle of 16/8 hours were maintained throughout the entire infection.

Four leaves per plant were infected with four spots each on the adaxial side. The progression of the infection and the development of lesions on the leaf surfaces were monitored at 24 hour intervals, from 48 hours post infection when primary lesions started forming. Lesion diameter at each infection spot was measured with a digital calliper (Mitutoya American Association, USA) and the infection was allowed to progress for four days for both of the *B. cinerea* strains. Day four was the last day the lesions were measurable. The technical repeats (four infection spots per leaf) were averaged per biological repeat (plant line). Paired t-tests were used to determine significant differences between the transgenic and wild type infections. Graphs were constructed using GraphPad PRISM 5 for windows (©1992-2007 GraphPad Software, La Jolla California USA, www.graphpad.com).

5.2.2.3 Detached leaf infection assays with the biotrophic fungus, *E. necator*

Grapevine powdery mildew (*E. necator*) was maintained on potted wild type *V. vinifera* (cv. Sultana) and wild type *V. vinifera* (cv. Red Globe) plants. These heavily infected source plants were maintained in a room at 25°C to promote optimal growth of the grapevine powdery mildew fungus.

As outlined in Table 5.2, three transgenic Hc-AFP1 and Rs-AFP2 individually transformed plant lines per cultivar *V. vinifera* (cv. Sultana) and *V. vinifera* (cv. Red Globe), as well as one transgenic *V. vinifera* (cv. Sultana) Vvi-AMP1 plant line and the respective untransformed controls were utilized for the infection assay according to a method described by Feechan et al. (2011) (refer to Figure 5.1). The plants selected for this infection assay were healthy with no disease symptoms. A total of 8 young (leaf 2 and 3); glossy leaves of approximately 6 cm in diameter were selected per plant line from 4-5 individual plants per line.

Leaf surfaces were sterilized after harvest by soaking in 0.25% (w/v) $CaCl_2O_2$ (Merck© 2018 Darmstadt, Germany) solution for 5 minutes with gentle shaking, followed by three subsequent rinsing steps in sterile dH_2O for 5 min each with gentle shaking. Leaves were then placed on sterile tissue paper and gently patted down with sterile tissue paper to remove most of the moisture. Leaves were then left to dry in a laminar flow cabinet for ±5-10 minutes to remove the remaining moisture. Using a sterile scalpel, the lower tip of the petiole of each leaf was removed to result in a 1 cm petiole. Leaves were then placed on 1% (w/v) water agar plates (120 x 120 mm), with the adaxial side facing up and the 1 cm petiole inserted in the medium.

The young, glossy, detached and sterilised leaves were inoculated with *E. necator* by gently tapping a heavily infected leaf, obtained from the source plant, above the open plates, according to the method described by Feechan et al. (2011). The infected leaves were incubated in the dark with closed lids to ensure 100% humidity for the first 24 hours after inoculation. Hereafter plates were removed from the dark and subjected to a 16/8 hour light/dark cycle. From 48 hours post infection, lids were opened once daily to reduce humidity inside the culture dishes. These conditions favoured the pathogen and ensured the optimal growth and development of *E. necator*.

Forty-eight hours after inoculation, two leaves per transgenic plant line and untransformed controls were visualized under a Leo® 1430VP Scanning electron microscope to monitor fungal structures and infection progression. Leaf discs of 15 mm in diameter were punched out and mounted with carbon tape for observation at 500x magnification.

Two more leaves per plant line and untransformed controls were removed at 48 hours after inoculation for trypan blue staining (according to the method of Feechan et al. 2011). Five leaf discs of 15 mm in diameter were punched out from these two leaves and stained in trypan blue staining solution for 1 hour in a boiling water bath as described by Koch and Slusarenko (1990). The discs were then subsequently decolourized in 2.5 g/mL chloral hydrate solution (Merck© 2018 Darmstadt, Germany) overnight before visualization under a Nikon Opti-photo 2 Hoffman

modulation contrast transmitted light upright microscope (Nikon, Japan) at 40x magnification. Germinated conidia were counted and categorized according to the infection structures formed namely: presence/absence of appressoria, haustoria, or haustoria with visible programmed cell death, as described by Feechan et al. (2011). The percentages of each infection mechanism were calculated in order to quantify the relative level of resistance or susceptibility of each plant line (Figure 5.1).

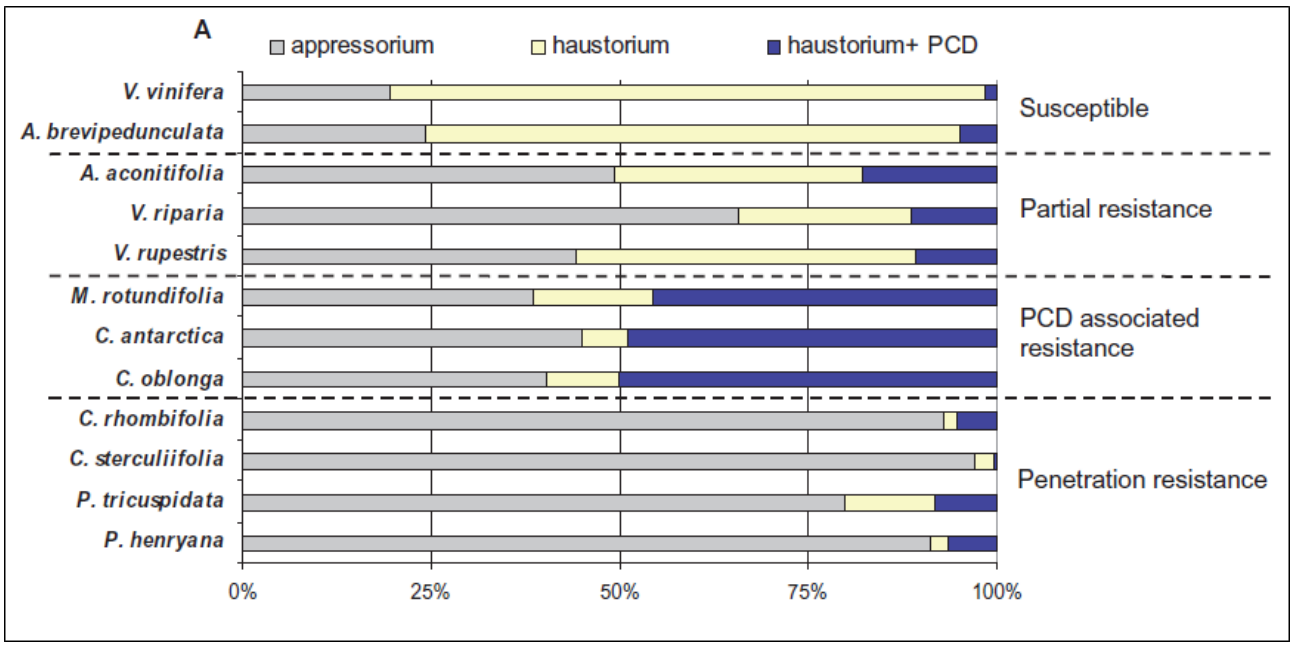

Figure 5.1. The susceptible and resistance phenotypes described for various grapevine species against *E. necator* at 48 hours post infection according to a method described by Feechan et al. (2011). The frequency of *E. necator* penetration attempts, followed by appressorium formation without penetration, successful penetration and haustorium formation or haustorium formation together with programmed cell death (PCD) are characteristic features scored to describe the defence phenotypes and are shown in percentage (adopted from Feechan et al. 2011).

On the remaining four infected leaves per plant line, the fungal infection was allowed to progress till 14 days post infection. The fungal growth on these four infected leaves per plant line was visualized under the stereomicroscope daily and the development of fungal structures and sporulation of *E. necator* also evaluated daily. This evaluation consisted out of a daily scoring of each leaf on a scale of 1-9 from 4 to 14 dpi according to the Global Resistance Index as described by Miclot et al. (2012). The Global resistance index is depicted and summarized in Table 5.3. All light microscope and stereomicroscope pictures were taken using a 5.0 M pixels Microscope digital eyepiece (Lasec South Africa (Pty) Ltd) and ScopePhoto (ScopeTek ScopePhoto,2003-2010, Microsoft®Windows®, USA) software.

Table 5.3 Grapevine powdery mildew resistance indexes adopted from (Du Plessis 2012; Miclot et al. 2012). Pictures were taken under a stereomicroscope with 100x magnification.

Resistance index	Description	Global response
9	Mycelium rare, no sporulation	
7	Mycelium scattered, no sporulation	
5	Mycelium widespread, low density, weak sporulation	
3	Mycelium widespread, dense, moderate sporulation	
1	Widespread, dense sporulation	

At 14 dpi, one leaf disc (15 mm in diameter) per leaf of the remaining 4 leaves per plant line was stained with trypan blue and visualized under a light microscope as described above. Furthermore, one leaf disc of four individual infected leaves (4 in total), per plant line were excised and collected. These leaf discs were then combined in 10 ml sterile distilled water containing 0.1% (w/v) Tween20 (Merck© 2018 Darmstadt, Germany). Form each conidial suspension, five aliquots were taken and conidia counted using a Bright-Line haemocytometer (Merck© 2018 Darmstadt, Germany). The average conidial concentration for each leaf was calculated according to Feechan et al. (2011). Graphs were constructed using GraphPad PRISM 5 for windows (©1992-2007 GraphPad Software, La Jolla California USA, www.graphpad.com). Significant differences of the average conidial concentration between the wild type and transgenic lines were determined using a factorial ANOVA, followed by a Fisher LSD post hoc test with significant differences analysed at $P \leq 0.05$, using statistical software Statistica, version 13 (Dell Inc., Tulsa, OK, USA).

5.2.2.4 Survival assay with the soft scale insect, *P. ficus*

Hardened off plants were prepared as described above. One day prior to the survival assay, plants were moved to the infection room to allow for sufficient acclimatization. Room temperature and a light/dark cycle of 16/8 hours were maintained throughout the entire challenge. As outlined in Table 5.2, three transgenic *V. vinifera* (cv. Sultana) Hc-AFP1 and Rs-AFP2 individually transformed plant lines, as well as one transgenic *V. vinifera* (cv. Sultana) Vvi-AMP1 plant line and untransformed controls were utilized for the mealybug challenge. The plants selected for this infection assay were healthy with no disease symptoms. Second and first instar *P. ficus* were kindly obtained from the ARC Infruitec-Nietvoorbij, Stellenbosch. These mealybugs are reared on butternut squash at the ARC Infruitec-Nietvoorbij insect rearing facility. Two plants per line, with four leaves per plant, were used. Five mealybugs were gently transferred with a small brush to each leaf. Each plant therefore contained 20 mealybugs for the infection. The survival assay was conducted over five days according to a method of Allsopp (2015). The number of mealybugs that remained active and feeding on the leaves were recorded daily. The data was expressed as percentage survival and represent the average of two independent experiments. Graphs were constructed using GraphPad PRISM 5 for windows (©1992-2007 GraphPad Software, La Jolla California USA, www.graphpad.com). Significant differences between the wild type and transgenic lines were determined using a one-way ANOVA, followed by a Fisher LSD post hoc test with significant differences analysed at $P \leq 0.05$, using statistical software Statistica, version 13 (Dell Inc., Tulsa, OK, USA).

5.3 RESULTS

5.3.1. *In silico* analysis of the expression patterns of grapevine DEFL genes in response to biotic stresses

The analysis of gene expression data showed that differential DEFL gene expression occurred in response to the treatment of the soft scale insect, *P. ficus* (mealybugs) (Figure 5.2), as well as in response to *B. cinerea* in Marselan and Trincadeira cultivars (Figures 5.3 and 5.4). From this analysis it was clear that defensins are constitutively expressed as reported in Chapter 4, but that the biotic stresses caused differential expression patterns in the DEFLs on a temporal level (period post challenge), (Figure 5.2, panels B and C respectively show the genes significantly increased in expression at 6 and 96 hours post challenge with an insect pest); per tissue (Figure 5.3) and per developmental stage (Figures 5.3 and 5.4) post infection with *Botrytis cinerea*. Notably, the Vvi-AMPs were upregulated in leaves challenged with mealybugs, together with a number of snakins and other DEFLs, but did not respond to *B. cinerea* infection in berries, except for increased expression in the skin of ripe berries in cv. Marselan (Figure 5.3C). Some members of the snakin family responded strongest to the infection in the berry tissues, as well as DEFL10.1 and 10.2. Furthermore, it was clear that the snakins and DEFL genes showed the most dramatic changes in

expression between different berry developmental stages, indicating that these genes responded to the infection in a developmentally regulated manner (Figures 5.3 and 5.4).

5.3.2 Whole plant infection assays of *V. vinifera* (cvs. Sultana and Red Globe) transgenic population ectopically expressing plant defensins Hc-AFP1 and Rs-AFP2 respectively with the necrotrophic fungus *B. cinerea*.

In order to determine if the ectopic expression of the respective plant defensins Hc-AFP1 and Rs-AFP2 in two *V. vinifera* cvs. (Sultana and Red Globe) conferred enhanced resistance towards the necrotrophic fungus *B. cinerea,* hardened off transgenic lines (and their controls) were challenged by two different *B. cinerea* strains. Whole plant infection assays were conducted in a time-course spanning five days (Figure 5.5) according to a previously optimised grapevine-*Botrytis* pathosystem. The take rate for the infections assays were 100% since all infection spots developed lesions, whereas mock inoculations remained symptomless. The infections spots developed into primary lesions within the first two days post infection and ultimately secondary spreading lesions (refer to Figures A5.1-6 in Addendum A for images of the infections). Measurements were only taken after 2 dpi as a sustained high humidity during the first 48 hours of the infection assay is essential for *B. cinerea* to establish successful infection. None of the transgenic populations displayed increased resistance to *Botrytis* infections at the end of the assay period (4 dpi) when infected with the hyper-virulent grape strain (Figure 5.5). Both the Sultana and Red Globe populations overexpressing the Rs-AFP2 peptide showed a reduced lesion diameter at 2 dpi against the grape strain (Figure 5.5 B and E), but the lesions ultimately developed to similar sizes, or even bigger sizes in the rest of the period, compared to the controls. The infections with the B05.10 strain yielded a statistically significant reduction in lesion diameter in the lines tested for Rs-AFP2 in both the Sultana and Red Globe populations at 4 dpi, but none of the other lines tested showed any reduced lesion sizes against this strain. Fungal reproductive structure development was displayed between 2-4 dpi for the Sultana control and transgenic lines (Addendum A to Chapter 5).

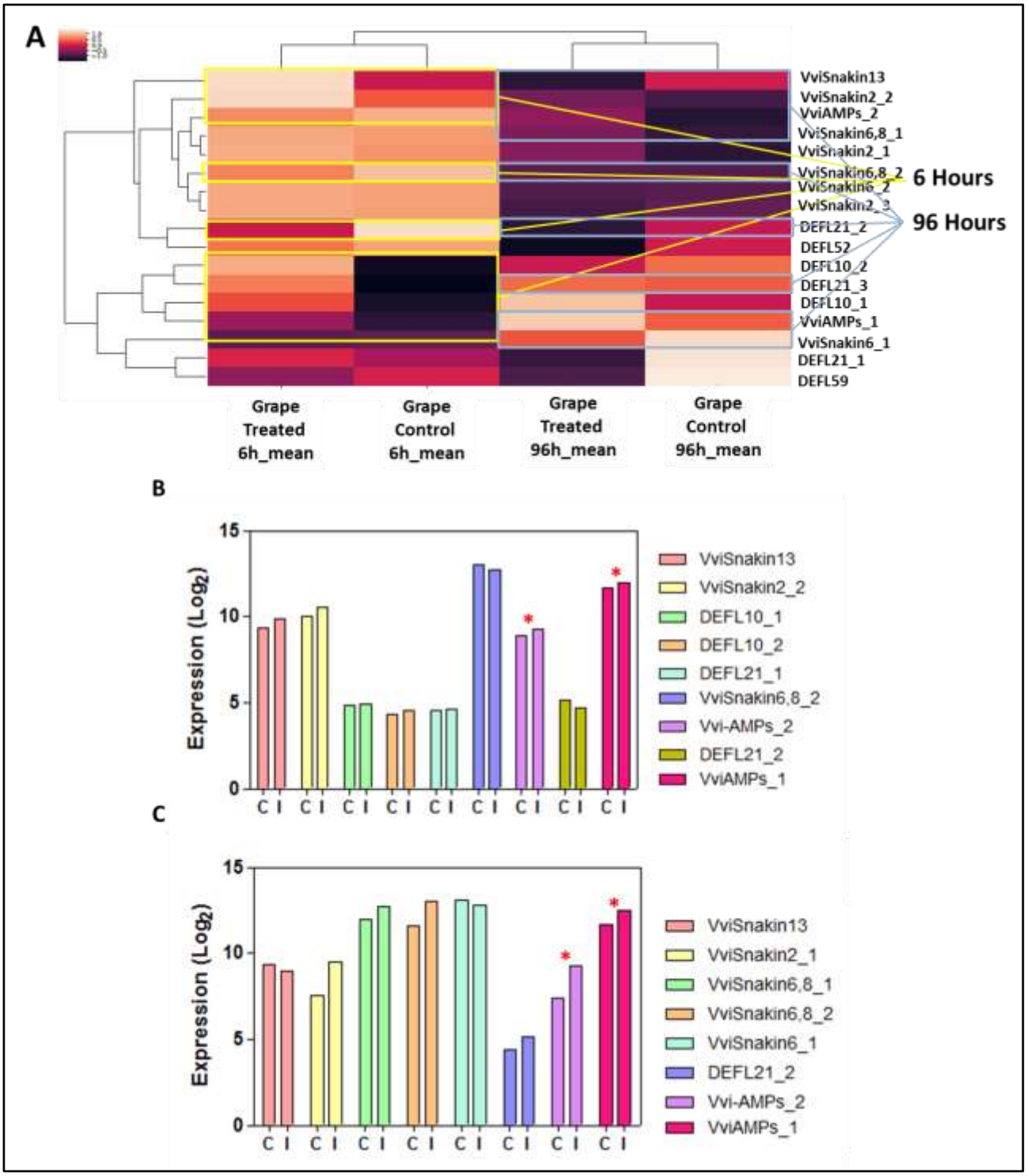

Figure 5.2. (A) Heat map depicting the tissue specific relative expression (Z-score normalized) of DEFL genes in grapevine leaves after exposure to *P. ficus.* The yellow boxes indicate the DEFL genes strongly upregulated at six hours post challenge/infection (also refer to panel B of the figure); whereas the blue boxes indicate the DEFL genes strongly upregulated at 96 hours post infection (also refer to panel C of the figure). (B) Expression patterns (log_2 fold change) of DEFL genes displaying the most dramatic fold changes after six hours and (C) 96 hours. The "C" and "I" on the x-axis refers to uninfected control plants and infected plants, respectively. The red asterisk indicates the expression patterns of the Vvi-AMPs.

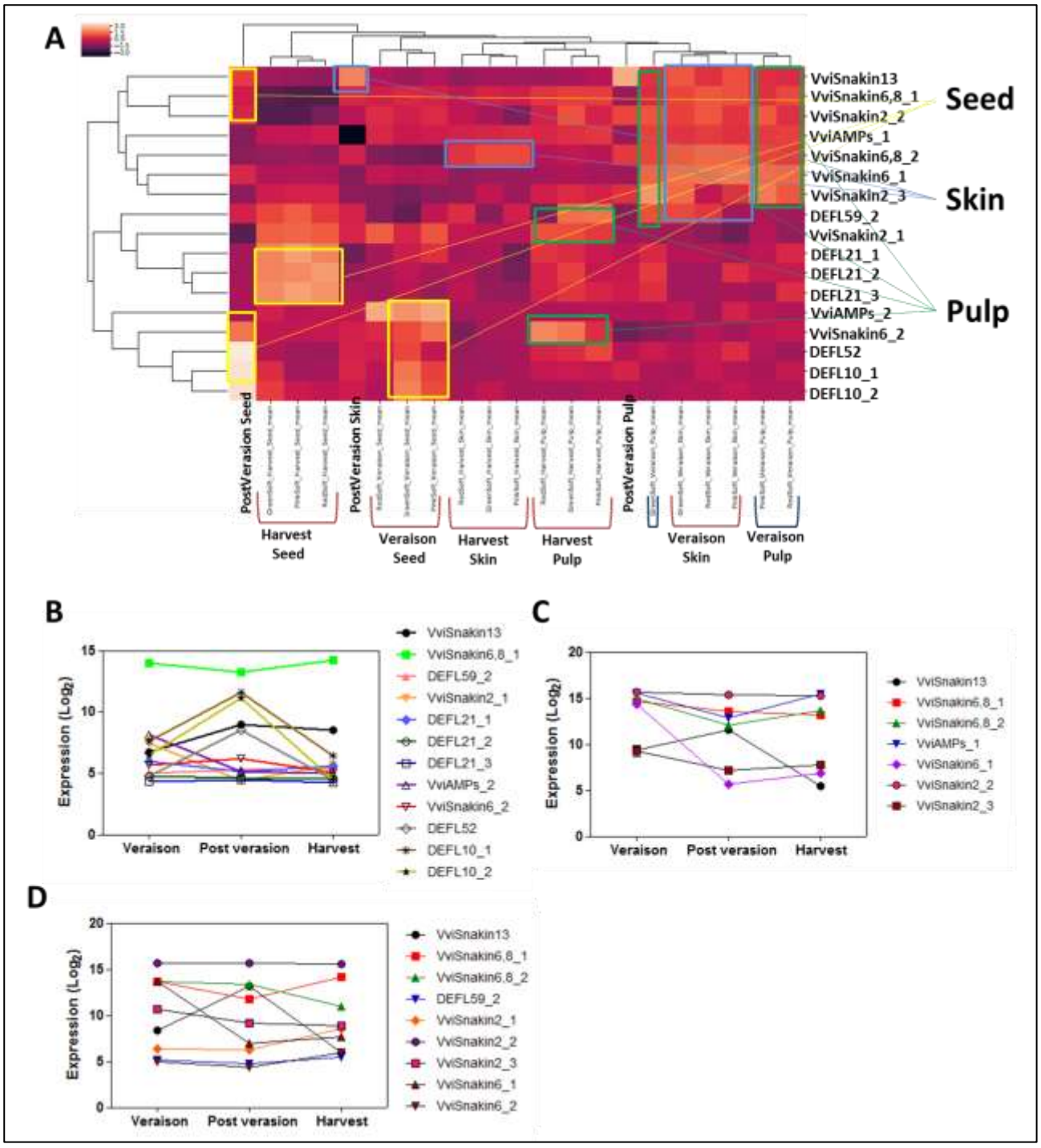

Figure 5.3 (A) Heat map depicting the tissue specific relative expression (Z-score normalized) of the DEFL genes in response to *B. cinerea,* relative to the uninfected in berry tissues in cv Marselan. The yellow boxes indicate the DEFL genes strongly affected in the seed tissue of the berry (also refer to panel B of the figure); whereas the blue boxes indicate the DEFL genes strongly affected in the skin tissue of the berry (also refer to panel C of the figure) and the green boxes indicate the DEFL genes strongly affected in the pulp of the berry (also refer to panel D of the figure). (B) Line graphs depicting DEFL gene expression patters (Log_2 fold change) with the most dramatic fold changes in berry seed, (C) skin and (D) pulp.

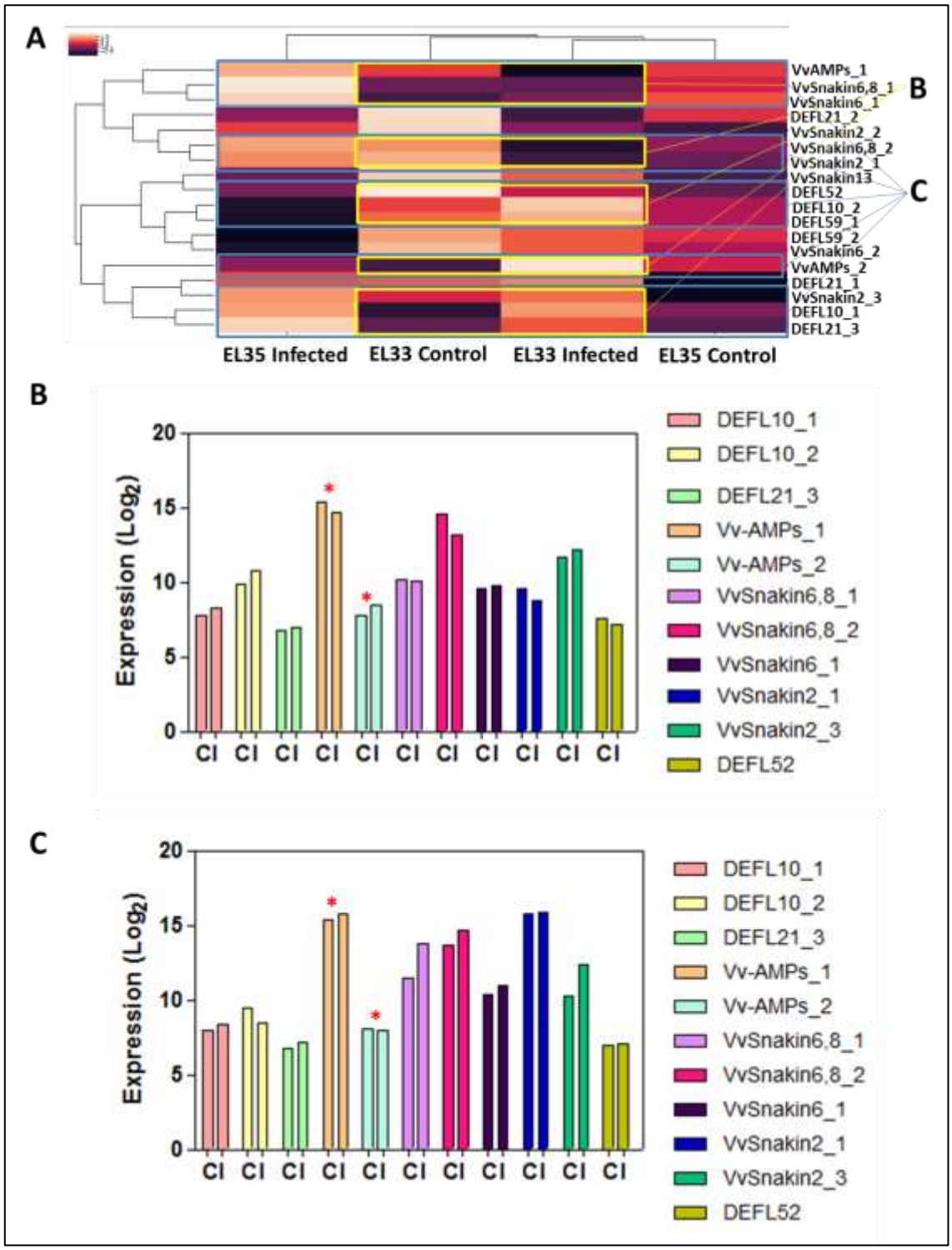

Figure 5.4. (A) Heat map depicting the relative expression (Z-score normalized) of DEFL genes in berry tissue of green berries (EL 33; yellow boxes) and veraison (EL35; blue boxes) of cv. Trincadeira, in response to *B. cinerea* infection. Column graphs depicting DEFL gene expression patters (Log_2 fold change) with the most dramatic fold changes in control (C) and infected (I) green berries (EL 33, panel B) and veraison berries (EL35, panel C) in response to *B. cinerea* infection. The red asterisk indicates the expression patterns of the Vvi-AMPs.

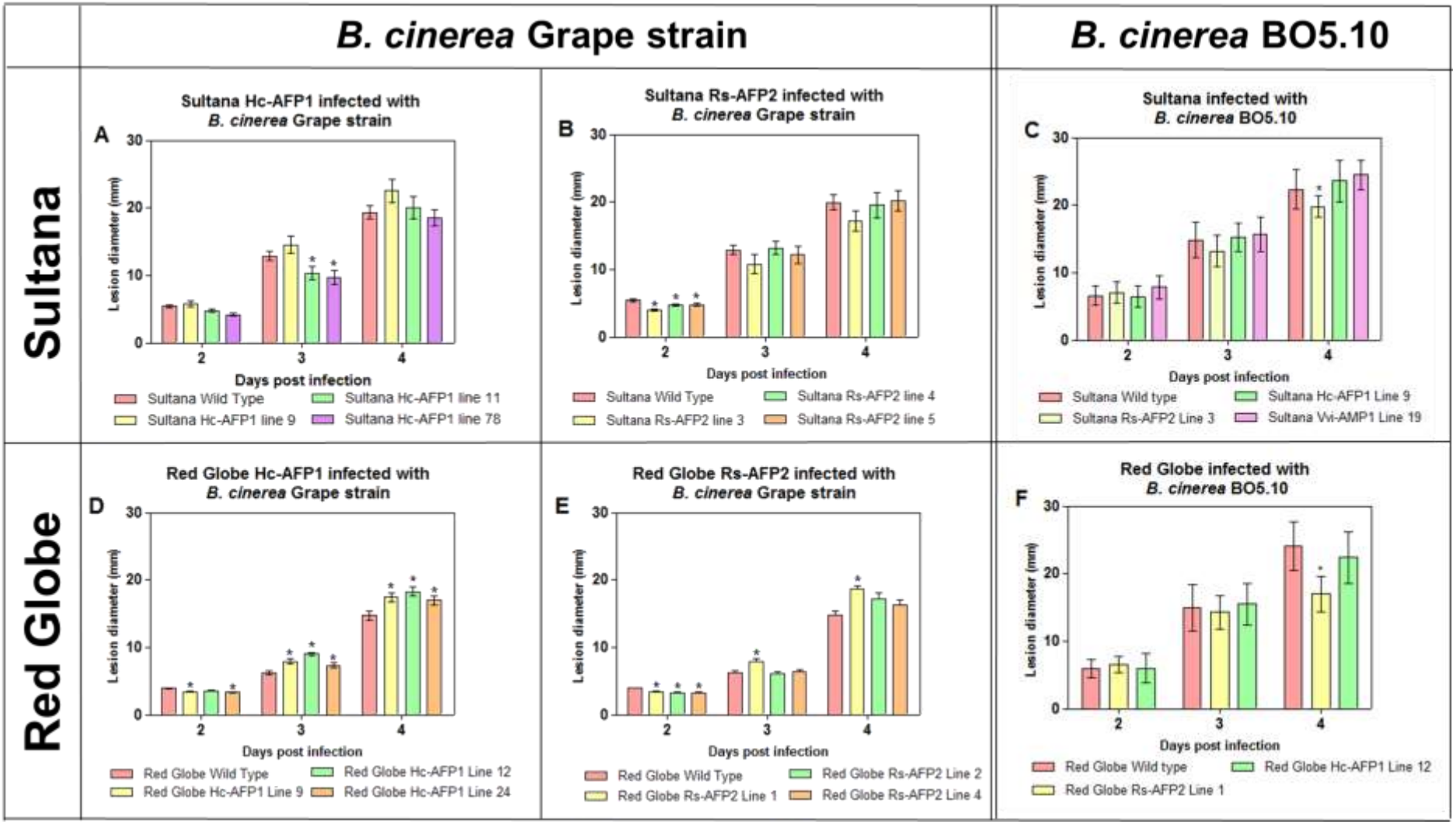

Figure 5.5. Whole plant infection assay with of transgenic *V. vinifera* (cv. Sultana and Red Globe) lines and the untransformed wild type Sultana control lines with two *B. cinereal* strains (the grape strain and B05.10). Lesion development of Sultana transgenic lines, ectopically expressing (A) Hc-AFP1; (B) Rs-AFP2 and Red Globe transgenic lines ectopically expressing (D) Hc-AFP1; (E) Rs-AFP2 infected with *B. cinerea* "grape strain" compared to the untransformed wild types. Lesion development of transgenic (C) *V. vinifera* (cv. Sultana) Hc-AFP1, Rs-AFP2 and Vvi-AMP1 lines; and (F) *V. vinifera* (cv. Red Globe) Hc-AFP1 and Rs-AFP2 lines infected with *B. cinerea* "B505.10" compared to the untransformed wild types. Three individuals from each plant line with four leaves per plant infected with four spots per leaf (1000 spores per spot). Error bars indicate standard deviation and asterisks indicate statistical difference from the Sultana and Red Globe wild types ($p<0.05$).

5.3.3. Detached leaf infection assays with the biotrophic fungus, *E. necator*

5.3.3.1 Assessment of the development of E. necator infection in Sultana and Red Globe transgenic lines

In order to describe and compare the overall development of *E. necator* (powdery mildew) infection on transgenic and control Sultana lines a method previously described by Miclot et al. (2012) was used that involved monitoring the development of the fungal structures from 4 dpi to 14 dpi under a stereomicroscope at 100X magnification and scaling it according to a global resistance index (Table 5.3) . From this analysis it was clear that the progression of powdery mildew was more rapid in the untransformed Sultana and Red Globe control lines, compared to the transgenic lines (Figure 5.6). The Sultana Vvi-AMP1 line was previously described to have increased resistance against powdery mildew (Du Plessis 2012) and was included here as a control. All transgenic and control lines eventually reached the sporulation stage of the infection, however, the tempo and the severity differed. Moreover, the final spore loads at the end of the infection confirmed that the transgenic lines all restricted the infections, yielding significant reduction in the number of spores formed during the infection. The Red Globe Rs-AFP2 line 4 was a non-expressor of the transgene and it behaved similarly than the untransformed control (Figure 5.6 F), whereas another non-expressor (Red Globe Hc-AFP1 line 9), also showed significant reduction in spore-load.

5.3.3.2. Assessment of germination and penetration of E. necator conidia on the control and transgenic lines with the Scanning electron microscope (SEM)

The germinated *E. necator* conidia were assessed at 2 dpi under a SEM in order to determine if there were any visible differences between the germinated conidia on the control and transgenic plant lines. It is clear form figure 5.7 and 5.8 that there were clear differences between the untransformed Sultana and Red Globe control and transgenic lines. The conidia on the leaf surface of the Sultana and Red Globe control lines were at a more advanced development stage compared to those on the transgenic lines (confirming the results from the Global Resistance Index analysis (Figure 5.6). Most of the conidia on the leaf surface of the control lines developed an appressorium and successfully penetrated the epidermal cells and developed secondary hyphae. The majority of the conidia on the leaf surfaces of the transgenic Vvi-AMP1 line 19 only showed the development of mostly abnormal appressoria, thick germ tubes that displayed multiple penetration attempts and no developed secondary hyphae, as previously described (Du Plessis 2012). The Hc-AFP1 and Rs-AFP2 lines in the Sultana and Red Globe backgrounds also displayed similar abnormalities with thick germ tubes and multiple penetration attempts. Almost all of the germinated conidia on the leaf surfaces of the Red Globe Rs-AFP2 line 4 (non-expressor of the transgene) displayed a developed appressorium with successful penetration, haustorium development and subsequent secondary hyphae development. These germinated conidia were at a more advanced development

stage than the rest of the transgenic lines and displayed a similar phenotype observed for the untransformed Red Globe control.

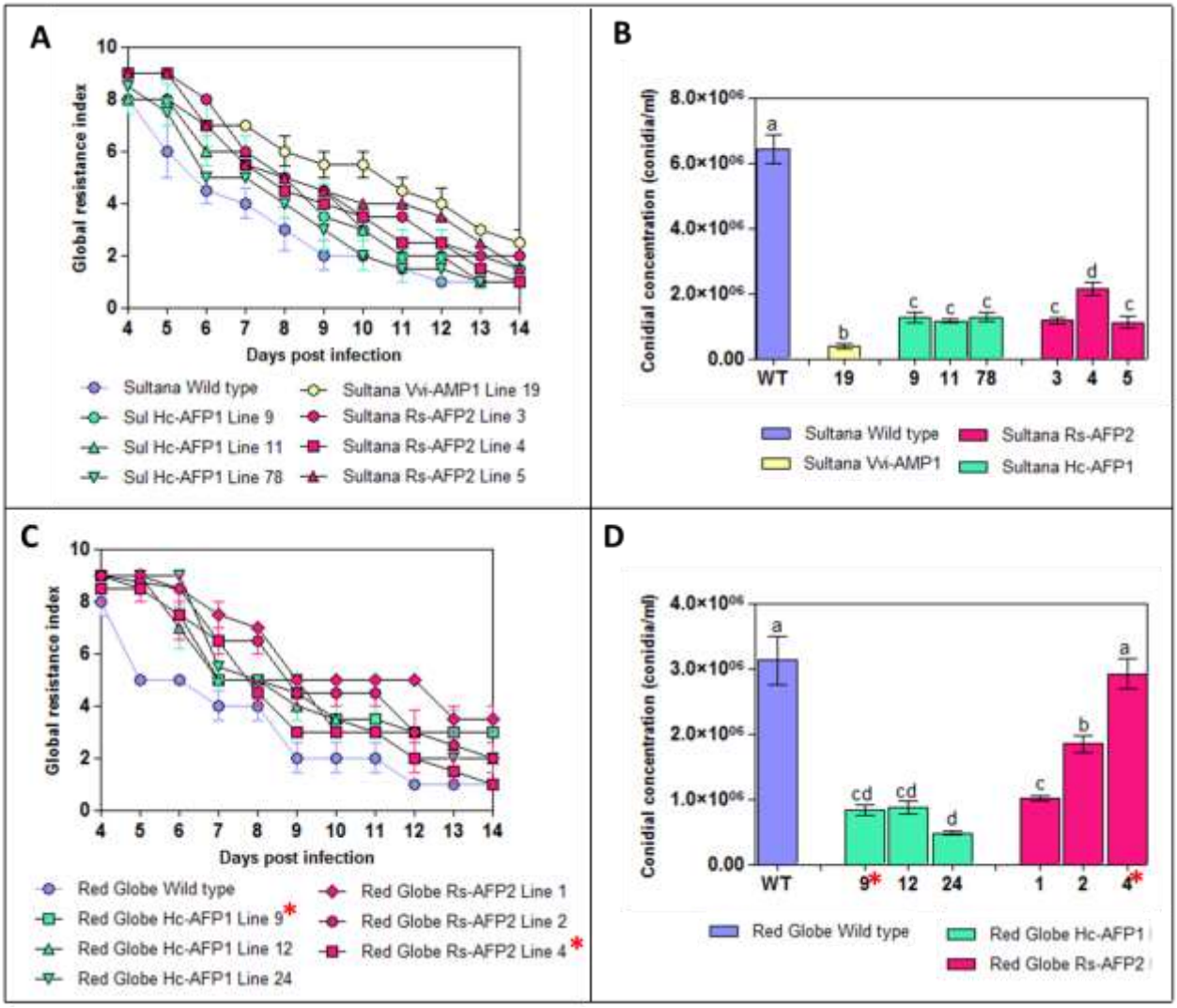

Figure 5.6 Time course of the *E. necator* development according to the global resistance index (Miclot et al. 2012) of (A) transgenic *V. vinifera* (cv. Sultana) lines ectopically expressing Hc-AFP1, Rs-AFP2 and (C) transgenic *V. vinifera* (cv. Red Globe) lines ectopically expressing Hc-AFP1 and Rs-AFP2 in comparison with the untransformed control lines and Sultana Vvi-AMP1. All bars indicate mean and standard error. Spore concentrations at the end of the infection (14 dpi) for (B) *V. vinifera* (cv. Sultana) and (D) *V. vinifera* (cv. Red Globe) transgenic lines. The non-expressor of the transgene plant lines are Red Globe Hc-AFP1 line 9 and Red Globe Rs-AFP2 line 4 and are indicated with red asterisks. Conidial concentration is the average of five technical repeats. Error bars indicate the standard error of mean between all detectable haemocytometer readings (n=3) with different letters within the same parameter are significantly different at $P \leq 0.05$.

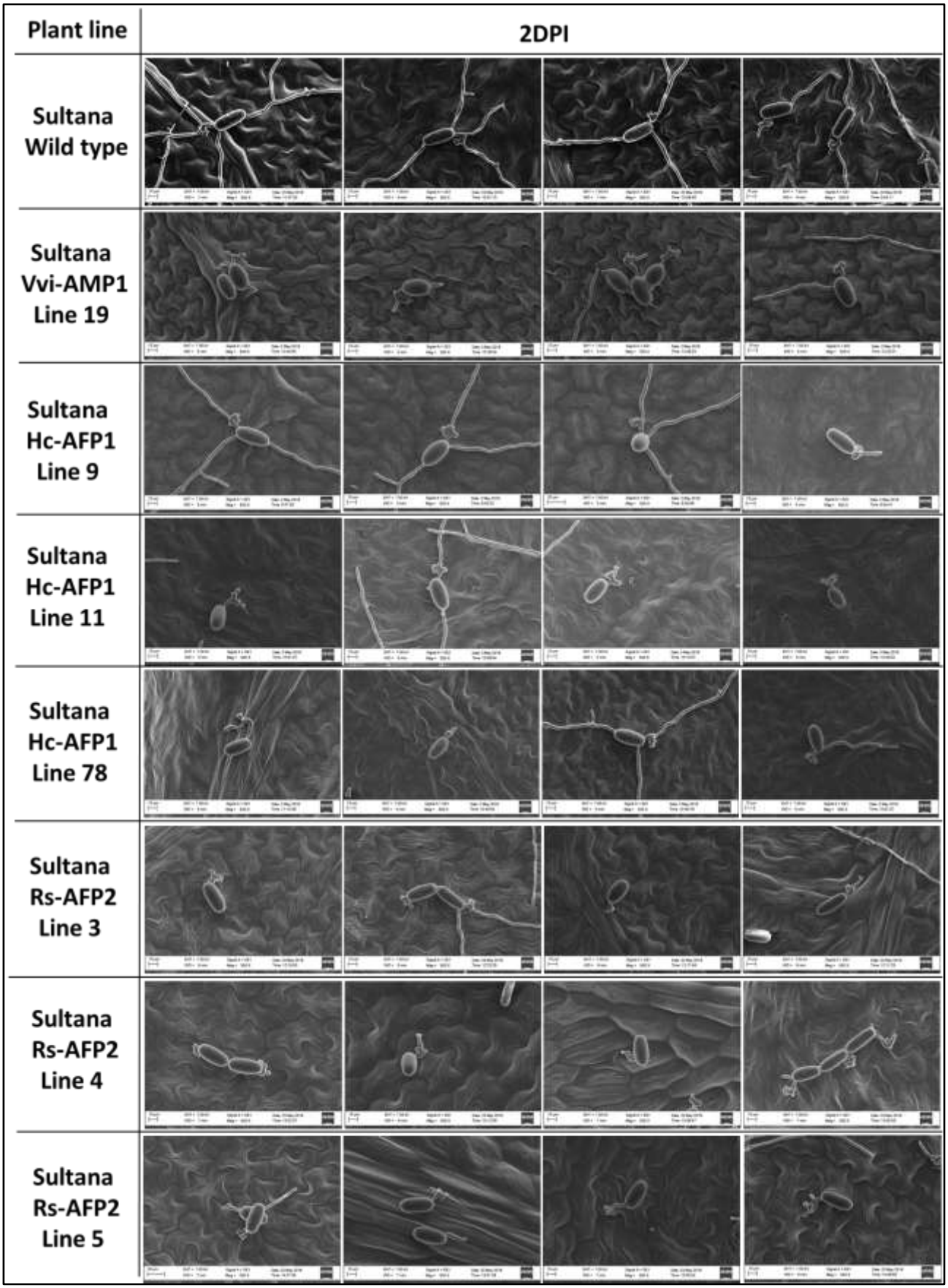

Figure 5.7. Representative scanning electron microscope images of germination and infection structures of *E. necator* on the leaf surfaces of transgenic grapevine ectopically expressing Vvi-AMP1, Hc-AFP1 and Rs-AFP2 respectively and an untransformed control line, at 48 hours post inoculation (at 500x magnification).

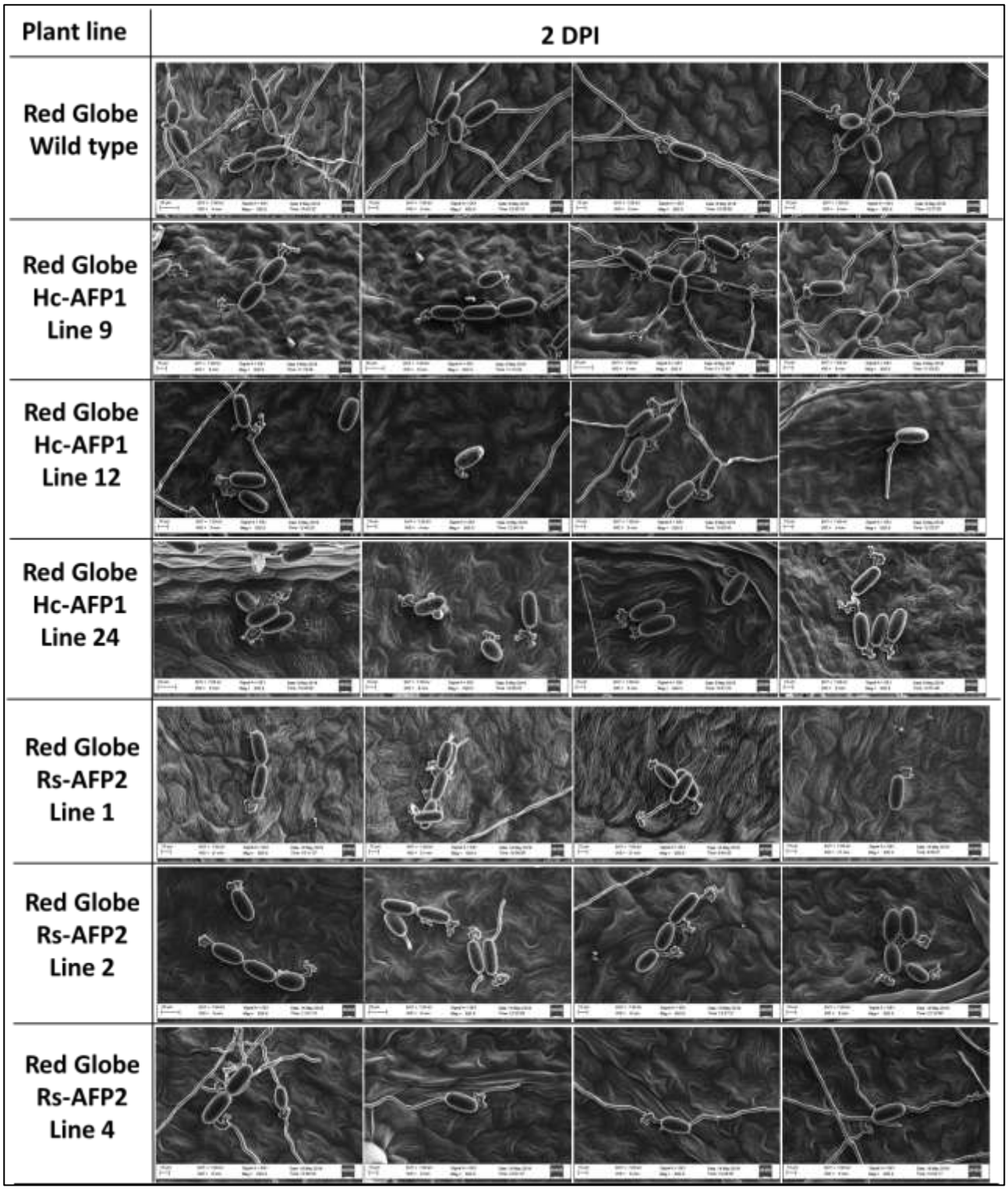

Figure 5.8. Representative scanning electron microscope images of germination and infection structures of *E. necator* on the leaf surfaces of transgenic grapevine ectopically expressing Hc-AFP1 and Rs-AFP2 respectively and an untransformed control line, at 48 hours post inoculation (at 500x magnification).

5.3.3.3 Characterization of resistance mechanisms of transgenic Sultana lines to E. necator infection.

The resistance mechanisms of the different transgenic plant lines were characterized through a method described by Feechan et al. (2011). Leaf material were harvested at 2 dpi and stained with

trypan blue in order to distinguish the fungal structures and dead host tissue. Germinated conidia were characterized according the infection stage, namely the development of an appressorium and or haustorium, together with the presence or absence of PCD. The frequency of each of these infection mechanisms was calculated in order to scale the level of susceptibility or resistance. Two mechanisms of resistance have been characterized, namely penetration resistance or PCD induction. It was clear that the untransformed Sultana control lines showed high susceptibility towards *E. necator* infection with 77% of germinated conidia leading to the development of haustoria within penetrated epidermal cells and subsequent development of secondary hyphae and low levels of PCD (2%) was observed (Figure 5.9). This high level of successful penetration resulted in a dense network of sporulating hyphae at 14 dpi (Figure 5.10). Sultana Vvi-AMP1 line 19 exhibited strong penetration resistance and slightly increased PCD and reduced fungal networks at 14 dpi (Figure 5.10), similar to what was previously described for this line by Du Plessis (2012). The Sultana Hc-AFP1 population exhibited strong penetration resistance towards *E. necator* infection. Only 52% of germinated conidia developed haustoria within penetrated epidermal cells of two of these plant lines (Figure 5.9). This subsequently resulted in only thinly scattered sporulating conidia on the leaf surface of these plant lines at 14 dpi (Figure 5.10). This transgenic population displayed very little PCD and it was clear that the major mechanism of resistance of this population was penetration resistance. Although there was slight differences in the frequencies of infection mechanisms of the inoculated *E. necator* between the different transgenic Sultana Hc-AFP1 lines, all these lines reduced the spore concentration significantly at the end of the infection (Figure 5.6). The Sultana Rs-AFP2 population also displayed strong penetration resistance in all of the plant lines tested. Furthermore this population also displayed an increased PCD resistance, with one line, namely Sultana Rs-AFP2 line 3 displaying 22% PCD due to the penetration of the epidermal cells (Figure 5.9). This transgenic population displayed a higher level of PCD resistance in all of its plant lines compared to the Sultana Hc-AFP1 population. Furthermore, this combined penetration and PCD resistance mechanisms of the Sultana Rs-AFP2 population resulted in only thinly scattered sporulating conidia at 14 dpi (Figure 5.10).

When comparing the transgenic Sultana populations overexpressing the Hc-AFP1 and Rs-AFP2 peptides, it was clear that although there were differences in levels of penetration resistance and/or PCD observed for the individual lines/construct and between constructs, the analysis indicated enhanced resistance (Figure 5. 9) and reduced fungal structures (Figure 5.10). Also, as mentioned before, all these lines significantly reduced the spore concentration of the fungus at the end of the infection (Figure 5.6).

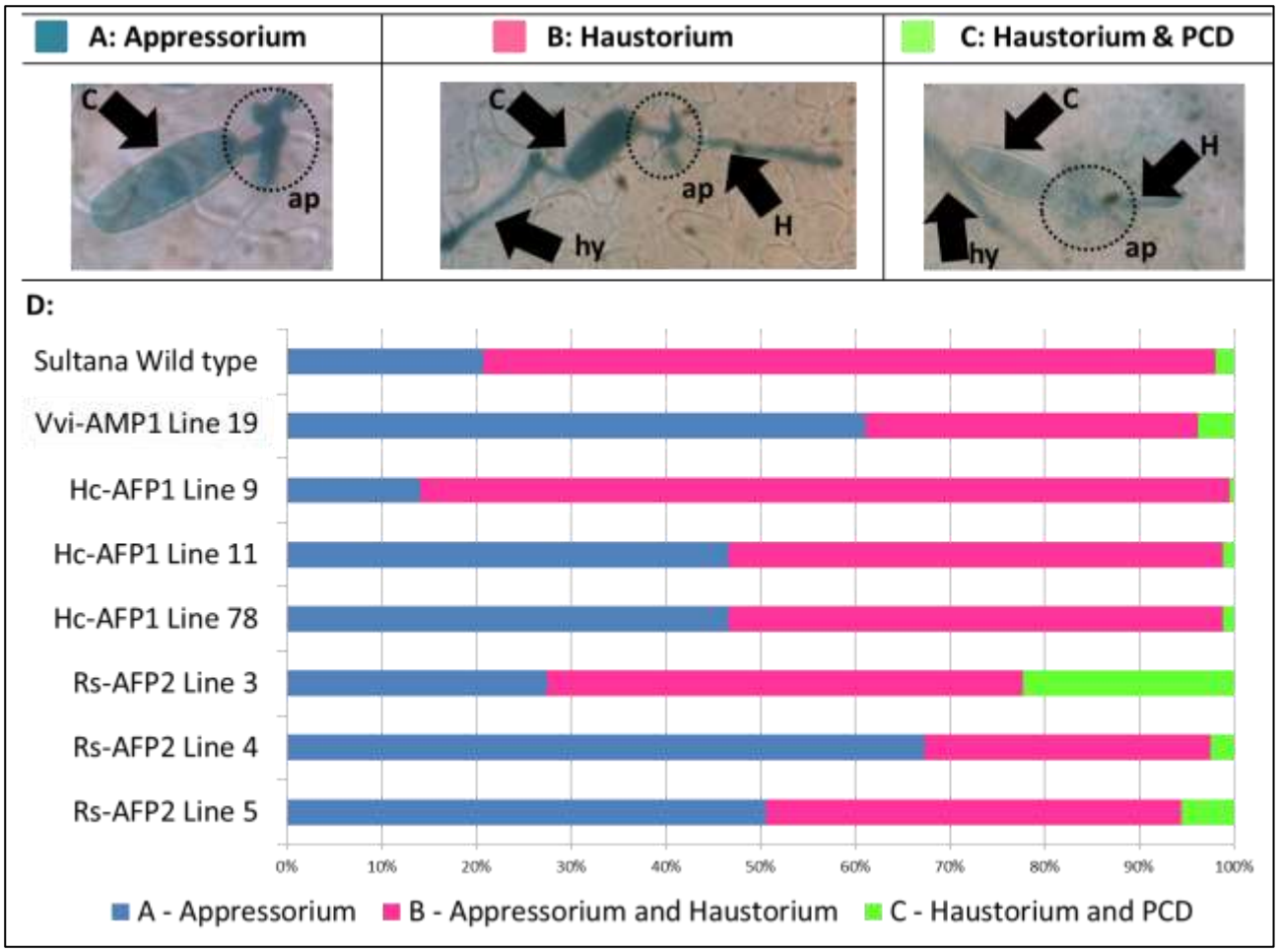

Figure 5.9. The susceptibility of various *V. vinifera* (cv. Sultana) lines overexpressing Hc-AFP1 and Rs-AFP2 respectively in comparison to Sultana wild type and Sultana Vvi-AMP1 line 19 in reaction to the infection with *E. necator* conidia in a detached leaf infection assay 48 hours post-inoculation. This susceptibility evaluation is based on a method described by Feechan et al. (2011). After trypan blue staining and visualization under a light microscope (40x) the following criteria was used to classify the infection mechanism and subsequently determine the susceptibility of the plant lines towards infection by *E. necator*. (A) Classification "appressorium" is assigned when there is visible formation of an appressorium without successful penetration and fungal development. (B) Classification "haustorium" is assigned when there is a visible appressorium formation, successful penetration and subsequent haustorium and secondary hyphae. (C) Classification "haustorium and PCD" is assigned when there is a visible formation of an appressorium with successful penetration, haustorium formation and subsequent programmed cell death (PCD). (D) The frequency of *E. necator* penetration attempts on the various *V. vinifera* (cv. Sultana) lines, which resulted in appressorium formation but no penetration, successful penetration and haustorium formation or a haustorium followed by programmed cell death (PCD) of the penetrated epidermal cell. Broken circles indicate the position of an appressorium. Ap, appressorium; c, conidium; hy, hyphae; H, haustorim.

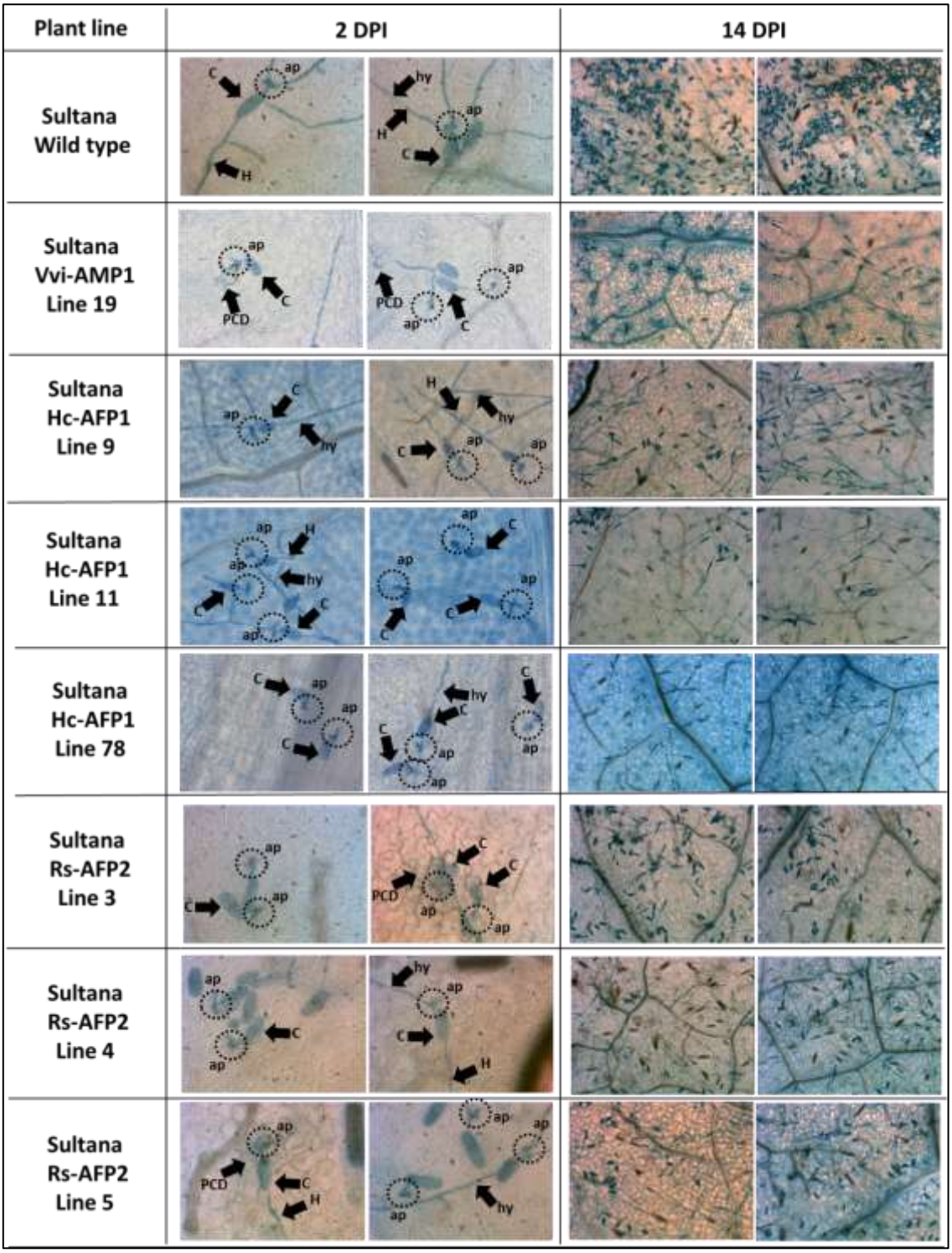

Figure 5.10. Comparison of the infection stages of *E. necator* on infected Sultana transgenic grapevine lines ectopically expressing Hc-AFP1 and Rs-AFP2 respectively in comparison with Sultana wild type and Sultana Vvi-AMP1 line 19 at 2 and 14 days post infection (dpi). All pictures are taken under a light microscope with 40x magnification used at 2 dpi and 10x magnification used at 14 days post infection. Broken circles indicate the position of an appressorium. Ap, appressorium; c, conidium; hy, hyphae; H, haustorium.

5.3.3.4 Characterization of resistance mechanisms of transgenic Red Globe lines to E. necator infection.

The untransformed Red Globe control presented a susceptible phenotype with 66% of germinated conidia successfully penetrating the epidermal cells with haustoria development and subsequent secondary hyphae development (Figure 5.11). Only 30% of germinating hyphae developed appressoria without successful penetration whereas only 4% of germinating conidial lead to PCD of the penetrated epidermal cells. This susceptible phenotype was also reflected in the dense network of sporulating hyphae at 14 dpi (Figure 5.12). The Red Globe Hc-AFP1 transgenic population showed a high level of penetration resistance for all the plant lines tested. Interestingly, the non-expressing line 9 displayed similar infection features to that of the expressing line 12. In addition to the observed penetration resistance, one of these plant lines namely Red Globe Hc-AFP1 line 24 also displayed a high level (47%) of PCD development in penetrated epidermal cells. The trypan blue stain at 14 dpi of the transgenic Hc-AFP1 lines revealed thinly scattered sporulating conidia on the leaf surface of these transgenic lines with Hc-AFP1 line 24 having the smallest amount of visible hyphae and conidia (Figure 5.12). These results are in accordance with the significantly reduced spore concentration of the fungus at the end of the infection (Figure 5.6).

Transgenic Red Globe Rs-AFP2 lines 1 and 2 also displayed high levels of penetration resistance and significantly increased levels of PCD compared to the untransformed control (Figure 5.11). These two transgenic lines presented thinly scattered mycelia and spores at the end of the infection when stained with trypan blue (Figure 5.12). The non-expressing Red Globe line 4 did not show higher penetration resistance or increased PCD (Figure 5.11) and a dense network of sporulating hyphae, comparable to that observed for the untransformed control lines was observed at the end of the infection period (Figure 5.12). These results are in accordance with the spore concentration of the fungus at the end of the infection (Figure 5.6).

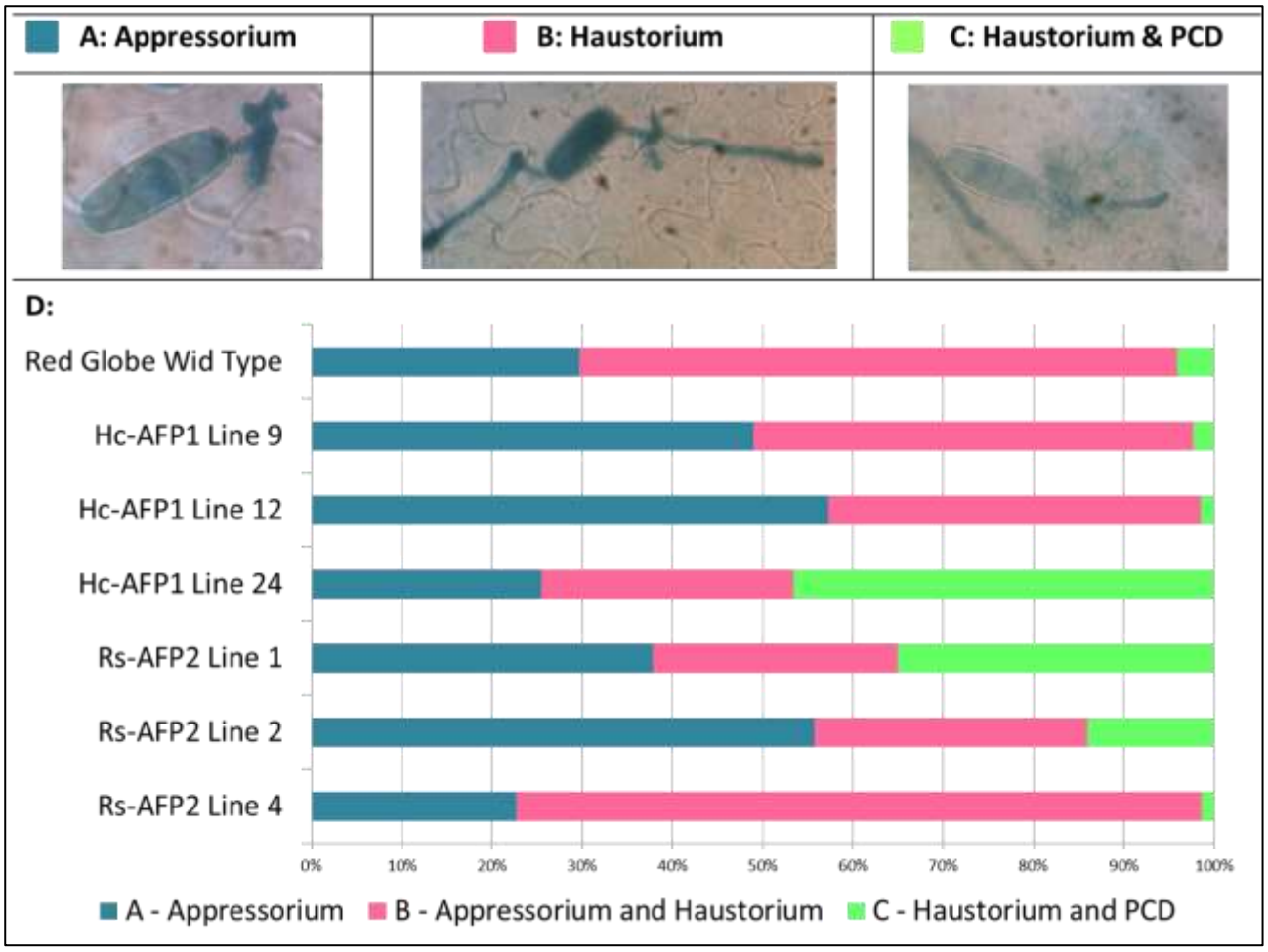

Figure 5.11. The susceptibility of various *V. vinifera* (cv. Red Globe) lines overexpressing Hc-AFP1 and Rs-AFP2 respectively in comparison to Red Globe wild type in reaction to the infection with *E. necator* conidia in a detached leaf infection assay 48 hours post-inoculation. This susceptibility evaluation is based on a method described by Feechan et al. (2011). After trypan blue staining and visualization under a light microscope (40x) the following criteria was used to classify the infection mechanism and subsequently determine the susceptibility of the plant lines towards infection by *E. necator*. (A) Classification "appressorium" is assigned when there is visible formation of an appressorium without successful penetration and fungal development. (B) Classification "haustorium" is assigned when there is a visible appressorium formation, successful penetration and subsequent haustorium and secondary hyphae. (C) Classification "haustorium and PCD" is assigned when there is a visible formation of an appressorium with successful penetration, haustorium formation and subsequent programmed cell death (PCD). (D) The frequency of *E. necator* penetration attempts on the various *V. vinifera* (cv. Red Globe) lines, which resulted in appressorium formation but no penetration, successful penetration and haustorium formation or a haustorium followed by programmed cell death (PCD) of the penetrated epidermal cell. Broken circles indicate the position of an appressorium. Ap, appressorium; c, conidium; hy, hyphae; H, haustorim.

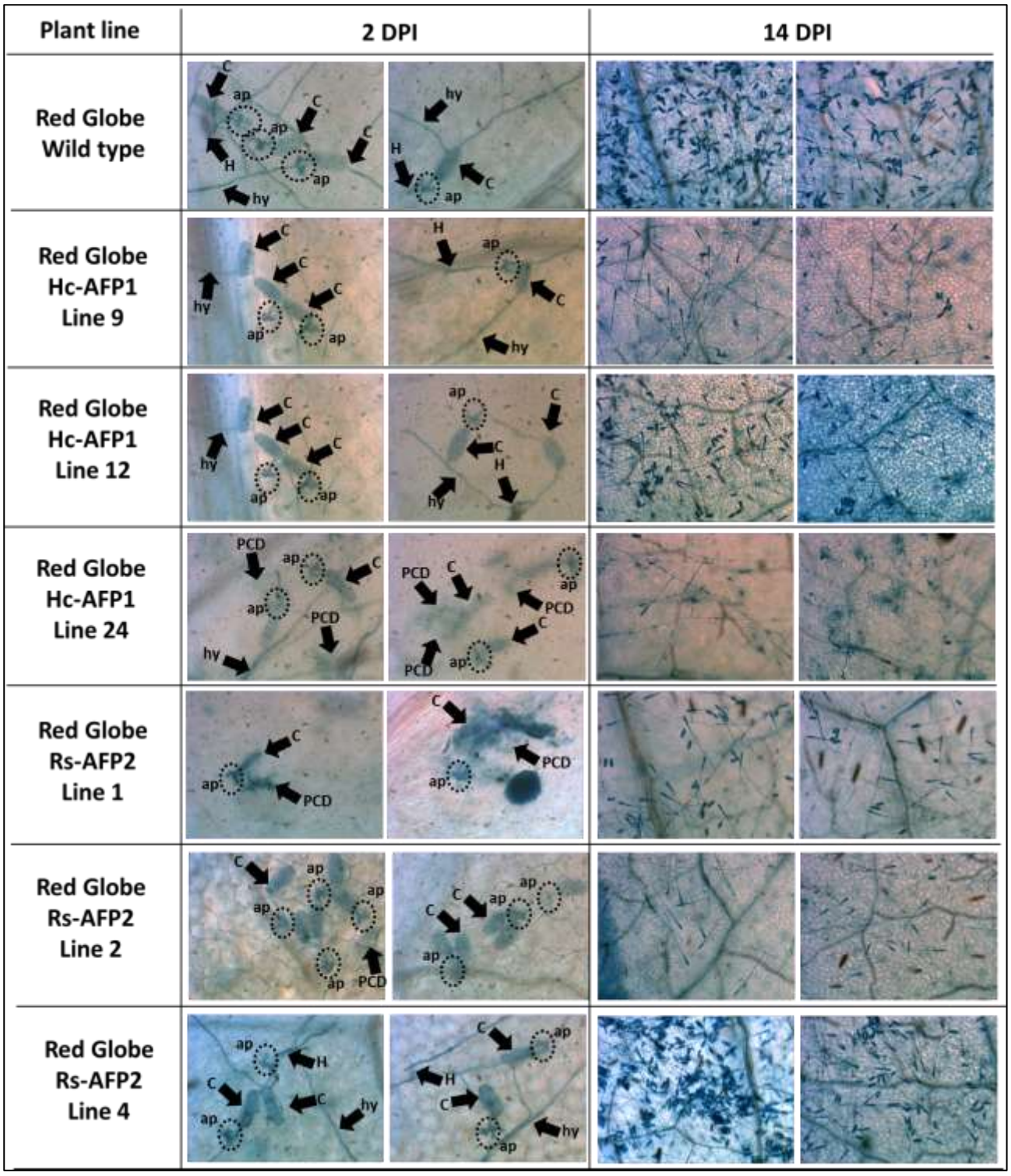

Figure 5.12. Comparison of the infection stages of *E. necator* on infected Red Globe transgenic grapevine lines ectopically expressing Hc-AFP1 and Rs-AFP2 respectively in comparison with Red Globe wild at 2 and 14 days post infection (dpi). All pictures are taken under a light microscope with 40x magnification used at 2 dpi and 10x magnification used at 14 days post infection. Broken circles indicate the position of an appressorium. Ap, appressorium; c, conidium; hy, hyphae; H, haustorium.

5.3.4. Whole plant survival assay of the soft scale insect *P. ficus* on transgenic and control grapevine plants

A selection of transgenic and control plant lines were challenged with mealybugs and the survival rates of the insects determined (Table 5.4 and Figure 5.13). The survival of the mealybugs on the different plants lines were monitored daily for 5 consecutive days. From these results it was clear that there was an 18% reduction in the survival of the mealybugs on the untransformed Sultana control plants after the first day post transfer (DPT). However, the survival percentage remained at 82% throughout the rest of the survival assay. The line with the highest reduction in survival percentage was Hc-AFP1 line 78 were 72% of the mealybugs died after 1 dpt. The rest of the transgenic lines showed a gradual daily decrease in survival in the mealybugs up to 5 dpi where all the transgenic Hc-AFP1 and Rs-AFP2 lines displayed significantly lower survival percentages compared to the untransformed control (Figure 5.21). Sultana Hc-AFP1 line 78 displayed the strongest activity towards the mealybugs with a survival percentage of only 20% at the end of the incubation period.

Table 5.4. The daily survival percentages of *P. ficus* on Sultana control and transgenic lines in a whole plant survival assay. The percentages with SEM represent the results of two independent experiments combined with two biological repeats per plant line with 4 technical repeats per plant.

Plant line	% Survival				
	1 DPT	2 DPT	3 DPT	4 DPT	5 DPT
Sultana Wild type	97±2.7	81±5.7	82±5.8	82±5.8	82±5.8
Sultana Vvi-AMP1 Line 19	58±9.7	51±9.2	48±10.8	43±17.7	36±10.1
Sultana Hc-AFP1 Line 9	52.±8.0	40±9.3	40±9.3	38±9.5	26±10.5
Sultana Hc-AFP1 Line 11	67±9.7	60±8.8	51±9.8	35±10.2	43±11.1
Sultana Hc-AFP1 Line 78	40±6.5	28±4.9	28±5.3	22±4.0	20±4.5
Sultana Rs-AFP2 Line 3	75±8.2	48±9.8	48±9.5	42±8.6	37±9.0
Sultana Rs-AFP2 Line 4	65±8.2	46±8.0	43±8.4	40±9.3	37±9.0
Sultana Rs-AFP2 Line 5	57.5±5.9	34±5.7	32±4.8	32±4.8	28±4.6

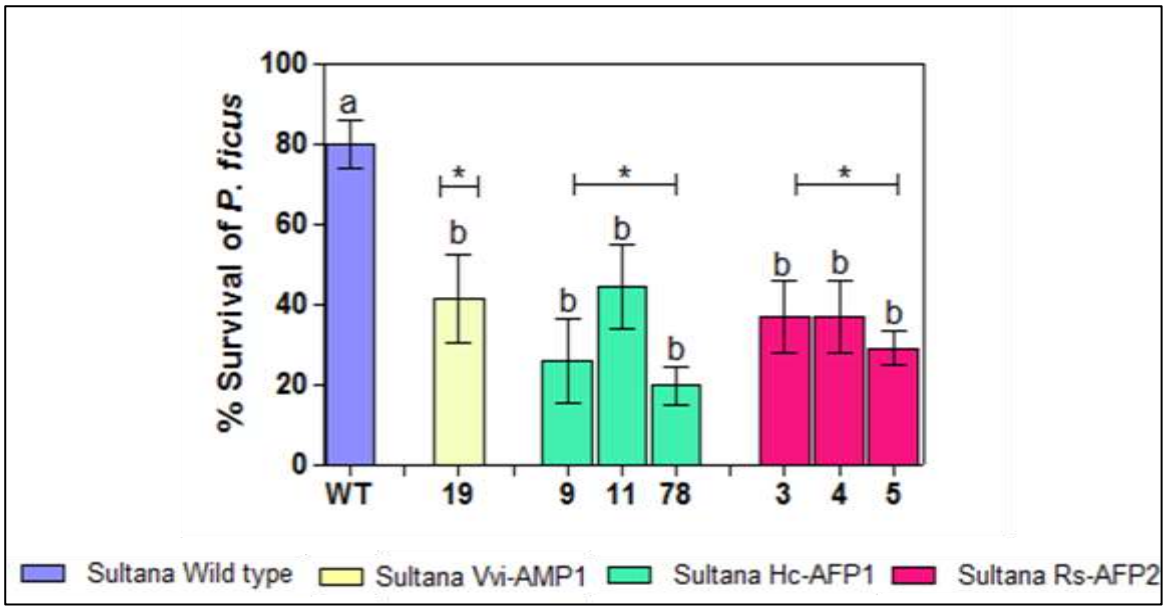

Figure 5.13. The percentage survival of *P. ficus* at 5 dpi in a whole plant infection assay of V. *vinifera* (cv. Sultana) transgenic lines ectopically expressing Vvi-AMP1, Hc-AFP1 and Rs-AFP2 respectively and the untransformed Sultana control. The numbers indicate the specific transgenic lines tested. Percentage survival is the average of four technical repeats and two biological repeats of two independently performed experiments. Error bars indicate the standard error of mean.

5.4 DISCUSSION

We performed *in planta* analyses on a selection of grapevine transgenic lines expressing plant defensin peptides in two different cultivars to characterise and describe any potential biotic defence phenotypes in these transgenic lines. The possible resistance phenotypes to the well-known grapevine pathogens *B. cinerea* and *E. necator*; as well as the important pest, *P. ficus*, the vector for the grapevine leafroll 3 virus, were examined.

The peptides that formed part of this study (Vvi-AMP1, Rs-AFP2 and Hc-AFP1) had already been characterised extensively *in vitro* and proven to have antifungal activities (Terras et al. 1992b, 1995; Tredoux 2011; De Beer and Vivier 2011; Du Plessis 2012; Barkhuizen 2013). The Vvi-AMP1 peptide is a non-morphogenic defensin with activity against *Fusarium oxysporum, F. solani, B. cinerea* and *Verticillium dahlia,* using a membrane permeabilisation mechanism (De Beer and Vivier 2008). The Hc-AFP1 peptide was reported to be a potent morphogenic peptide against *B. cinerea* and *F. solani*, also using a membrane permeabilization mechanism (De Beer and Vivier 2011; Barkhuizen 2013). This plant defensin was also found to be part of the natural defence mechanism in the root tips of its native host, where it was secreted to protect the root tip (as part of the root-border cells) from surrounding pathogens (Weiller et al. 2016). Moreover, the Hc-AFP peptides were shown to be very sensitive to the presence of divalent cations as the biological activity of Hc-AFP1 was strongly antagonised in the presence of cations (Barkhuizen 2013). Investigation showed that the cations influences the structural conformation of the peptide, causing it to adopt an inactive, unordered conformation (Barkhuizen 2013). The *in vitro* activity of Rs-AFP2 has been described towards a wide range of fungal pathogens (Terras et al. 1992b, 1995). This

peptide inhibits a wide range of fungal pathogens through membrane permeabilisation and the stimulation to produce reactive oxygen species (ROS) and programmed cell death (Cools et al. 2017; Parisi et al. 2018). Comparing the IC_{50} values reported for these peptides against the pathogens they all inhibit, Rs-AFP2 has the strongest *in vitro* antifungal activity with an IC_{50} value of 2 μg/mL towards *B. cinerea*, followed by Vvi-AMP1 with 15 μg/mL and then Hc-AFP1 with a IC_{50} value above 25 μg/mL. From transgenic overexpression studies, the Rs-AFP2 peptide caused increased resistance against *Magnaporthe oryzae*, *Rhizoctonia solani, Alternaria longpipes, Pseudomonas syringe* pc. *Tabaci, Alternaria solani, F. oxysporum, Phytohtora infestans, Rhisoctonia solani* and *A. solani* in transgenic populations of rice, apple, tobacco, tomato and canola (Terras et al. 1992a, b, 1995; Bondt et al. 1998; Parashina et al. 2000; Jha and Chattoo 2010), whereas the Vvi-AMP1 peptide did not cause any increase in disease resistance in tobacco or grapevine populations against *B. cinerea* (De Beer 2008), but a strong resistance phenotype was reported against the biotrophic powdery mildew pathogen in grapevine (Du Plessis 2012).

Here the analyses showed that the defensin overexpressing populations did not exhibit improved resistance against two strains of *Botrytis*, despite the strong *in vitro* activities reported for all the peptides against this pathogen. From the DEFL gene expression analysis, it was seen that some putative grapevine defensin genes, particularly those encoding the snakins, DEFL 10.1 and 10.2 displayed slight upregulation when berries were infected with *Botrytis*, whereas the Vvi-AMPs notably maintained their basal expression levels or were even slightly downregulated. From our whole plant infection assays with the *Botrytis* strains, it was noted that at the early stages of the infections, the primary lesions were diminished in the transgenic lines, but that the secondary lesions spread at the same speed, or even faster than on the control plants. The same results were reported for transgenic Sultana Vvi-AMP1 lines infected with the *B. cinerea* “grape strain” (Du Plessis 2012). It is well known that there exists a great variability in virulence among different *B. cinerea* isolates (Armijo et al. 2016). The *B. cinerea* BO5.10 laboratory strain was less virulent than the *B. cinerea* “grape stain” and all the transgenic lines were more susceptible towards the *B. cinerea* “grape strain” compared to the *B. cinerea* BO510 strain (based on lesion sizes).

B. cinerea infect plant cells by means of penetration through an appressorium and penetration pegs. The appressorium secretes lytic enzymes to cross the epithelial wall. After a primary lesion has been established, the fungus secretes cell wall degrading enzymes such as endopolygalacturanases, pectin methylesterases, cellulases and hemicellulases that decompose the plant tissues in order for the fungus to consume these cells. *B. cinerea* further secretes toxins and more importantly oxalic acid that leads to the acidification of the locally infected region (Armijo et al. 2016). The two different genetic backgrounds (Sultana and Red Globe) did display slight differences in their responses, with the Red Globe proving to be overall slightly more susceptible than Sultana, as reported by Boso and Kassemeyer (2008). Both, however, displayed the typical maceration lesions linked to an active and successful infection by *Botrytis*. The defence responses

of the hosts (programmed cell death through ROS production during the hyper sensitive response) would favour the necrotrophic fungus, as it would thrive on the dead tissue generated through the activation of PCD (Heath 2000).

Interpreting the results of this study against this background, we are proposing the following scenarios that could partly explain the lack of increased resistance phenotypes observed in the transgenic populations: (i) it is possible that the known acidification of *Botrytis* at the infection sites, particularly as the infection progressed, could have negatively impacted or even inactivated the plant defensins, who are known to have optimal pH ranges of between 4.0 and 10.0 (De Oliveira Carvalho and Gomes 2011). This could also explain the rather similar responses (observed in the different infection assays), irrespective of the different peptides, because the peptides were possibly rendered inactive at the infection sites as the pathogens started to modulate the cellular environment of the host. (ii) Furthermore the cell degrading/macerating action of the *B. cinerea* fungus is known to cause the release of cations that can antagonise the biological activity of these defensin peptides *in planta* (given their proven sensitivity to the prevalence of divalent cations) (Broekaert et al. 1995; Thevissen et al. 1999; Aerts et al. 2008; De Oliveira Carvalho and Gomes 2011; Vriens et al. 2016). The validity of these scenarios would require additional experimental work, including activity assays of these peptides in low pH ranges.

In contrast, all the transgenic lines provided enhanced protection against the biotrophic powdery mildew pathogen. The powdery mildew fungus, *E. necator* is an obligate biotrophic fungus and is dependent on a host cell in photosynthesis-active tissues to complete its life cycle (Gadoury et al. 2012). Once the conidium lands on plant tissue, it germinates and forms an infection structure, namely the appressorium. The fungus penetrates the plant cell through the formation of a penetration hyphae/peg from the lower surface of the appressorium. Once the plant epidermal cell is penetrated the fungus forms a feeding structure called the haustorium. This feeding structure facilitates the exchange of molecules between the fungus and host. Generally, plants have two main defence mechanisms against biotrophic fungal pathogens, namely penetration resistance and programmed cell death (PCD)-mediated resistance. Penetration resistance blocks the entry to the cell wall and cell membrane, preventing the formation of a haustorium. The PCD mediated resistance occurs in an already penetrated epidermal cell where the cell induces PCD on the infected cell to isolate the infection and blocks further growth and development by termination of the supply of nutrients. The innate immune response responsible for these resistant mechanisms includes the pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is the first line of defence of the plant cell and is activated through the recognition of pathogen specific molecules through pattern recognition receptors in the plasma membrane. Once these pathogen specific molecules have been detected, a series of protein kinase cascades are activated that triggers several defence responses that include the secretion of antimicrobial and cell wall restructuring peptides. Some powdery mildew strains have

found a way to bypass the PTI through effector proteins that supress the PTI and become virulent. However, the plant has developed a counter strike mechanism that involves resistance (R) proteins (R-genes) that specifically recognize the fungal effector molecules, leading to ETI. ETI involves the induction of various defence responses that include PCD (Lipka et al. 2008; Feechan et al. 2011; Qiu et al. 2015). The cultivated grapevine *V. vinifera* however contains no genetic resistance towards powdery mildew (Ramming et al. 2010; Feechan et al. 2011; Merdinoglu et al. 2018). Several R genes have been identified and described in the *non-vinifera* species. These genes encode proteins with nucleotide binding (NB) site-leucine-rich repeat (LRR) domains. To date 11 R-genes have been identified in grapevine. Some of these genes provide complete resistance towards powdery mildew whereas others only provide partial resistance towards the biotropic fungus. The RUN1 (Resistance to *Uncinula necator* 1) gene confers complete resistance towards powdery mildew and is associated with rapid PDC of the penetrated cell whereas the REN4 (Resistance to *Erysiphe necator* 4) gene only confers partial resistance towards powdery mildew through rapid PCD of the penetrated cell, as well as callus encasement of the haustorium (Ramming et al. 2010; Qiu et al. 2015; Merdinoglu et al. 2018).

All of the transgenic lines for each construct displayed an enhanced resistance towards the powdery mildew pathogen. In this study a combination of assays was used to describe the defence responses. These assays included the examination the physical appearance of the infection structures on the plant surfaces at the onset of infection, the progression of the disease, the evaluation of the resistance mechanisms (penetration and/or PCD) and the development of the fungal structures and spore load. Overall, the majority of the lines (irrespective of the type of peptide) led to morphological abnormalities in the fungal infection structures early on in the infection, most notably multiple penetration attempts and affected germ tubes. This abnormal appressorium phenotype of the powdery mildew pathogen was also reported for germinating conidia on transgenic Vvi-AMP1 lines, resistant *M. rotundifolia* (cv. Regale) population and a resistant barley mutant (Rubiales et al. 2001; Blanc et al. 2012; Du Plessis 2012). These impacts on the fungal infection structures could be linked to the direct activities of the secreted defensin peptides with the powdery mildew conidia interacted on the leaf surface. All plant defensin peptides contain secretion signals and they are known to be either secreted on the surface of plant organs and/or in the apoplast between cells (Osborn et al. 1995; Terras et al. 1995; Broekaert et al. 1995; Lay and Anderson 2005; Oomen et al. 2011). Almost all the lines displayed increased penetration resistance and significant reduction of feeding structures and overall colonisation of the tissue, including the development of secondary hyphae and a significant reduction in spore load at the end of the incubation period. A gene involved in the penetration resistance of *Arabidopsis* have been discovered, namely *Arabidopsis thaliana* penetration 1 (PEN1). This gene is a member of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein family that play a role in the trafficking of secretory vesicles to the plasma membrane that contain cargo required for penetration resistance. The *V. vinifera* orthologue of this gene has also been

discovered, namely *Vitis vinifera* penetration 1 (VviPEN1). The VviPEN1 protein was demonstrated to accumulate in the papillae formed beneath the penetration peg of the powdery mildew fungus (Feechan et al. 2013; Qiu et al. 2015). The VviPEN1 gene might be a useful target for deeper analysis of the penetration resistance observed. Interestingly, the non-expressor line, Red Globe Hc-AFP1 line 9 also showed an increase in penetration resistance and an overall reduction in spore load at the end of the incubation period suggesting that this enhanced penetration resistance could be the result of the integration of the defensin transgene within the genome, leading to the enhancement of natural penetration resistance conferred by the plasma membrane proteins VviPEN1. This theory will, however, require further investigation.

Interestingly, although slight increases were observed in the PCD triggered in most of the transgenic lines, it was possibly the combination of penetration resistance, and limiting of secondary structures that proved most effective. The Rs-AFP2 peptide, and specifically in the Red Globe background also produced very significant PCD reaction. This peptide has been known to initiate ROS production as part of its mode of action (Aerts et al. 2007; Cools et al. 2017; Parisi et al. 2018). As mentioned above, PCD resistance mechanism is associated with R-genes that are absent in the *Vinifera* backgrounds of the transgenic plant lines in this study, suggesting that this is due to the presence of the defensin peptides. Heterologous expression of a defensin peptide isolated from onion seeds, *Allium cepa* antimicrobial peptide 1 (Ace-AMP1) have also been demonstrated to confer enhanced resistance towards powdery mildew in roses (Li et al. 2004).

From what we know about the mode of action of Rs-AFP2 we presume that these peptides bind to a membrane target on the fungus (Cools et al. 2017; Parisi et al. 2018). For Rs-AFP2 this membrane target has been determined as the sphingolipid glycosylceramide (GlcCer), however, this target might differ for the other plant defensins. This binding will then induce rapid membrane responses involving Ca^{2+} influx and K^{+} efflux, resulting in an altered membrane potential. Furthermore, as a result of this binding to the membrane target, ROS will be produced. These ROS will cause destruction in the fungal cell, damaging proteins, lipids and DNA and ultimately lead to PCD as seen in some of these plant lines (Aerts et al. 2007; Cools et al. 2017; Parisi et al. 2018). Against the biotrophic pathogens, this is an effective strategy, but *Botrytis* is known to counter this defence response with antioxidants in the initial stages of infection and then actually thrive on the PCD induced in the host cells to form the secondary lesions.

Interestingly, a selection of lines also showed promising results when challenged with the soft scale insect *P. fic*us (mealybug). *P. ficus* is a key pest in vineyards and can attack and desiccated grapevine berry bunches, cause early leaf loss and subsequently weaken vines. More importantly, these pests are the responsible vectors of the grapevine leaf roll virus 3 (GLRaV3) (Walton and Pringle 2004; Daane et al. 2012; Almeida et al. 2013). The *in silico* analysis of the grapevine DEFL genes in response to *P. ficus* infection showed the upregulation of several DEFL genes, including the Vvi-AMP genes. Several plant defensins have been reported to have anti-insect activity. This is

achieved by their ability to either inhibit the α-amalyse activity of the insect gut and through protease inhibitory activity, namely the inhibition of trypsin (Wijaya et al. 2000; Chen et al. 2004, 2005; Lin et al. 2007; Pelegrini et al. 2008; Hu et al. 2018). No previous reports on insect activity of Hc-AFP1, Vvi-AMP1 and Rs-AFP2 exist. However, the expression of a fusion peptide consisting of Rs-AFP2 and *Trigonella foenum-graecum* defensin (Tfgd2) in transgenic tobacco demonstrated enhanced resistance towards the insect larvae of *Spodoptera litura* (Vasavirama and Kirti 2013). Furthermore, this wider spectrum of potential anti-insect activity of these peptides have to be further investigated. This potential anti-insect activity does, however, offer great potential for the protection of grapevine against mealybugs and indirectly offers great potential towards the spreading of the grapevine leaf roll virus 3 (GLRaV3).

This comprehensive analysis is one of very few studies where defensin peptides were analysed for their *in planta* phenotypes. In addition to this novel approach is the analysis of these transgenic plants against divergent types of pathogens and pest.

5.5. ACKNOWLEDGEMENT

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Addendum A to Chapter 5

This Addendum contains relevant and additional data not shown in Chapter 5.

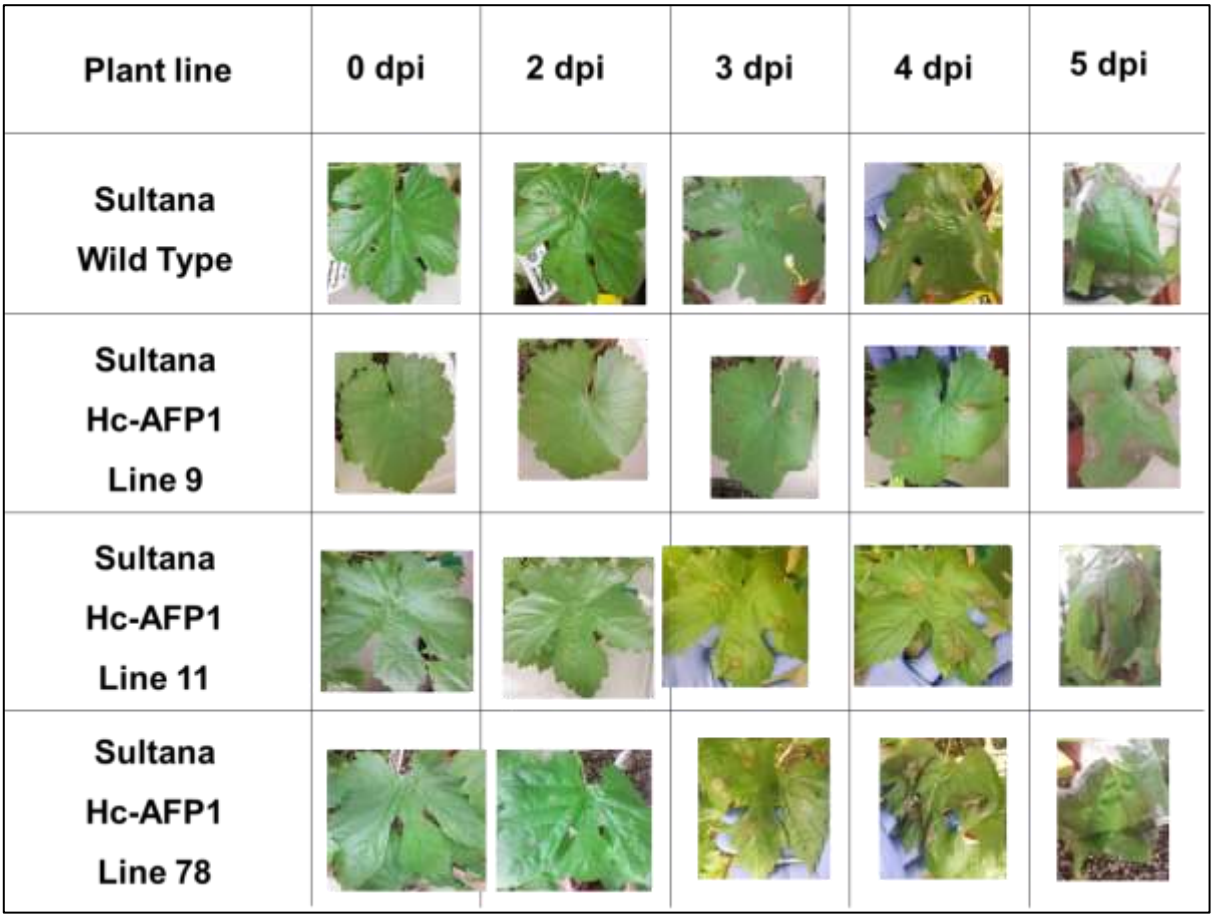

Figure A5.1. Whole plant infection assay of transgenic *V. vinifera* (cv. Sultana) Hc-AFP1 lines and the untransformed wild type Sultana control lines with *B. cinerea* "grape strain".

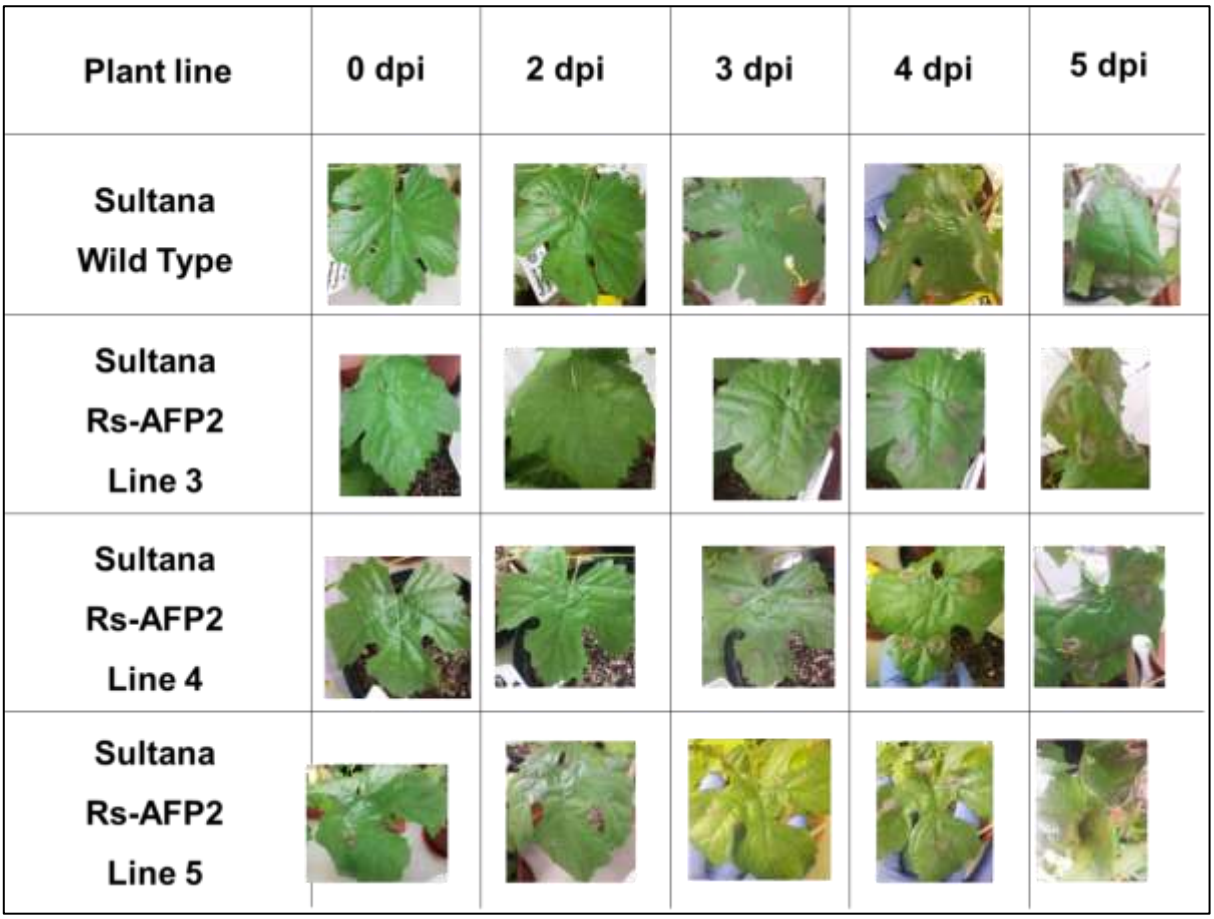

Figure A5.2. Whole plant infection assay of transgenic *V. vinifera* (cv. Sultana) Rs-AFP2 lines and the untransformed wild type Sultana control lines with *B. cinerea* "grape strain".

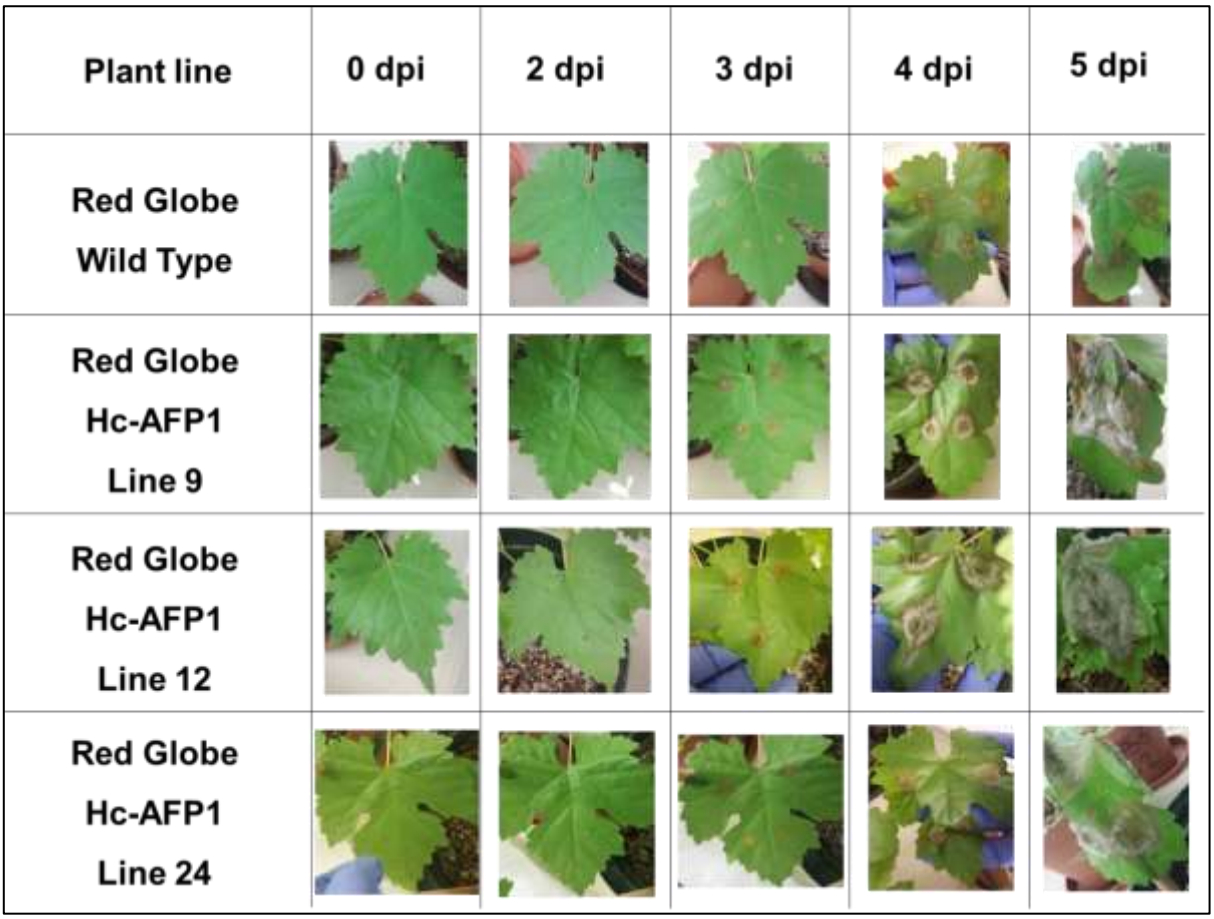

Figure A5.3. Whole plant infection assay of transgenic *V. vinifera* (cv. Red Globe) Hc-AFP1 lines and the untransformed wild type Sultana control lines with *B. cinerea* "grape strain".

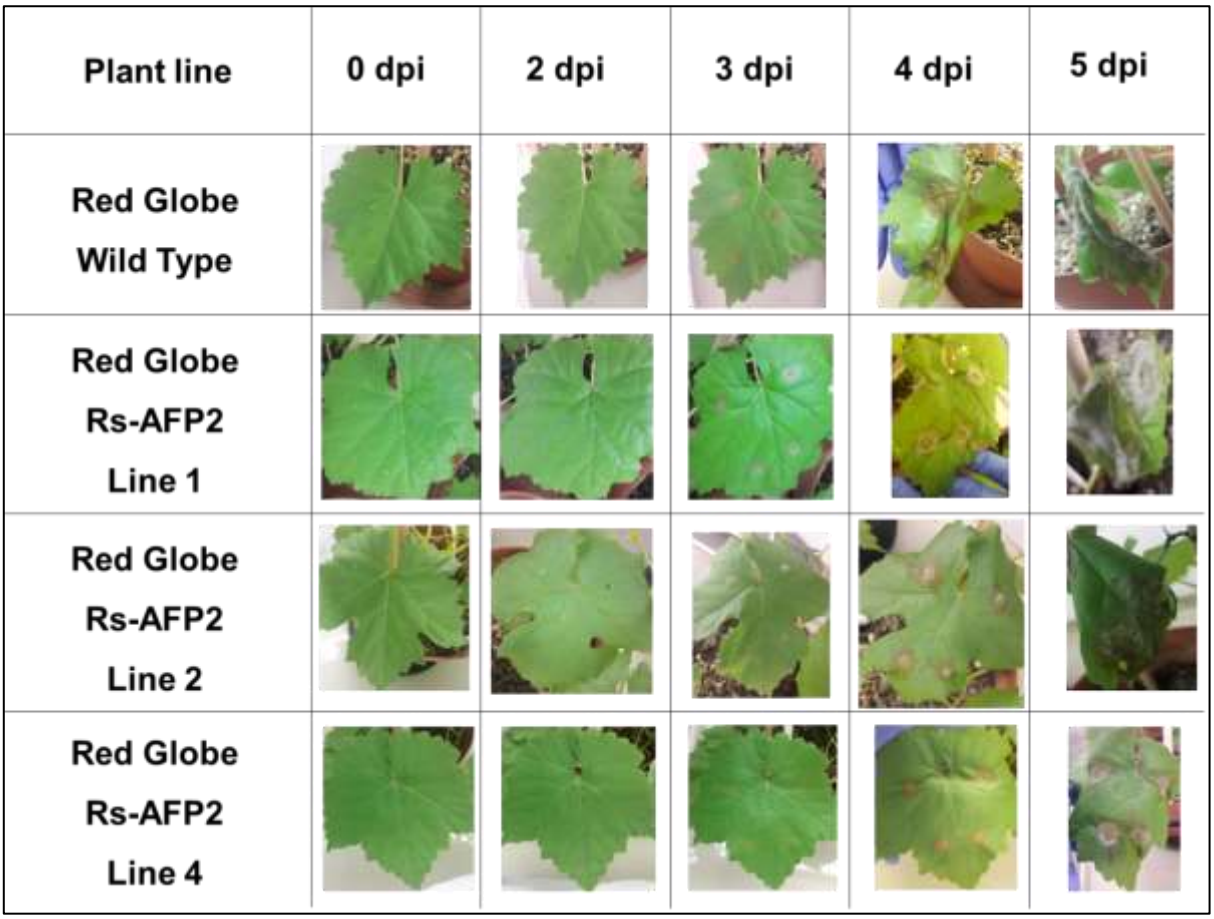

Figure A5.4. Whole plant infection assay of transgenic *V. vinifera* (cv. Red Globe) Rs-AFP2 lines and the untransformed wild type Sultana control lines with *B. cinerea* "grape strain".

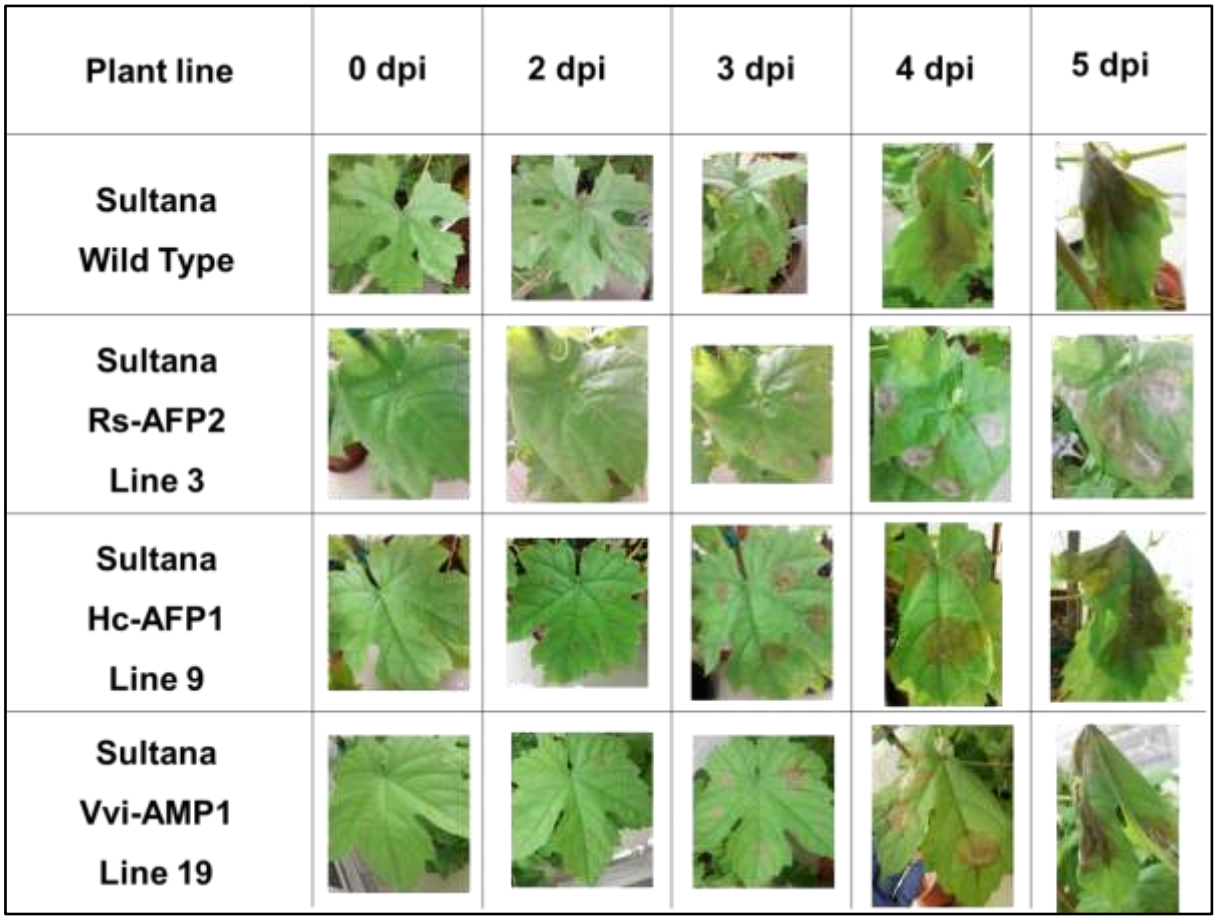

Figure A5.5. Whole plant infection assay of transgenic *V. vinifera* (cv. Sultana) Hc-AFP1, Rs-AFP2 and Vvi-AMP1 lines and the untransformed wild type Sultana control lines with *B. cinerea* BO5.10.

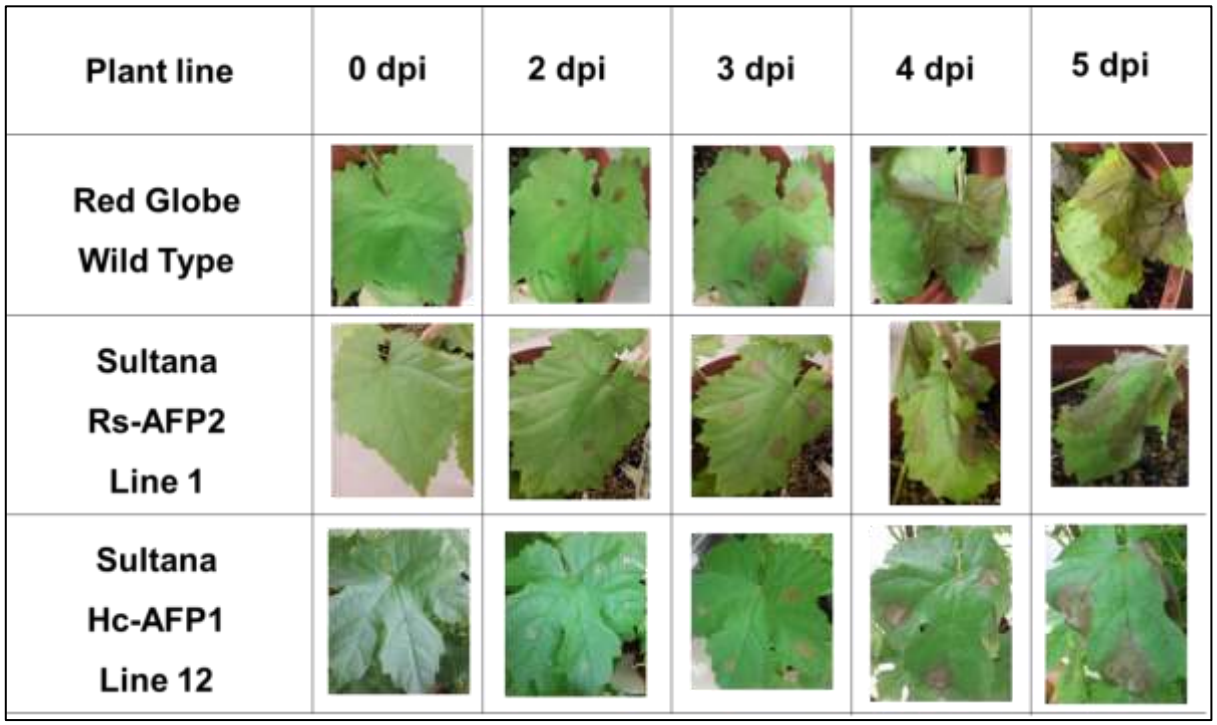

Figure A5.6. Whole plant infection assay of transgenic *V. vinifera* (cv. Red Globe) Hc-AFP1 and Rs-AFP2 lines and the untransformed wild type Sultana control lines with *B. cinerea* BO5.10.

Chapter 6

Research results

Phenotyping of transgenic grapevine lines ectopically expressing Vvi-AMP1, Hc-AFP1 and Rs-AFP2 defensin peptides for their response to drought stress

The authors contributed as follows to the work presented: HB performed all experiments, data-interpretation and compiling of results under the guidance of MAV and Dr Carlos Poblete-Echeverria; HB drafted and finalized the chapter with inputs from all authors; MAV conceived of the study.

RESEARCH RESULTS

Phenotyping of transgenic grapevine lines ectopically expressing Vvi-AMP1, Hc-AFP1 and Rs-AFP2 defensin peptides for their response to drought stress

6.1 INTRODUCTION

Grapevine has the ability to be cultivated in a range of climates. Most grapevine cultivation is done in semi-arid areas that experience seasonal drought. This poses a major threat to the cultivation of grapevine as climate change is predicted to increase drought conditions experienced in some areas. These areas have been referred to as global climate change hotspots. It is therefore highly relevant to understand the responses of grapevine cultivars and rootstocks to limiting water supply and quality (for example saltiness), as well as abiotic stress mitigation in general (Dal Santo et al. 2016; Tortosa et al. 2016; Puértolas et al. 2017). It is well known that grapevine scion and rootstock materials differ in their sensitivity to water limitation. Some cultivars are considered “sensible” and respond early and strongly to limit water loss, whereas others are more “risky” users and respond slower and less efficiently to the signals of limiting water supply. Progress has been made to understand the genetical, molecular and physiological basis of these behaviours (Koundouras et al. 2008; Gullo et al. 2018), and several molecular pathways, protein and metabolite role players have been identified (Chaves et al. 2010; Berdeja et al. 2015).

Plant defensins are not normally linked to drought resistance responses as primary role players, but there are several reports that have shown that the expression levels of some defensin peptides are upregulated in a range of hosts when abiotic stresses, including water and salinity stress are observed (Thomma et al. 1998; Van den Heuvel et al. 2001; Koike et al. 2002; Do et al. 2004; Archambault and Strömvik 2011; Germain et al. 2012; Mith et al. 2015; Hsiao et al. 2017; Mahnam et al. 2017). In grapevine, defensin peptides have not yet been functionally characterised for potential drought resistance phenotypes. Du Plessis (2012) performed an *in silico* analysis that showed that a selection of Defensin-like (DEFL) genes in grapevine cultivars were upregulated in response to water deficit stress, salinity stress, abscisic acid treatments and exposure to short photo-periods. Moreover, several abiotic stress responsive genes were co-expressed with Vvi-AMP1, a grapevine defensin peptide that was consistently upregulated in the water stress experiments that formed part of the *in silico* analysis conducted by Du Plessis (2012). A pepper defensin was similarly upregulated in the leaves of the host plant under drought stress conditions (Do et al. 2004).

These prior studies provided motivation for the analysis of seven transgenic grapevine populations, expressing three defensin peptides in two cultivars of grapevine, for potential drought resistance phenotypes. We performed active drying experiments on the following grapevine populations: Hc-

AFP1, Rs-AFP2 and Vvi-AMP1 overexpressed in *V. vinifera* cv. Sultana; and Hc-AFP1 and Rs-AFP2 overexpressed in in *V. vinifera* cv. Red Globe. In order to do this, we evaluated the intrinsic water use efficacy (WUE) of the different transgenic lines under non-stressed and stressed conditions (in an active drying experiment). Plants react to drought stress by increasing stomatal closure that in turn leads to a decrease in plant water potential and assimilation (Zúñiga et al. 2018). In order to improve plant resistance to drought stress, plants have to maximize soil moisture capture and limit water loss through stomatal transpiration and therefore conserve water by promoting WUE (Karaba et al. 2007). The water potentials and leaf gas exchange variables such as stomatal conductance (g_s) and net CO_2 assimilation (A_n) were measured and compared. Intrinsic WUE was calculated as the ratio between leaf net photosynthesis, or net CO_2 assimilation (A_n) and stomatal conductance (g_s) (Blum 2009; Medrano et al. 2015; Tortosa et al. 2016; Zúñiga et al. 2018).

6.2 MATERIALS AND METHODS

6.2.1 *In silico* analysis and data mining

The *in silico* pipeline described in Chapter 4 was used to further investigate the expression patterns of DEFL genes in response to abiotic stress (as a theoretical framework). The transcriptomic data included a water stress and heat stress experiment as examples of abiotic stresses and was obtained from a collection of publically available microarray expression studies (Table 6.1). All datasets were screened for expression patterns using the *in silico* pipeline described in Chapter 4. The resulting heat maps were thoroughly investigated for significant up or down regulation of the 10 DEFL-genes. Line and bar graphs of the expression data (log_2 fold change) were constructed using GraphPad PRISM 5 for windows (©1992-2007 GraphPad Software, La Jolla California USA, www.graphpad.com).

Table 6.1 Summary of the selected accessible *V. vinifera* microarray experiments form platform GPL17894 included in the analysis of the DEFL gene expression from NCBI.

Study ID	Description	Tissue	Reference
GSE78920	Transcriptomic network analyses of leaf dehydration responses identify highly connected abscisic acid (ABA) and ethylene signalling hubs in three grapevine species differing in drought tolerance	Grapevine leaves	(Hopper et al. 2016)
GSE86551	Dissecting the biochemical and transcriptomic effects of locally applied heat stress on developing Cabernet Sauvignon grape berries	Grape berries	(Lecourieux et al. 2017)

6.2.2 Plant growth conditions

The transgenic *V. vinifera* (cv. Sultana and cv. Red Globe) populations overexpressing Hc-AFP1, Vvi-AMP1 and Rs-AFP2 were obtained from the grapevine transformation and regeneration platform of the IWBT and maintained as described in Chapter 4.

6.2.3. Plants selected for drought stress experiments and experimental design for the water stress experiments

The hardened off plants of transgenic *V. vinifera* (cv. Sultana) and *V. vinifera* (cv. Red Globe) populations and their untransformed controls selected to be phenotyped for their response to drought stress are indicated in Table 6.2. Line 4 of the Red Globe Rs-AFP2 was a non-expressor of the transgene, whereas all other transgenic lines were previously shown to express the relevant transgenes.

Table 6.2 Hardened off plants selected for active drying experiments.

			Repeats per treatment	
Cultivar	**Peptide construct**	**Plant line**	**Full**	**Deficit**
Sultana	**Wild type**		4	4
	Vvi-AMP1	19	4	4
	Hc-AFP1	78	3	3
	Rs-AFP2	5	4	3
Red Globe	**Wild type**		4	3
	Hc-AFP1	12	4	4
	Rs-AFP2	1	4	4
	Rs-AFP2	4	3	3

The water stress experiments were conducted both in a growth room with low levels of artificial lighting with a 16h/8h day night cycle and constant irradiance at 15.68 μmol $m^{-2}s^{-1}$ photosynthetic active radicals (PAR) (refer to Figure A6.1 in Addendum A), as well as with controlled and stable temperature and humidity (Figure A6.2 and A6.3 in Addendum A). A separate set of experiments were done in a green-house with natural ambient light exposure (Figure A6.4 in Addendum A) and with temperature and humidity control (Figure A6.5 and A6.6 in Addendum A). Plants were moved to the different rooms two days before the experiments to allow enough time for plants to acclimatize. Light, temperature and humidity measurements were done with TinyTag LiCor sensors (Gemini data loggers Ltd, West Sussex, United Kingdom), measuring light between 400 nm and 700 nm. Between three and four plants per transgenic and untransformed control lines were exposed to active drying conditions and equal numbers were kept non-stressed as comparative controls. Pots were covered in plastic bags to prevent water loss through evaporation. At the start of the experiment, all plants were watered to full (field) capacity. To induce drought stress, the subset of plants intended to be stressed were exposed to active drying without any watering for the

entire period of the experiment (20 days). For non-stressed plants, soil water content was maintained between 28 and 50%. An empirical threshold of 28% soil water content was estimated for the specific soil in these experiments to represent well-watered (above 28%), or water limiting (below 28%) conditions. In the no stress treatments, water was added to maintain the soil water content above 28% throughout the course of the experiment. Soil water content was measured daily with GS1 soil water content sensors (Decagon Devices Inc., Washington, USA). Three measurements were made for each pot to get an average of the soil water content in the different areas within the pot and water was added to maintain the soil water content above the 28% threshold.

The Sultana and Red Globe WT and transgenic populations were first evaluated in the growth room (low light conditions) for their water stress phenotypes. After the drought stress experiments in these low light conditions were concluded, the same plants were re-watered to full capacity and allowed to recover for two days. Dead and wilted leaves were removed and the plants were moved to the greenhouse where it was grown under natural ambient light conditions (Figure A6.4 in Addendum A), with temperature control to keep the minimum and maximum above and below certain thresholds (Figure A6.5 and A6.6 in Addendum A). Plants that underwent the active drying experiment in the low light conditions became the control group (no stress) in the green house experiment, whereas the non-stressed groups from the low light environment were subjected to the active drying under the green house conditions. Pots were covered in plastic bags to prevent water loss through evaporation.

6.2.4. Physiological measurements

Photosynthesis measurements were executed using a Licor Li6400XT portable photosynthesis system (LI-6400, LI-COR Inc., Lincoln, Nebraska, United States). During measurements, leaf chamber temperature block was maintained at 22°C which was within range of the air temperature during the measurements. The molar air flow rate inside the chamber was set to 500 µmol. s^{-1} and the photosynthetic photon flux was set to 1000 µmol photons $m^{-2}s^{-1}$. All measurements were taken at a reference CO_2 concentration of 400 µmol mol^{-1}. Four mature, healthy, fully expanded leaves were selected per plant for photosynthesis measurements. After placing the Li-Cor cuvette on the leaf for a minimum of two minutes, stability of multiple parameters was monitored and logged. The intrinsic WUE (WUE = A_n/g_s) was determined from the leaf gas exchange measurements, CO_2 assimilation (A_n) and stomatal conductance (g_s). The maximum CO_2 assimilation (A_n) for each dataset was determined and A_n/A_{nmax} ratios were estimated to standardize the degree of change. Data points were manually inspected for negative values and values that fell outside of the normal ranges for stomatal conductance and CO_2 assimilation and filtered. Graphs were constructed in Microsoft Excel (version 14.07166.5000 (64-bit)), Microsoft Office Professional Plus, 2010.

6.2.5. Leaf and stem water potential measurements

At the end of each active drying drought stress experiment (at day 19), destructive leaf and stem water potential measurements were conducted. These measurements were conducted according to the pressure chamber method (Scholander et al. 1965) using a plant moisture tensiometer, pressure chamber instrument, Model 1505D (PMS Instrument Company, USA). Four leaves per plant were used for the measurements of the leaf water potential determinations. Stem water potential was measured on two non-transpiring leaves per plant that have been covered with both a plastic sheet and aluminium foil for two to six hours before measurements. The bagging of the leaves prevented leaf transpiration in order for leaf water potential to represent stem water potential (Begg and Turner 1970). Graphs were constructed using GraphPad PRISM 5 for windows (©1992-2007 GraphPad Software, La Jolla California USA, www.graphpad.com). Significant differences between the wild types, transgenic lines and treatments were determined using a Factorial ANOVA, followed by a Fisher LSD post hoc test with significant differences analysed at $P \leq 0.05$, using statistical software Statistica, version 13 (Dell Inc., Tulsa, OK, USA).

6.3 RESULTS

6.3.1. *In silico* analysis and data mining of grapevine DEFL genes in abiotic stress

Several grapevine DEFL genes showed upregulation in response to both the water stress experiment (Figure 6.1), as well as the local heat stress in berries (Figure 6.2).

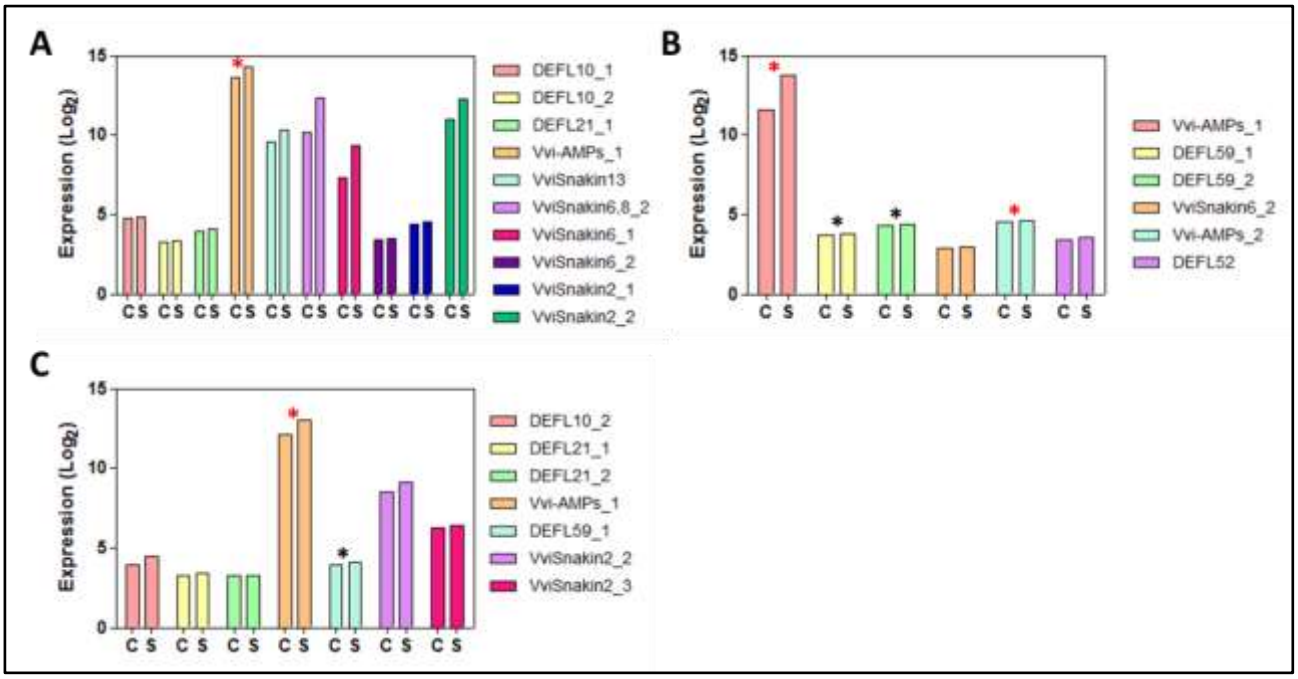

Figure 6.1 The expression patterns (Log_2 fold change) of DEFL genes upregulated in response to 24 hours of leaf dehydration in (A) *V. vinifera* cv. Cabernet Sauvignon, (B) *V. riparia* cv. Riparia Gloire and (C) *V. champinii* cv. Ramsey. "C" on the x-axes refers to non-stressed controls, whereas "S" refers to the leaf dehydration samples (after 24 hours of leaf dehydrations). Red and black asterisk refers to the expression of Vvi-AMP and DEFL59 genes respectively.

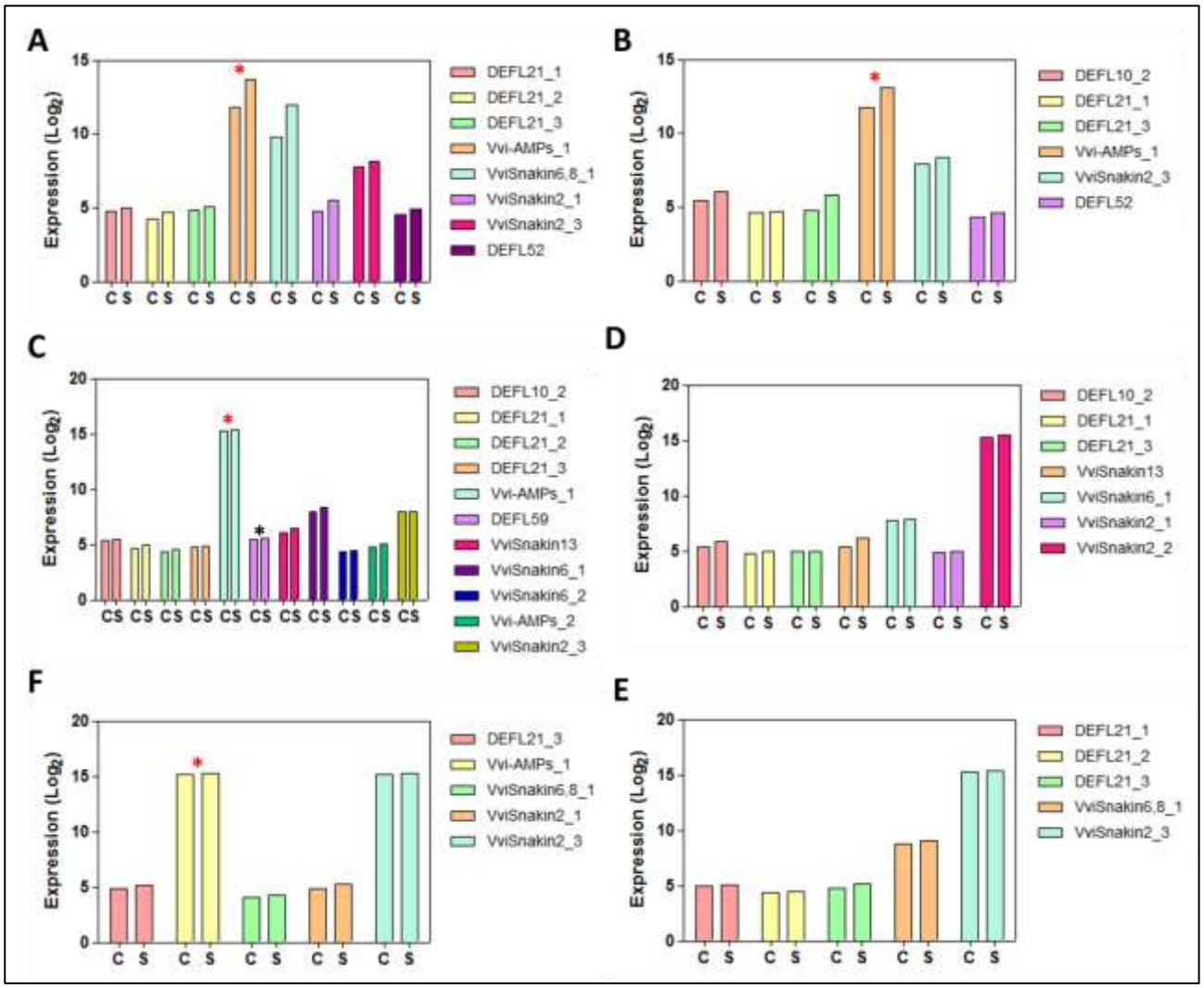

Figure 6.2. The expression patterns (Log_2 fold change) of DEFL genes upregulated in response to locally applied heat stress on Cabernet Sauvignon grape berries. (A) Green berries with locally applied heat stress for 7 days and (B) 14 days. (C) Veraison berries with locally applied heat stress for 7 days and (D) 14 days. (E) Ripening berries with locally applied heat stress for 7 days and (F) 14 days. The red asterisk indicates the expression of the Vvi-AMPs genes and the black asterisk indicates the expression of the DEFL59 genes.

The three cultivars that formed part of the water stress experiment did not show upregulation of the same DEFLs, but the VviAMPs_1 were upregulated in all three in response to 24 hours of leaf dehydration in Cabernet Sauvignon (intermediate drought tolerant), Riparia Gloire (drought sensitive), as well as Ramsey (drought tolerant) (Figure 6.1). Similarly, a number of the unknown DEFL's and snakins responded to the heat stress application of seven and 14 days. Interestingly, DEFL21_3 and VviAMPs_1 remained upregulated in each developmental stage after seven hours heat stress application (Figure 6.2).

6.3.2 Comparison of the physiological reactions of Sultana and Red Globe wild type plants when subjected to active drying experiments in low and ambient light conditions

The two genetic backgrounds were evaluated for their responses to the active drying treatments. Hardened off plants of *V. vinifera* cv. Sultana and cv. Red Globe were tested under both low (growth room) and ambient (greenhouse) light conditions. Figure 6.3 A and B shows the progression of the active drying experiments in terms of percentage of soil water content for the no

stress (watered) and stressed (water withheld) treatments respectively. The Red Globe plant pots (for both light conditions) consistently dried out slightly slower, reaching the 28% soil water content threshold slower compared to the Sultana cultivar (Figure 6.3 B and Table 6.3). When comparing the physiological measurements, it was clear that the levels of photosynthesis and stomatal conductance was very low in the low light environment and that Red Globe always recorded slightly higher values than Sultana (Figure 6.3 C). In the growth chamber, in the low light conditions, both cultivars only recorded significantly reduced stomatal conductance at day 14 of the active drying experiment, after showing increased levels at day 10, even though both cultivars had already reached soil water content below the 28% threshold. When comparing the stomatal conductance recorded in the higher light conditions in the greenhouse, Red Globe had a higher initial stomatal conductance compared to Sultana. Furthermore, at day 10 when both cultivars have already reached the 28% soil water content threshold, the Red Globe cultivar strongly reduced stomatal conductance compared to day six. The Sultana cultivar instead had a slight increase in its stomatal conductance compared to day 6 and only started to reduce stomatal conductance at day 10.

When comparing the calculated intrinsic water use efficiency (WUE) (Figure 6.3 D and Table 6.3) of the two cultivars under the two different light conditions, we see that all the curves showed a non-linear significant relationship between A_n/A_{nmax} and g_s for both datasets. The r^2 values in Table 6.3 indicate a good positive relationship between the data and regression lines with more than 60% of the values falling in the regression line. Furthermore, it was evident that the overall intrinsic WUE under low light conditions was highest for both cultivars, with Sultana being more efficient than Red Globe. When using the equation (A_n/A_{nmax} = a ln (g_s) + b) of the graph in Figure 6.3 D and comparing the CO_2 assimilation at a stress level of 0.04 mol H_2O m^{-2} s^{-1} stomatal conductance, Sultana had the highest CO_2 assimilation rate at 62% compared to Red Globe. The opposite was found under higher light: Red Globe showed a higher intrinsic WUE compared to the Sultana cultivar. At a stress level of 0.04 mol H_2O m^{-2} s^{-1} stomatal conductance in the ambient light conditions, the Red Globe had a CO_2 assimilation rate of 55% compared to the Sultana control with a CO_2 assimilation rate of 50%.

Due to the very low levels of photosynthesis and stomatal conductance recorded for the plants in the low light environment, their biological relevance was considered questionable and therefore, it was decided to focus on the results obtained with the experimental plants under the ambient light conditions when comparing the transgenic populations for their physiological responses to the imposed water stresses (see next section).

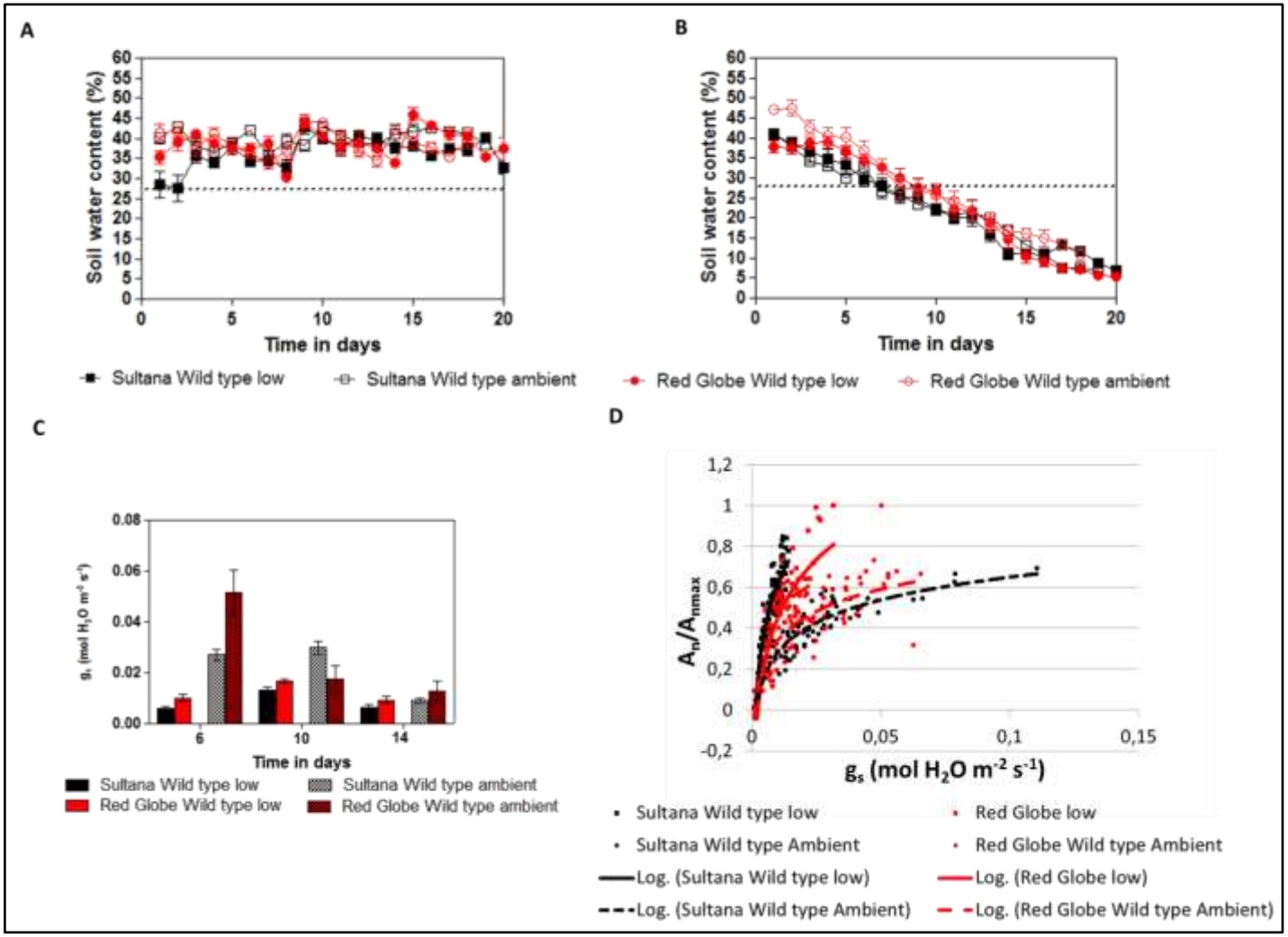

Figure 6.3 Comparison of the *V. vinifera* cv. Sultana and cv. Red Globe (untransformed) lines in reaction to active drying experiments under low and ambient light conditions. The daily soil water content readings for (A) fully irrigated plants, maintaining the soil water content above a threshold of 28% (non-stressed) and (B) plants that were subjected to active drying (water stress). (C) The stomatal conductance of the water stressed treated plants over time with error bars indicating standard error of mean (SEM) with n=9 or 12. (D) Relationship between the A_n/A_{nmax} ratio versus stomatal conductance (g_s) in the watered plants and those undergoing active drying. A_n is the actual CO_2 assimilation and A_{nmax} is the maximum value of CO_2 assimilation.

Table 6.3 The time recorded for *V. vinifera* cv. Sultana and cv. Red Globe wild type plants to reach a threshold of 28% soil water content in the active drying experiments when the plants were tested under either low light growth room conditions, or ambient light in a greenhouse. The WUE is represented by the relationship between A_n/A_{nmax} ratio versus stomatal conductance (g_s) and the values of the logarithmic equation slope, intercept and R^2 of this relationship are indicated.

	Soil water content		WUE		
Plant line and light condition	**Threshold reached (in days)**	**N**	**a***	**b***	**r^2**
Sultana WT Low	7 ± 3	12	0,3197	2,0899	0,7716
Red Globe WT Low	9 ± 3	12	0,3133	1,8911	0,7089
Sultana WT Ambient	6 ± 2	12	0,1606	1,0195	0,7339
Red Globe WT Ambient	9 ± 3	9	0,1561	1,058	0,6322

*$A_n/A_{nmax} = a\ ln\ (g_s) + b$

6.3.3 Analysis of the *V. vinifera* transgenic populations under non-stressed and stressed (active drying) conditions, in comparison with the untransformed controls

All the different plant lines/populations reached the same low level of soil moisture content towards the end of the experiment conducted in the green-house, but some lines displayed periods of "buffering", when there were a few days where the soil water content did not drop notably (see for example transgenic lines of Rs-AFP2 in the Sultana background, between days 3-7, and 10 – 13). The Sultana wild type and transgenic lines reached below the 28% threshold (theoretical onset of stressful water limitation for the soil used) faster compared to the Red Globe wild type and transgenic lines (Table 6.4). In both cultivars the Hc-AFP1 transgenic lines lost soil moisture the fastest. When comparing the different Red Globe transgenic lines, we see that all the transgenic lines lost soil moisture faster compared to the wild type line. The "buffering" effect seen in the Sultana wild type and transgenic lines was less pronounced in the Red Globe populations (The soil water content readings for the individual plants are indicated in Figures B6.1 and B6.4 in Addendum B to Chapter 6).

Table 6.4 The time recorded for the plant lines to reach a threshold of 28% soil water content in the active drying experiments when the plants were tested under greenhouse conditions.

Cultivar	Plant line	Soil water content below threshold reached (days)	N
Sultana	**Wild type**	7±2	12
	Vvi-AMP1 line 19	8±3	12
	Rs-AFP2 line 5	8±2	12
	Hc-AFP1 Line 78	6±1	9
Red Globe	**Wild type**	10±3	9
	Rs-AFP2 line 1	9±3	12
	Rs-AFP2 line 4	9±2	9
	Hc-AFP1 Line 12	8±3	12

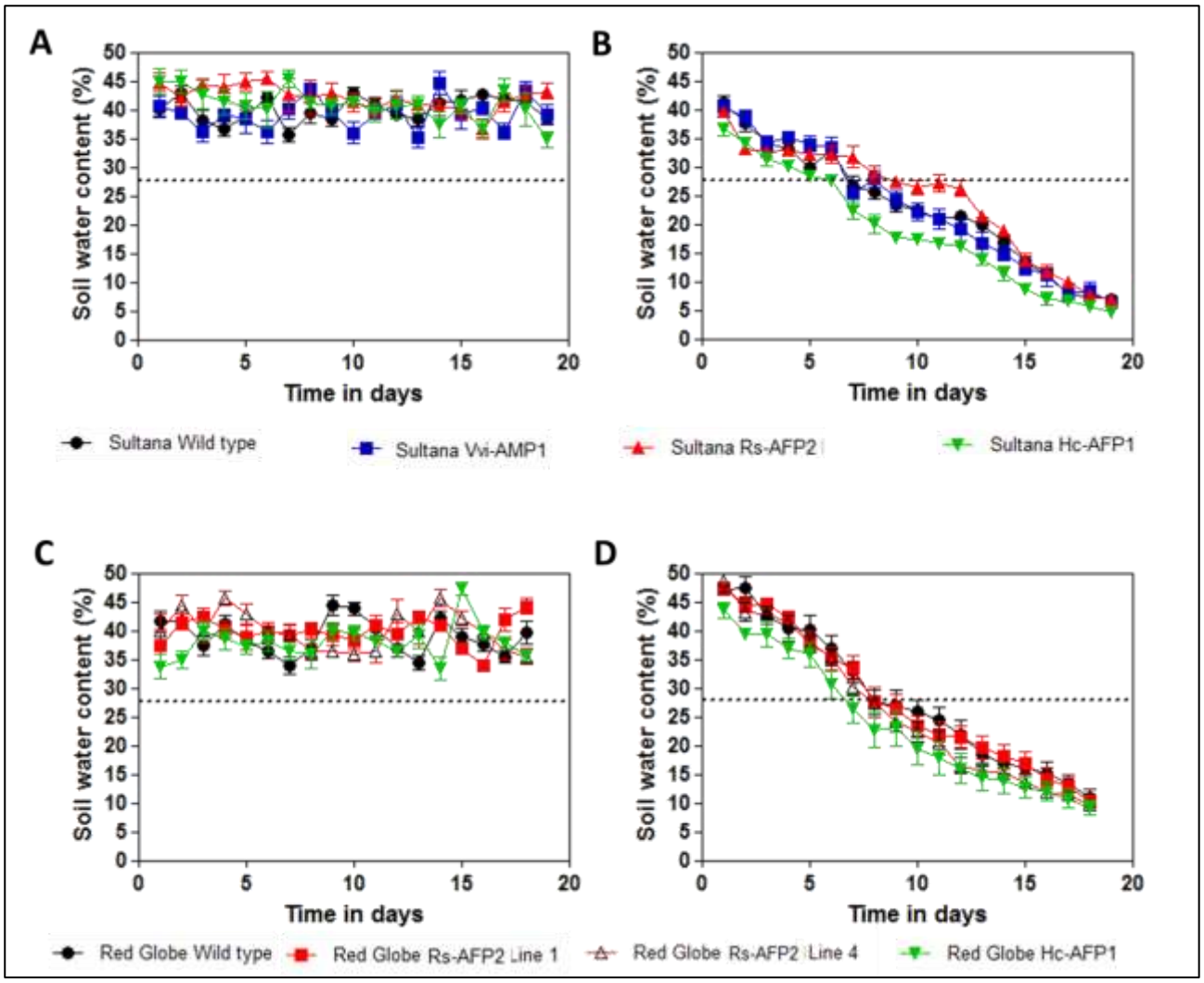

Figure 6.4 The daily soil water content readings of the Sultana and Red Globe populations kept in a green-house under well- watered condition (A and C), as well as during an active drying experiment (B and D) . Panels A and B show the results for the Sultana populations and panels C and D for the Red Globe populations. The error bars indicate standard error of mean (SEM) of three measurements taken per plant and 3 to 4 plants per treatment (n=9 or 12). The broken line indicates the soil water content threshold of 28%.

In order to evaluate the reaction of the different populations to the water deficit, the stomatal conductance of the plants undergoing active drying were considered over a 19 day period (Figure 6.5). The Red Globe cultivar reacted by closing stomata faster during the water stress experiment compared to the Sultana cultivar. The Red Globe wild type only dipped under the soil water content threshold at 10 days, while a reduction in stomatal conductance was already visible at that time. The Sultana wild type dipped under the threshold at 7 days, but only showed a reduced stomatal conductance at day 16. The Sultana Rs-AFP2 plant lines had a very low and constant stomatal conductance throughout the active drying experiment. Furthermore, the Sultana Hc-AFP1 lines reacted earlier to the active drying, reducing their stomatal conductance earlier compared to the Sultana wild type and Sultana Vvi-AMP1 lines. Red Globe Hc-AFP1 and Rs-AFP2 line 1 also showed a, earlier reaction towards the water deficit treatment, whereas the non-expresser Red Globe Rs-AFP2 line 4 reacted similarly to that of the Red Globe control at day 14 (Fig 6.5B).

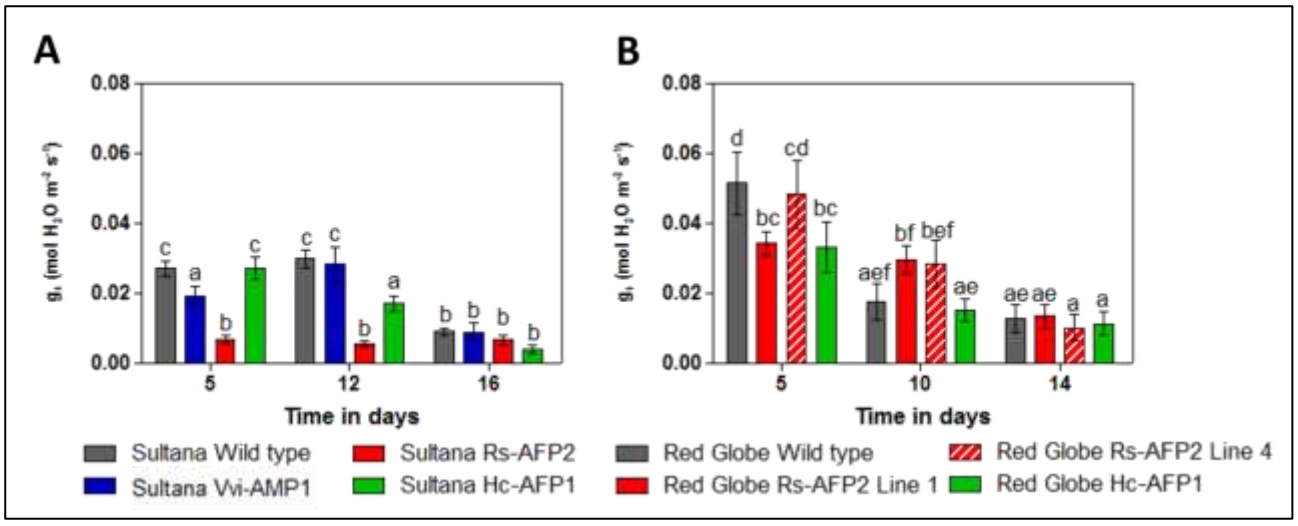

Figure 6.5 The stomatal conductance of the (A) *V. vinifera* cv. Sultana and (B) *V. vinifera* cv. Red Globe wild type and transgenic lines undergoing an active drying experiment in a greenhouse. Error bars indicate standard error of mean and with different letters within the same parameter are significantly different at P ≤ 0.05 with n=9 or 12.

The intrinsic water use efficacy of the Red Globe and Sultana populations were evaluated by plotting the relationship between the A_n/A_{nmax} ratio and stomatal conductance (g_s) (Figure 6.6A and B). The curves of the relationship between the A_n/A_{nmax} ratio and stomatal conductance (g_s) of the individual plant lines are indicated in Figure B6.2 in Addendum B to Chapter 6. All curves showed a non-linear significant relationship between A_n/A_{nmax} and g_s for both datasets. The R^2 values in Table 6.5 indicate a good positive relationship between the data and regression lines for all the curves (except for Red Globe Rs-AFP2 line 1), with more than 60% of the values falling in the regression line. The same genetic constructs did not lead to similar phenotypes in the Sultana versus the Red Globe backgrounds (Figure 6.5). When considering the Sultana populations (Figure 6.10A), it was clear that there were differences in the intrinsic WUE values between the Sultana wild type and transgenic lines. When using the equation (A_n/A_{nmax} = a ln (g_s) + b) of the graph in Figure 6.10A and comparing the CO_2 assimilation at a stress level of 0.04 mol H_2O $m^{-2}s^{-1}$ stomatal conductance, the Sultana wild type lines had the lowest CO_2 assimilation rate at 50% and in turn the lowest WUE compared to the Sultana transgenic lines. Furthermore, Sultana Vvi-AMP1 lines behaved similar to the WT and showed a very similar CO_2 assimilation rate of 51% at the same level of stomatal conductance. Sultana Rs-AFP2 demonstrated the highest CO_2 assimilation rate of 70% at a stress level of 0.04 mol H_2O m^{-2} s^{-1} stomatal conductance, followed by Sultana Hc-AFP1 with a CO_2 assimilation rate of 60%.

The results obtained for the Red Globe populations (Figure 6.5B) indicated smaller differences between the wild type and the transgenic lines, with some of the transgenic lines behaving very similar to the wild type (the curves of the relationship between the A_n/A_{nmax} ratio and stomatal conductance (g_s) of the individual plant lines are indicated in Figure B6.5 in Addendum B to Chapter 6.). As mentioned above, the Red Globe Rs-AFP2 line 1 had a very low r^2 value with only 55% of the values falling within the regression line and making it difficult to make any concrete

conclusions regarding this plant line. At a (stress) level of of 0.04 mol H_2O m^{-2} s^{-1} stomatal conductance, the Red Globe Hc-AFP1 line had the highest CO_2 assimilation percentage (64%) and therefore the highest intrinsic WUE compared to the Red Globe Wild type and other transgenic lines. Furthermore, at the same level of stomatal conductance, the non-expressing Red Globe line 4 had a CO_2 assimilation of 61% with the Red Globe wild type at 56% CO_2 assimilation. At the same level of stress, the Red Globe transgenic Rs-AFP2 line 1 had a very similar CO_2 assimilation rate compared to the control (54%). When comparing the intrinsic WUE of the Hc-AFP1 and Rs-AFP2 constructs, we see that the Hc-AFP1 had an increased intrinsic WUE in both genetic backgrounds compared to the wild types. The Rs-AFP2 showed an increase in intrinsic WUE in the Sultana genetic background compared to the wild type, whereas in the Red Globe genetic background the Rs-AFP2 lines displayed a decrease in the intrinsic WUE compared to the wild type.

Table 6.5 The logarithmic equation slope, intercept and R^2 values of the relationship between A_n/A_{nmax} ratio versus stomatal conductance (g_s) in Figure 6.6 of *V. vinifera* cv. Sultana and cv. Red Globe wild type and transgenic lines during an active drying experiment conducted under greenhouse conditions. A_n is the actual CO_2 assimilation and A_{nmax} is the maximum value of CO_2 assimilation.

Cultivar	Plant line	a*	b*	r^2
Sultana	**Wild type**	0.1606	1.0195	0.7339
	Vvi-AMP1	0.1212	0.9001	0.6342
	Hc-AFP1	0.1873	1.2046	0.6463
	Rs-AFP2	0.2338	1.4549	0.8545
Red Globe	**Wild type**	0.1561	1.058	0.6322
	Rs-AFP2 Line 4	0.191	1.2283	0.7211
	Hc-AFP1	0.2108	1.3203	0.8471
	Rs-AFP2 Line 1	0,1534	1,0302	0,5488

The water status of the *V. vinifera* cv. Sultana and cv. Red Globe wild type and transgenic plant lines were determined at the end (Day 19) of the active drying experiment by measuring the leaf and stem water potential. No significant differences were observed in the stem and leaf water potentials of the plants that were well-watered throughout the experiment (Figure 6.7 “full”), although there were differences in their CO_2 assimilation rates (numbers added to the bar graphs represent the CO_2 assimilation rates measured at day 19 for the different plant lines). For the measurements made from plants at the end of the active drying (Figure 6.7 “deficit”), the overall trends between the leaf (Figure 6.7A and C) and stem (Figure 6.7B and D) water potential readings were similar.

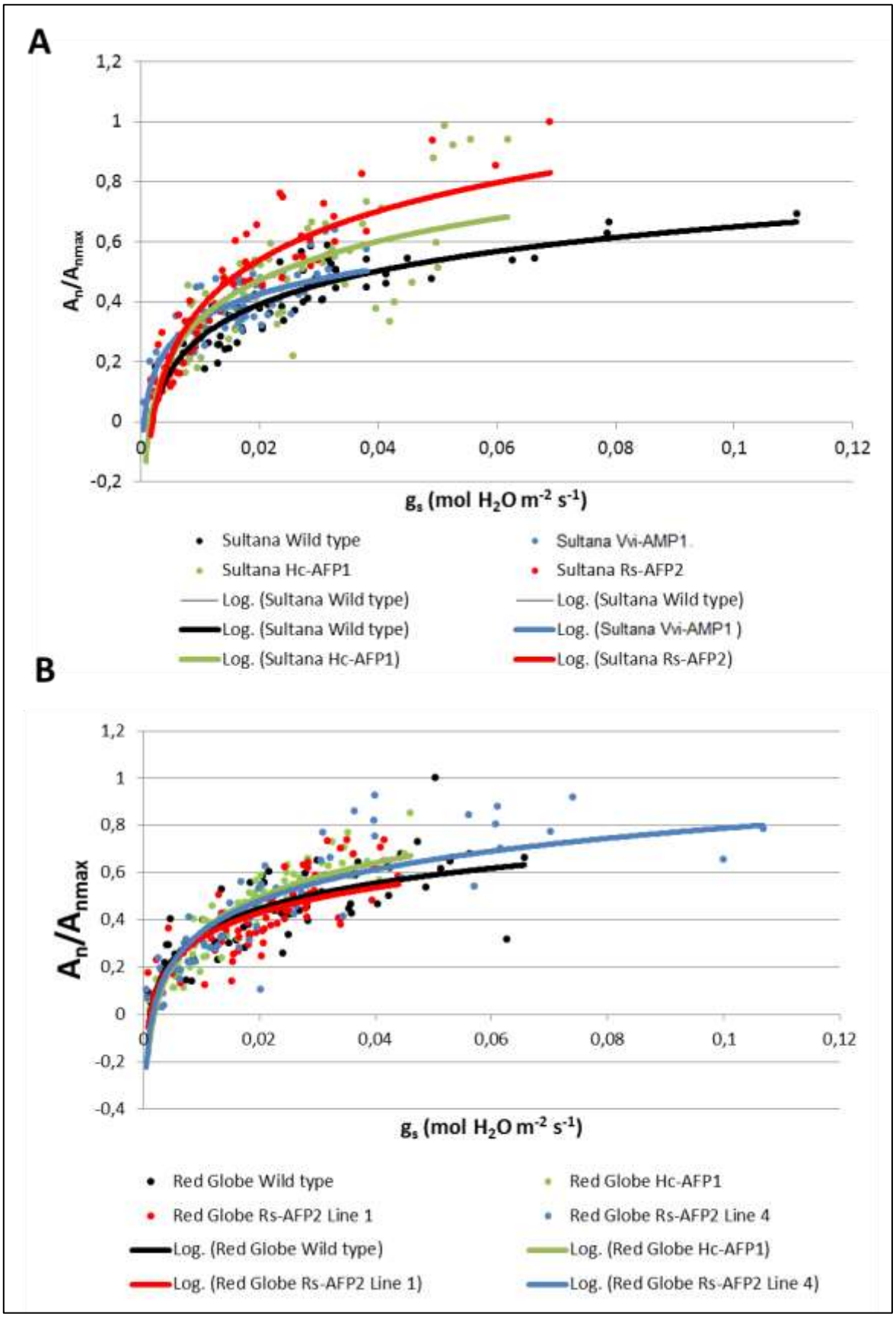

Figure 6.6 Relationship between the A_n/A_{nmax} ratio versus stomatal conductance (g_s) presented by a logarithmic trend line of (A) *V. vinifera* cv. Sultana wild type and transgenic lines and (B) *V. vinifera* cv. Red Globe wild type and transgenic lines undergoing an active drying experiment in a greenhouse. A_n is the actual CO_2 assimilation and A_{nmax} is the maximum value of CO_2 assimilation.

Under stressed conditions at the end of the experiment, it was clear that the Sultana Rs-AFP2 line had the highest hydration status compared to the rest of the Sultana lines (Figure 6.7A and B). Furthermore this transgenic line also had the highest CO_2 assimilation rate and therefore the highest intrinsic WUE under this stressed conditions. Sultana Vvi-AMP1 and Hc-AFP1 lines, had the lowest stem and leaf water potential, indicating low available water and a severely stressed condition. The stem and water leaf potential readings reflected the physical state of the plants where Sultana Vvi-AMP1 and Hc-AFP1 had the most wilted leaves compared the Sultana wild type and Sultana Rs-AFP2 at the end of the experiment (Figure D6.3 in Addendum D). The water potential readings of the Red globe wild type and transgenic lines demonstrated that the Red Globe Hc-AFP1 line had the lowest hydration status compared to the other Red Globe lines. In contrast, the Red Globe wild type and non-expresser Rs-AFP2 line 4 had the highest water status at the end of the active drying experiment. The Hc-AFP1 construct showed similar low hydration status in both cultivars at the end of the experiments.

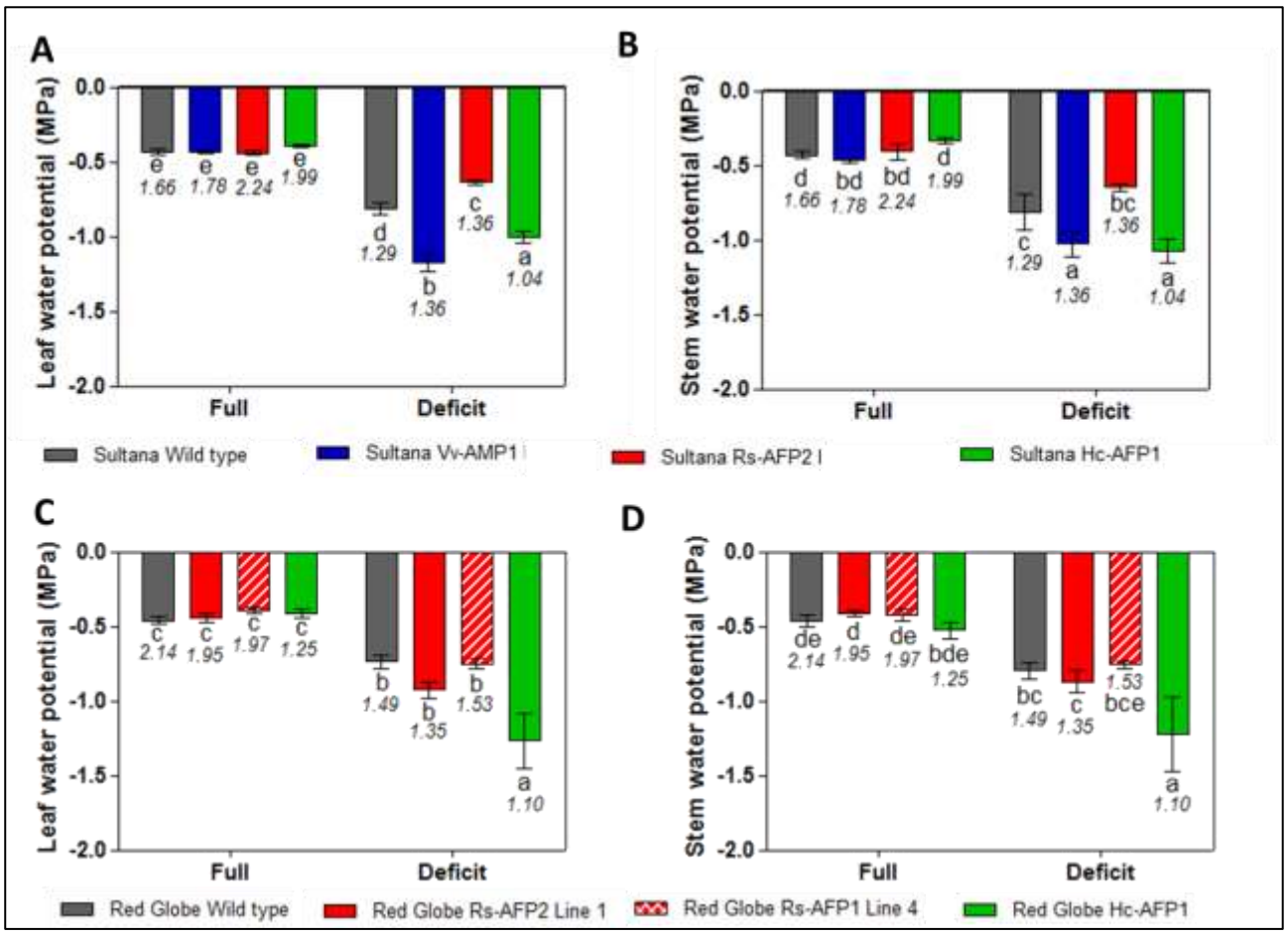

Figure 6.7 (A and C) Leaf and (B and D) stem water potential of (A and B) *V. vinifera* cv. Sultana and *V. vinifera* cv. Red Globe wild type and transgenic lines at day 19 of the active drying experiment Error bars indicate standard error of mean and with different letters within the same parameter are significantly different at $P \leq 0.05$. The mean CO_2 assimilation rate (µmol CO_2 m^{-2} s^{-1}) is indicated for each plant line above the respective column.

6.4 DISCUSSION

Very few studies have reported on the expression of plant defensins and DEFL genes in reaction to drought and other abiotic stresses, but some transcriptomic data is available pointing to their potential roles in this regard (Do et al. 2004; Du Plessis 2012). A link between heat shock and plant defensins have been functionally established in *Arabidopsis*. Plant defensin genes have been identified as the targets of heat shock factor dependent negative regulation. Knockout mutants of *Arabidopsis* plants lacking the heat shock factors B1 (*hsfB1)* and B2 (*hsfB2b)* displayed an upregulation in the *Arabidopsis* plant defensin 1 (*Pdf1*) expression. Furthermore, these mutant plants showed an altered formation of their heat shock factor A2 dependent binding motifs, suggesting that *hsfB1* and *hsfB2b* may interact with a class A heat shock factor in the regulation of the heat shock response. These results provide an interesting link between defensins and abiotic stresses, an aspect that is still largely unexplored (Kumar et al. 2009). For drought stress, the majority of the data for a possible link between peptides and the drought response is “guilt by association”. Several gene expression studies (Maitra and Cushman 1994; Do et al. 2004) found defensins to be upregulated in response to water deficit and the same was observed in grapevine (Du Plessis 2012). The data presented by Du Plessis (2012) showed that a selection of defensin-like genes in grapevine cultivars were upregulated in response to water deficit stress (Du Plessis 2012). The meta-analysis of the gene expression of DEFL genes reported in this study also corroborated the prior study since it showed that several DEFL genes were upregulated in response to leaf dehydration and heat stress conditions. This analysis was, however, limited in that some of these DEFL genes did not map to a unique sequence within the V1 transcriptome and only included two experiments. Future studies that include gene expression studies will be useful in order to confirm the results of the *in silico* analysis. How exactly the peptides would function during drought responses are still unclear and it is also not known if defensin promoters are directly controlled by signalling molecules (such as ABA) during water stress. The typical motifs, such as the dehydration response element (DRE), abscisic acid responsive element (ABRE), etc. found in many gene promoters that respond to water stress have not been found in defensin promoters analysed in this regard (Manners et al. 1998; Shinozaki et al. 2003; Lata and Prasad 2011).

Although the role/functions of defensins in the grapevine’s responses to water stress are not clarified, from the screening study presented here, it was evident that the ectopic expression of plant defensins could positively impact on grapevine stomatal behaviour under water stress conditions, resulting in improved hydration status and improvement of the intrinsic WUE of plant lines expressing defensins. Transgenic grapevine populations overexpressing four different defensin peptides, in two genetic backgrounds were used to describe the response of these transgenics when they were exposed to increasing limiting water in an active drying experiment. The focus was not on any particular stage of the drought response, but rather to profile the responses of the lines during the course of the process to move from a non-stressed state to an

increasing level of stress. This was done by measuring the physiological response of the wild types and transgenic lines and comparing them, in context with the diminishing soli water content. Active drying experiments have been widely used for characterising the effects of drought stress on the water use efficiency, dry mass and physiology in various crops (Ray and Sinclair 1997; Liu and Stützel 2004; Achten et al. 2010) and have also been used in grapevine (Tombesi et al. 2015). It is important to note that the biomass of the plants could not be brought into the evaluation, eliminating the ability to normalise for potential biomass differences and calculations of overall WUE on a whole plant level (instead the intrinsic WUE was reported). Moreover, the transgenic plant lines were juvenile and the scion cultivars were tested on their own roots, making it difficult to find comparable data in literature, since most published analyses were conducted on mature plants and in most cases, grafted vines.

It was clear that the Sultana and Red Globe cultivars displayed different intrinsic WUE, with Red Globe using the water more efficiently under greenhouse conditions that mimicked outside variable conditions (in light and temperature) more accurately (Figure 6.3). We initially conducted the drought stress experiments under low light intensity conditions in a highly controlled environment (in terms of light, temperature and humidity, specifically since these conditions would favour logistics for the gas exchange measurements). These measurements are time consuming as it takes a minimum of two minutes for the instrument to stabilize per measurement. We considered it important to test all the different genotypes within the same experiment with the required repeats, resulting in numerous gas exchange measurements that took a minimum of four hours to conduct. For this reason we decided to initially conduct these experiments under constant light conditions to limit the variability in gas exchange measurements that could be influenced by the changing light conditions over a four hour period (in ambient light). The stomatal conductance values recorded in the low light conditions were, however, extremely low; for example the levels in the non-stressed plants were lower than those typically recorded for severely water stress plants. The CO_2 assimilation for these plants under the low light intensity conditions was also very low and therefore the data from the growth room (low light conditions) was not suitable for a comparison of the physiological responses of the plants to water stress. Interestingly, a preliminary analysis of samples obtained from the unstressed plants and the lines undergoing the active drying treatments confirmed that typical marker genes for the molecular responses to water deficit, yielded predictable patterns of gene expression indicating the activation of a stress response (results not shown, personal communication with Ms Sarah McMurty of the IWBT who performed a time-course RT-PCR analysis of drought stress biomarker genes involved in abscisic acid metabolism). The RT-PCR data showed that irrespective of the light conditions, the plants did mount a predictable water stress response, but given the fact that our comparisons are dependent on the physiological measurements, the data from the green-house experiments were considered more suitable for the comparisons.

The ambient light in the green house supported significantly higher levels of photosyntheitic rates and stomatal conductance in all lines. The data of all the datasets were normalized by the maximum photosynthetic CO_2 assimilation rate of each specific dataset in order to standardize the degree of change and create a percentage output to compare the different datasets with each other and other data sets in literature. It is important to note that the photosynthetic CO_2 assimilation rates of these grapevine plants, even when tested under ambient light intensity conditions were still lower than photosynthetic CO_2 assimilation rates that have been reported for grapevine. It is possibly linked to the plants being juvenile and the fact that they have never been exposed to high light conditions. Furthermore, Björkman and Holmgren (1963) showed that photosynthetic apparatus form plants grown in exposed habitats are able to utilize stronger light more effectively compared to plants form less exposed environments.

The normal reaction of plants towards water stress is to decrease stomatal conductance resulting in a decrease in plant water potential and intensity of photosynthetic CO_2 assimilation. Stomatal conductance is the earliest response to drought stress even at mild drought stress conditions. Three different phases of stomatal conductance depending on the severity of the drought stress have been defined for grapevine. These include: mild ($0.5\text{-}0.7 > g_s > 0.15$ mol H_2O $m^{-2}s^{-1}$), moderate ($0.15 > g_s > 0.05$ mol H_2O $m^{-2}s^{-1}$) and severe ($g_s < 0.05$ mol H_2O $m^{-2}s^{-1}$) water stress levels (Flexas and Medrano 2002; Medrano et al. 2002; Zúñiga et al. 2018). The stomatal conductance of the non-grafted juvenile grapevine plants were lower compared to these established levels of stomatal conductance, but it was possible to characterize the response of the different plant lines in response to the drought stress conditions tested. The transgenic Sultana Rs-AFP2 and Hc-AFP1 lines showed an earlier response in reaction to the drought stress by reducing their stomatal conductance, whereas the Sultana Vvi-AMP1 and wild type responded slower/later. These results were consistent with the intrinsic WUE calculations and the earlier reaction of the Hc-AFP1 and Rs-AFP2 transgenic lines can possibly explain their increased WUE. Furthermore, the Red Globe Hc-AFP1 transgenic lines also showed a rapid response to the drought stress condition by the reduction of stomatal conductance. Early stomatal closure in response to water stress is a vital component to consider as it can contribute to drought tolerance. In a study on cowpeas it was demonstrated that early stomatal closure could be partially responsible for drought avoidance by this early regulation in leaf water potential as the employment of early stomatal closure leads to less water loss and a higher water potential compared to other crops employing a osmotic adjustment mechanism (Bates and Hall 1981; Mccree and Richardson 1987).

The genotype had a very strong influence in the behaviour of the different transgenic lines as the Sultana and Red Globe population did not show the same responses for the different constructs. The transgenic expression of Rs-AFP2 and Hc-AFP1 increased the intrinsic WUE in Sultana, but the Rs-AFP2 expression in Red Globe did not have the same effect, whereas the Hc-AFP1 lines still performed marginally better in terms of intrinsic WUE in Red Globe. Furthermore, the Red Globe population (including the WT) mounted a strong response on the physiological level to the

water stress (under ambient light), with a strong reduction in stomatal conductance and photosynthesis, also maintaining a good hydration status. The Rs-AFP2 lines in the Red Globe background was previously shown to reduce many of the growth parameters, particularly root growth (Chapter 4). Rs-AFP2 peptides have been shown to have a negative effect on root growth with a well-developed model (Allen et al. 2008; Parisi et al. 2018). The observed reduced WUE could therefore perhaps be linked to reduced root growth of the Red Globe Rs-AFP2 lines, reducing the efficiency of the plants to utilise the available soil water, also contributing to the lower hydration status as observed. These aspects must be confirmed by also factoring in biomass differences to calculate the overall WUE in follow-up experiments.

The intrinsic WUE data generated did mostly correspond to the actual hydration status of the plants. Interestingly, these measurements were relatable to established grapevine water status responses (even in the low light conditions). These responses were reported as mild (Ψ_s > -1.0 MPa), moderate (-1.0 MPa > Ψ_s <-1.2 MPa) and severe (Ψ_s <-1.4 MPa) water stress (Zúñiga et al. 2018). The leaf and stem water potentials are usually a result of the soil water deficit. The leaf water potential is however dependent on the adaptive mechanisms of the plant such as stomatal closure and cannot be used as a sensitive measure of water stress (Jones 2007).

These promising results provide the first experimental evidence that defensins may have functional roles in the water stress response in grapevine. It is clear that there is much scope for more comprehensive analysis of these phenotypes in future studies. The genetic backgrounds clearly play a significant role in modulating the final reactions of the specific cultivar and this needs to be rigorously considered in water-stress experiments. The characterised transgenic lines may also prove useful to explore the possible unique changes to the molecular and transcriptional responses of the defensin-expressing plants under water stress conditions to ultimately understand how defensins participate in this abiotic stress response.

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Addendum A to Chapter 6

This Addendum contains relevant and additional data not shown in Chapter 6.

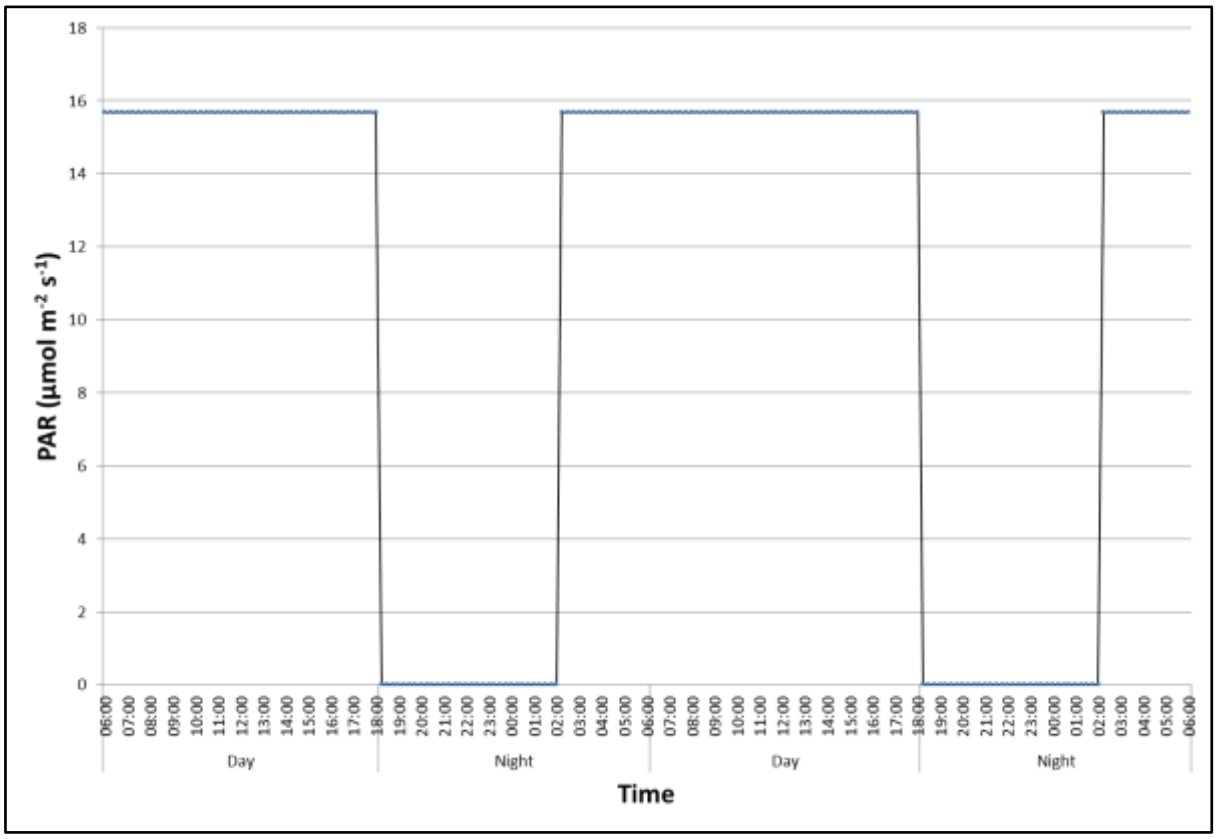

Figure A6.1 Photosynthetically active radiation (PAR) profiles measured for two consecutive days under the constant low light intensity conditions maintained in the growth room.

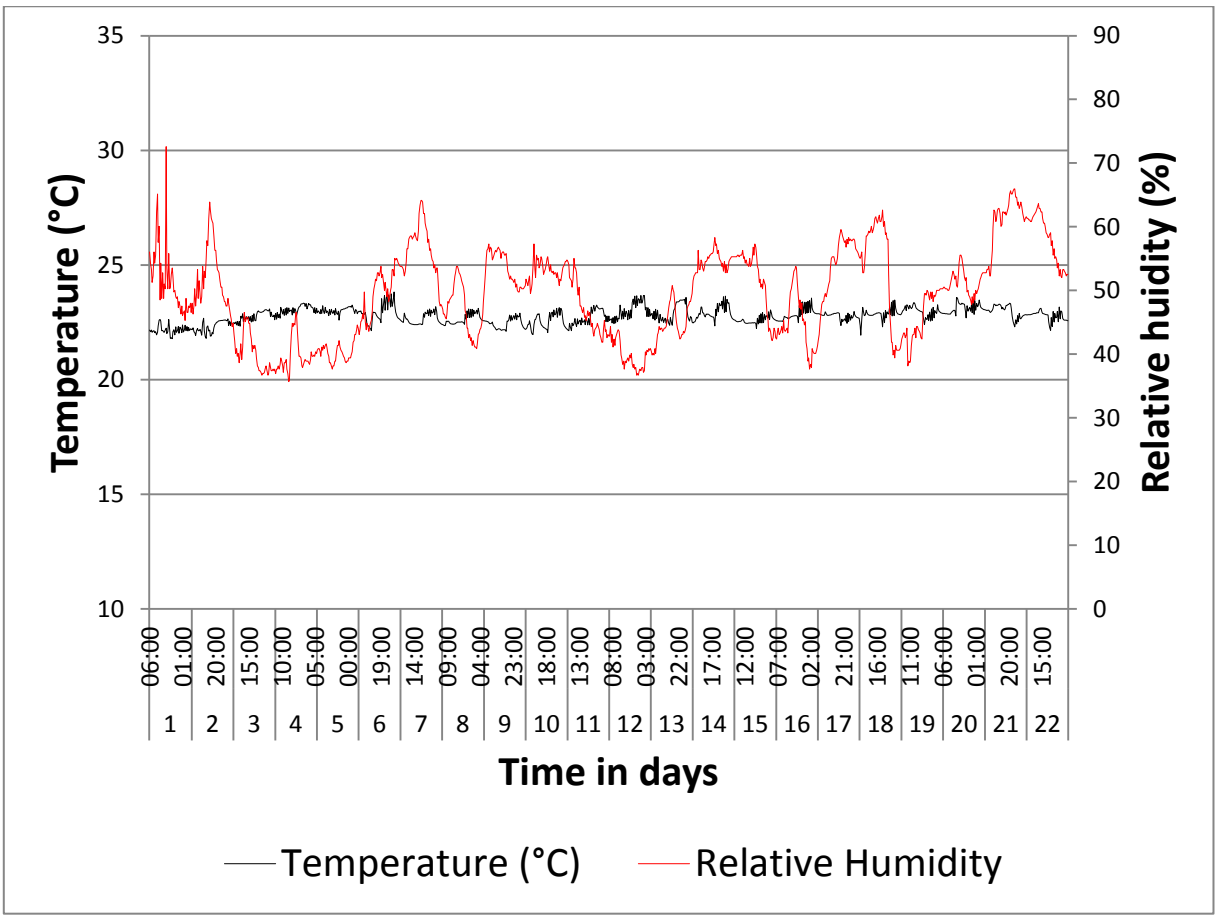

Figure A6.2 The daily temperature and relative humidity conditions maintained in the growth room during the active drying experiment conducted on the Sultana populations.

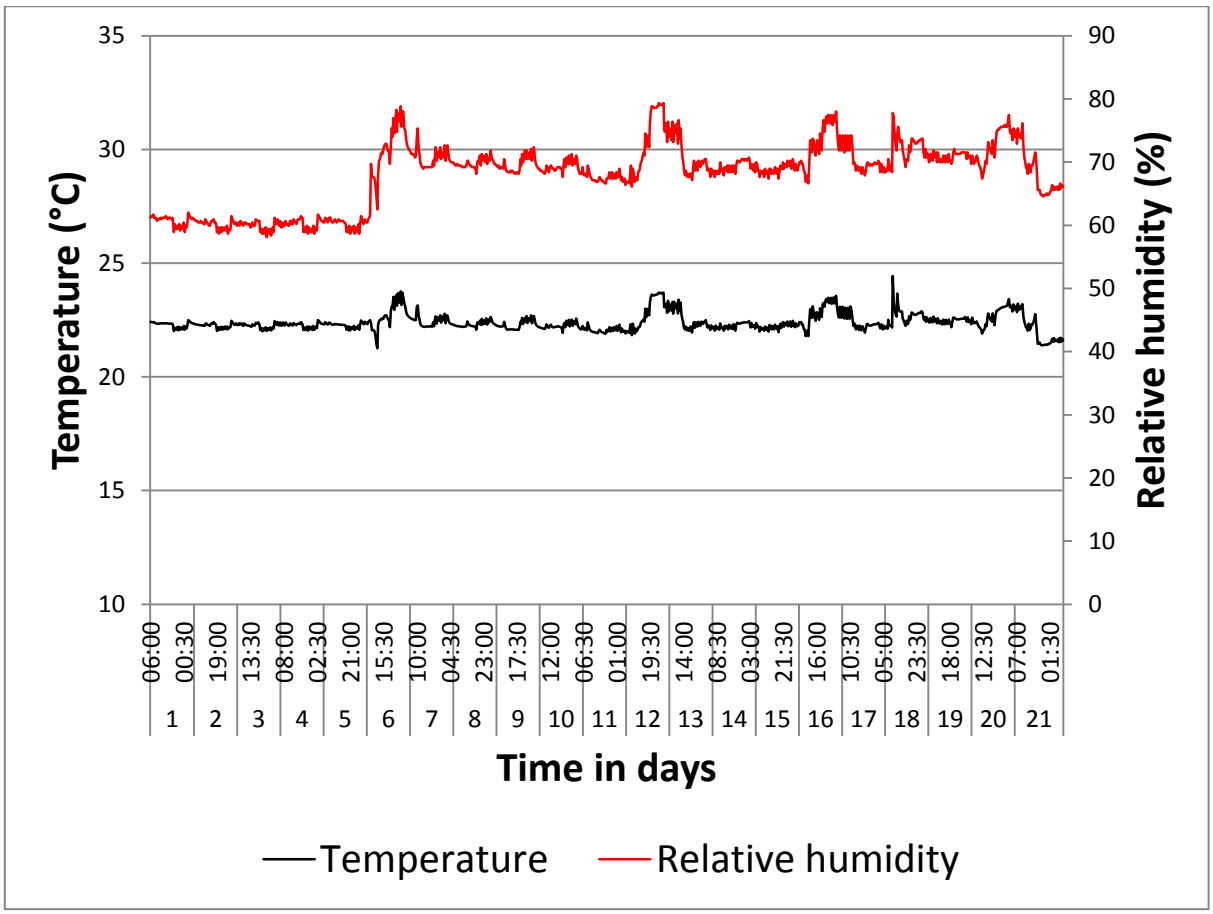

Figure A6.3 The daily temperature and relative humidity conditions maintained in the growth room during the active drying experiment conducted on the Red Globe populations.

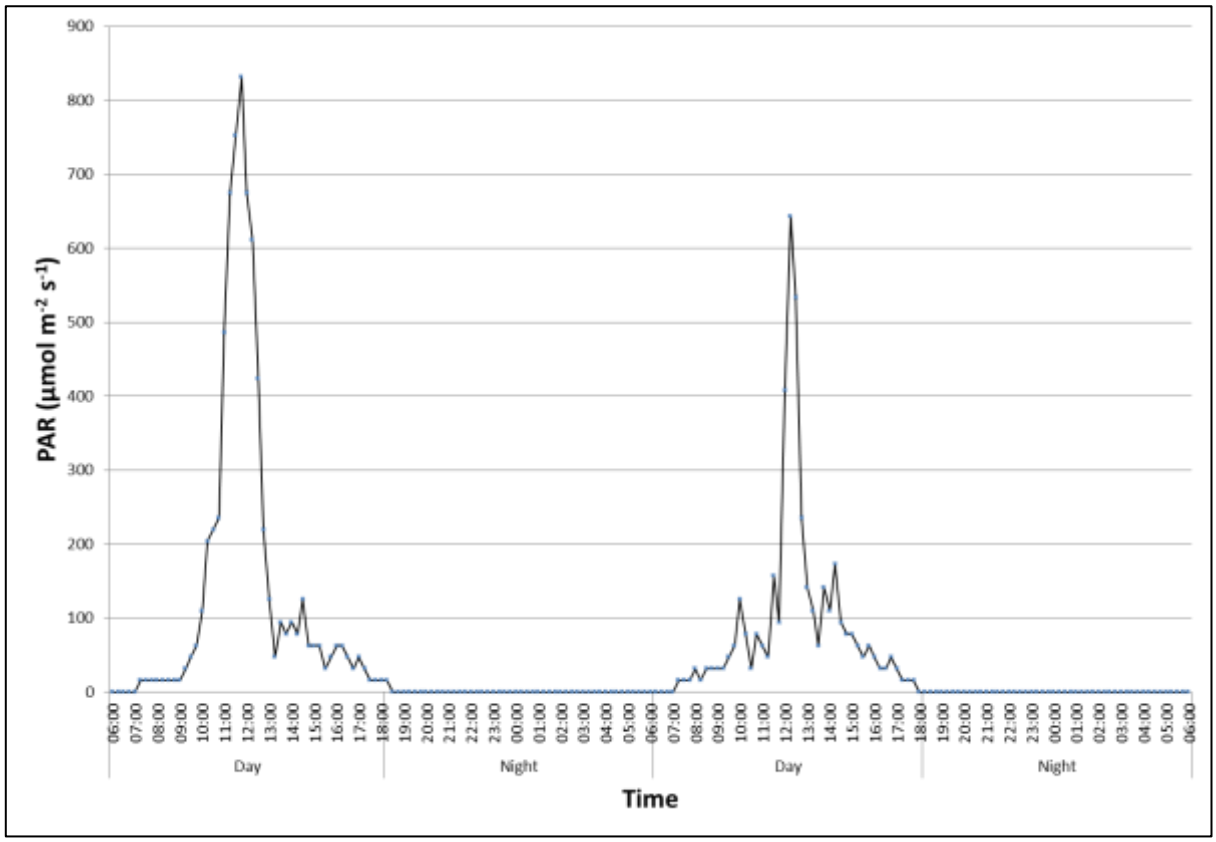

Figure A6.4. Photosynthetically active radiation (PAR) profiles measured for two consecutive days under the ambient light conditions maintained in the green-house.

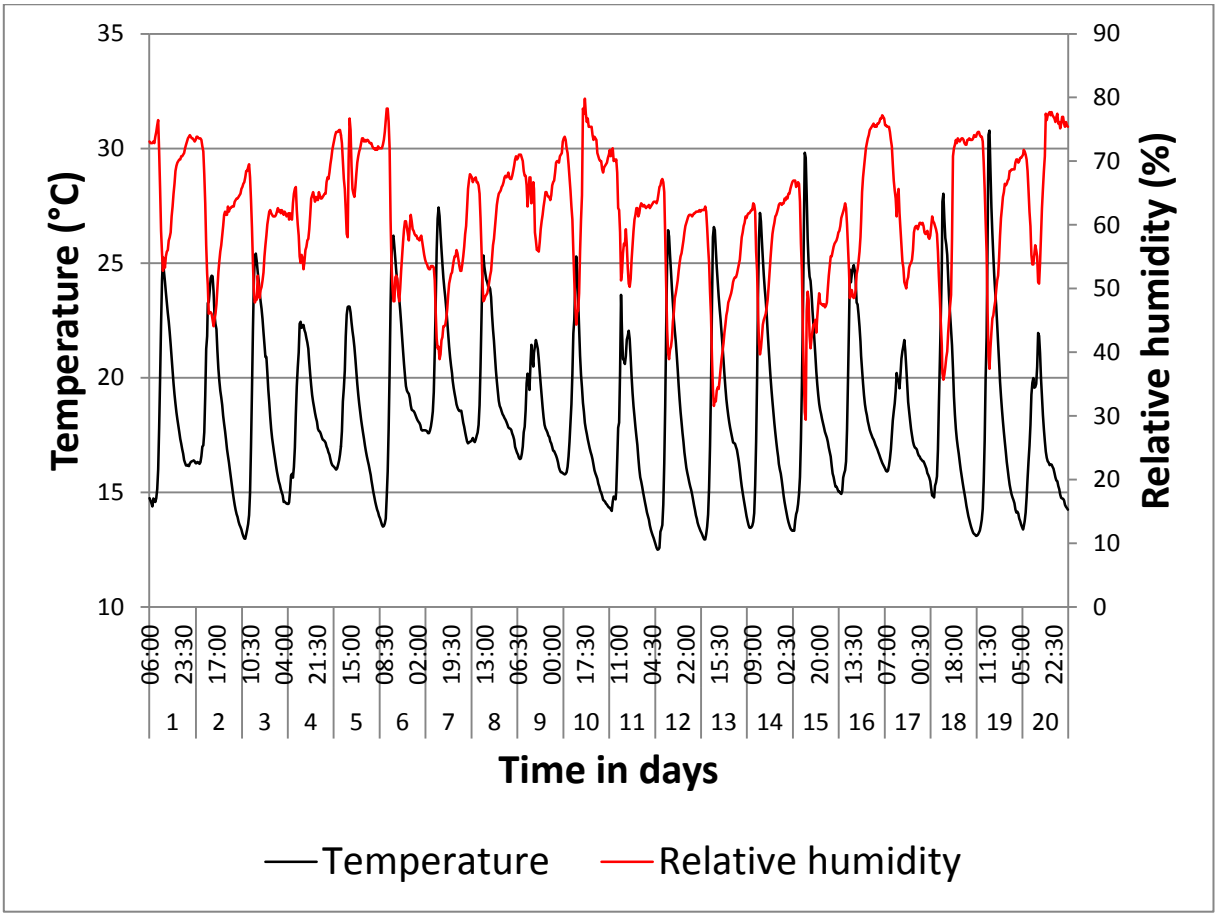

Figure A6.5 The daily temperature and relative humidity conditions maintained in the greenhouse during the active drying experiment conducted on the Sultana populations.

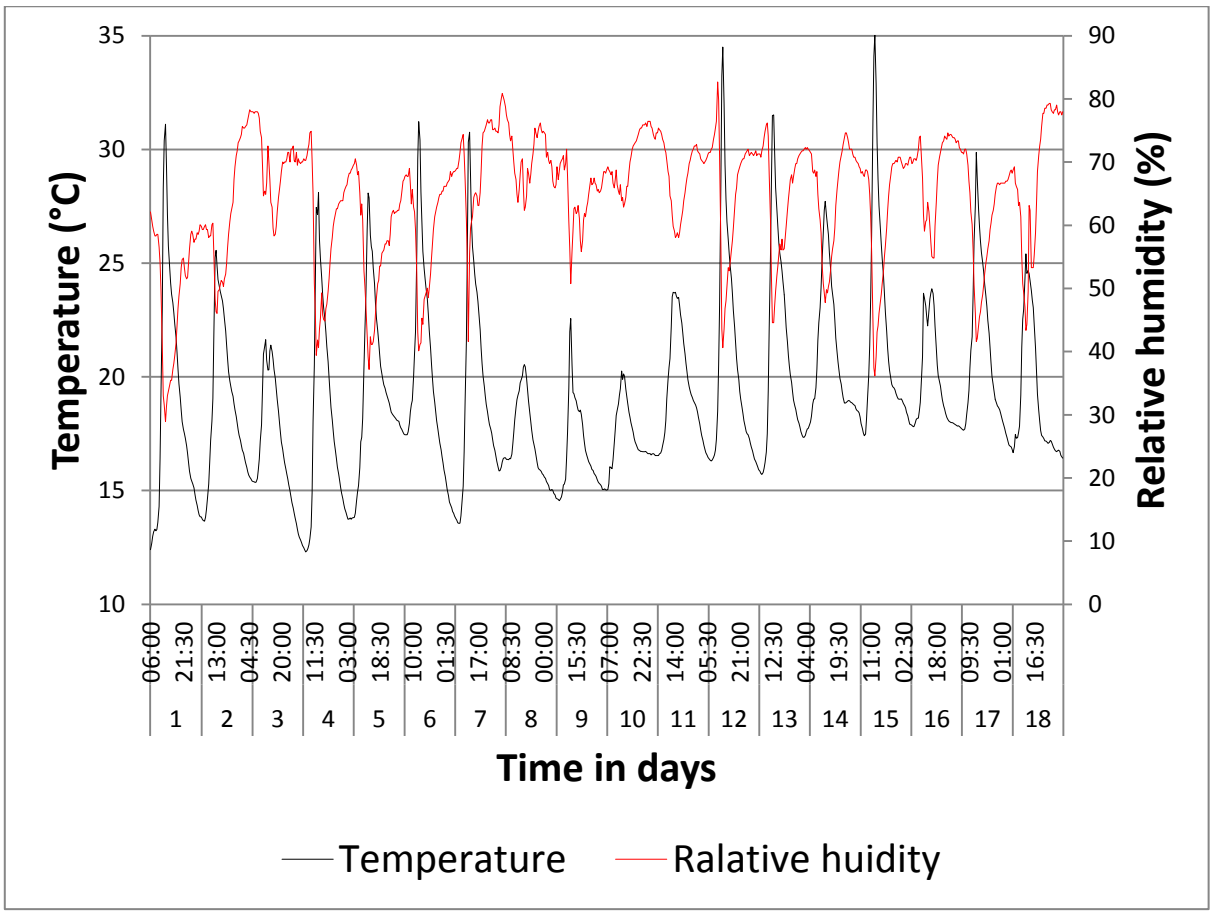

Figure A6.6 The daily temperature and relative humidity conditions maintained in the greenhouse during the active drying experiment conducted on the Red Globe populations.

Addendum B to Chapter 6

This Addendum contains relevant and additional data not shown in Chapter 6.

B6.1 *V. vinifera* cv. Sultana transgenic population ambient light conditions active drying water stress experiment

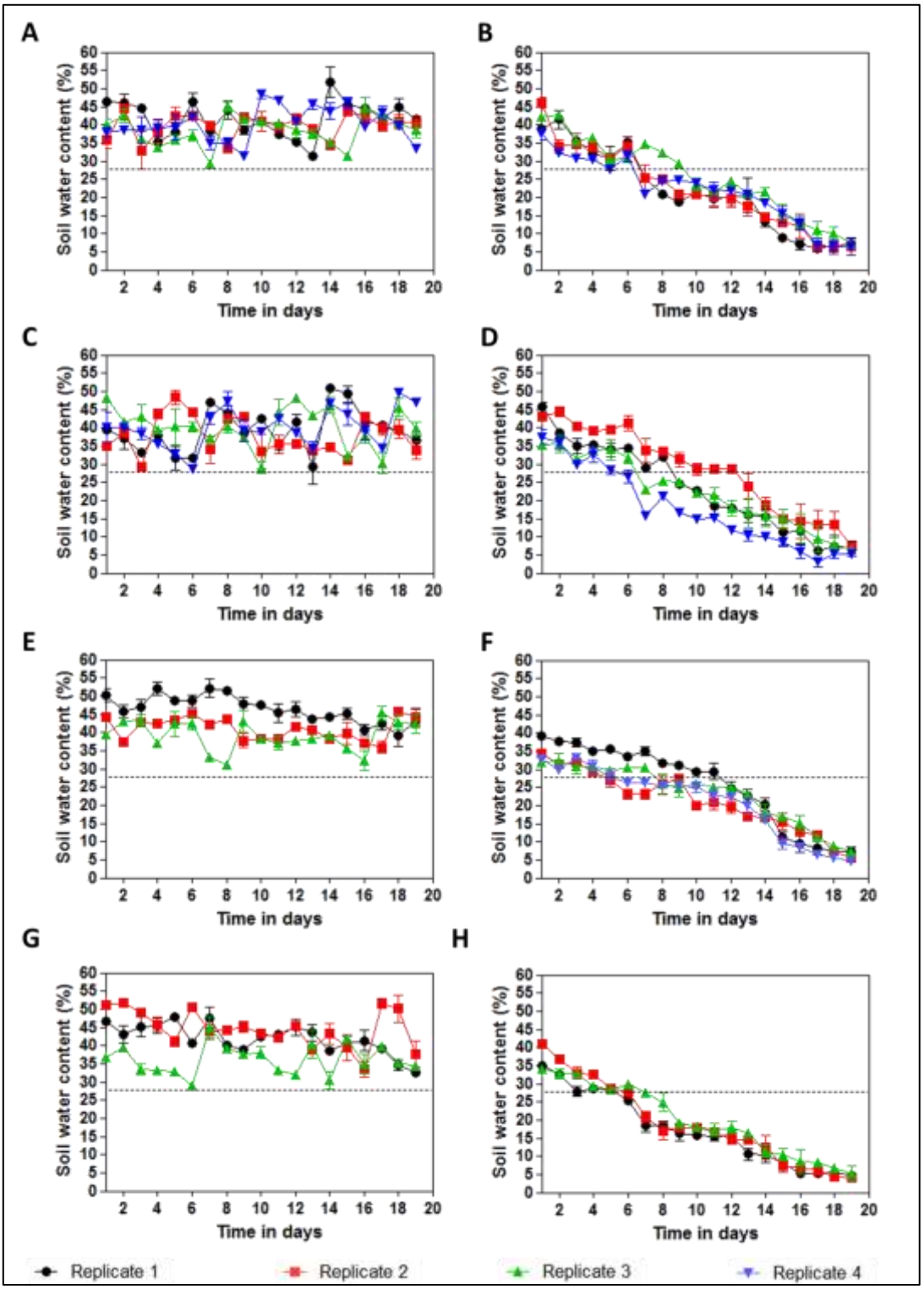

Figure B6.1 The daily soil water content readings of the individual plants of the Sultana population under well- watered condition (A, C, E and G), as well as during an active drying experiment (B, D, F and H). Panels A and B show the results for the Sultana wild type, panels C and D for Sultana Vvi-AMP1, panels E and F show the results for Sultana Rs-AFP2 and panels G and H show the results for Sultana Hc-AFP1. The error bars indicate standard error of mean (SEM) of three measurements taken per plant (n=3). The broken line indicates the soil water content threshold of 28%.

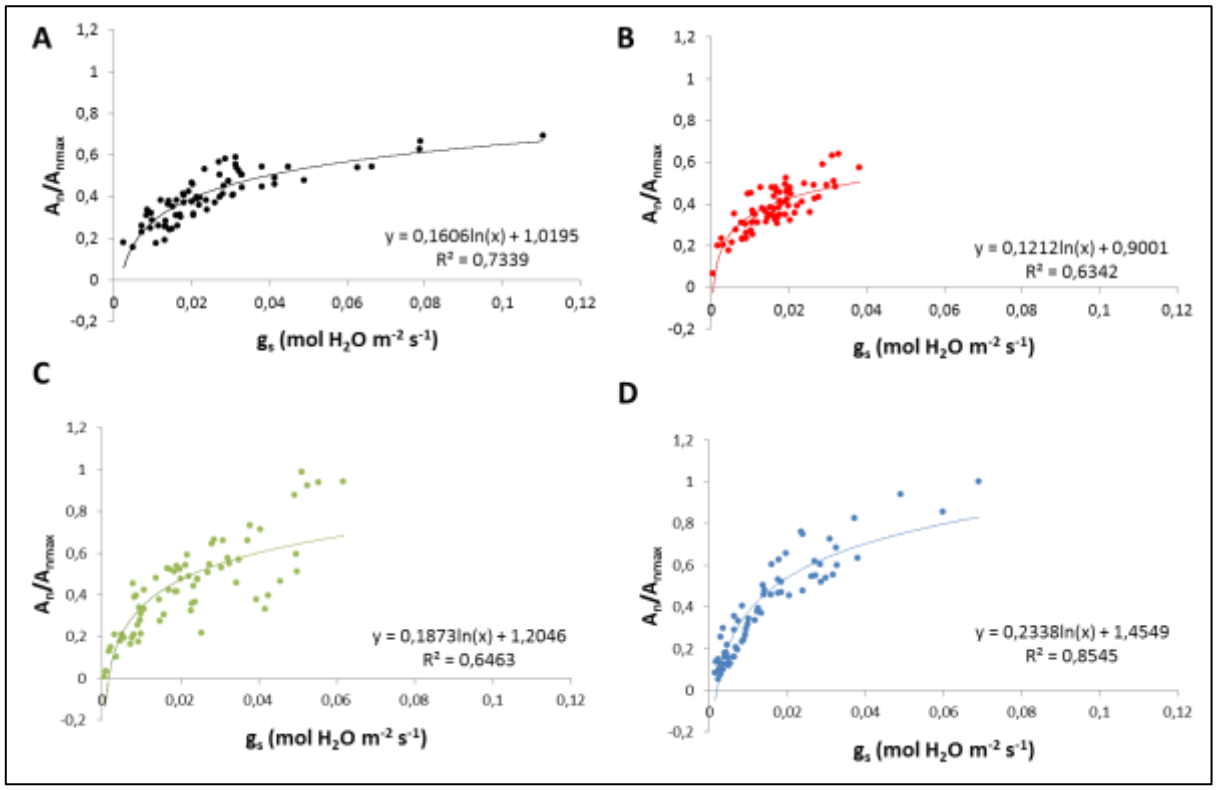

Figure B6.2. Relationship between the A_n/A_{nmax} ratio versus stomatal conductance (g_s) of *V. vinifera* cv. Sultana (A) Wild type, (B) Sultana Vvi-AMP1, (C) Sultana Hc-AFP1 and (D) Sultana Rs-AFP1 lines undergoing an active drying experiment in a greenhouse. A_n is the actual CO_2 assimilation and A_{nmax} is the maximum value of CO_2 assimilation.

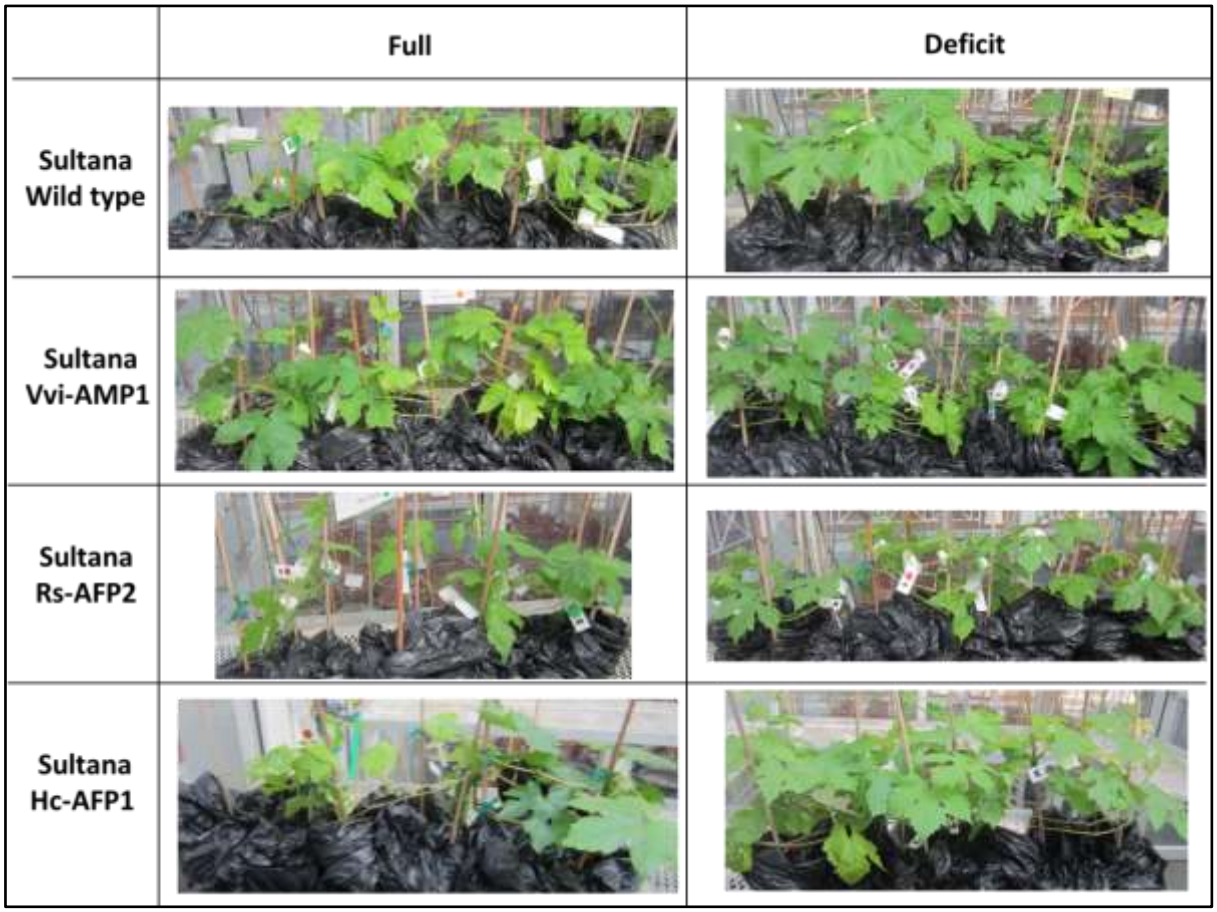

Figure B6.3 Pictures of Sultana wild type and transgenic plants exposed to well-watered and active drying treatments in greenhouse conditions at the end of the active drying experiment.

B6.2 *V. vinifera* cv. Red Globe transgenic population ambient light conditions active drying water stress experiment

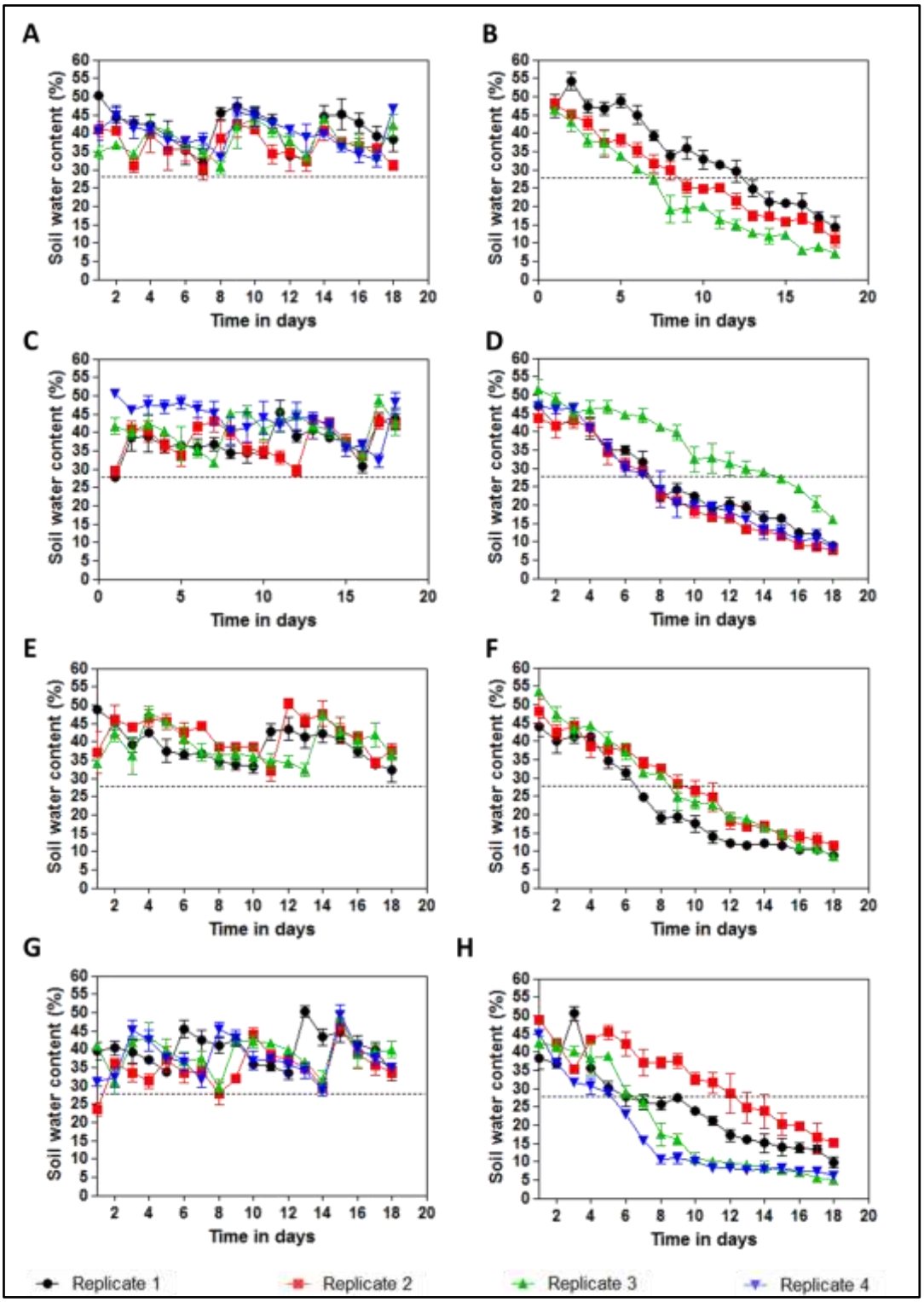

Figure B6.4 The daily soil water content readings of the individual plants of the Red Globe population under well- watered condition (A, C, E and G), as well as during an active drying experiment (B, D, F and H). Panels A and B show the results for the Red Globe wild type, panels C and D for Red Globe Rs-AFP2 line 1, panels E and F show the results for Red Globe Rs-AFP2 line 4 and panels G and H show the results for Red Globe Hc-AFP1. The error bars indicate standard error of mean (SEM) of three measurements taken per plant (N=3). The broken line indicates the soil water content threshold of 28%.

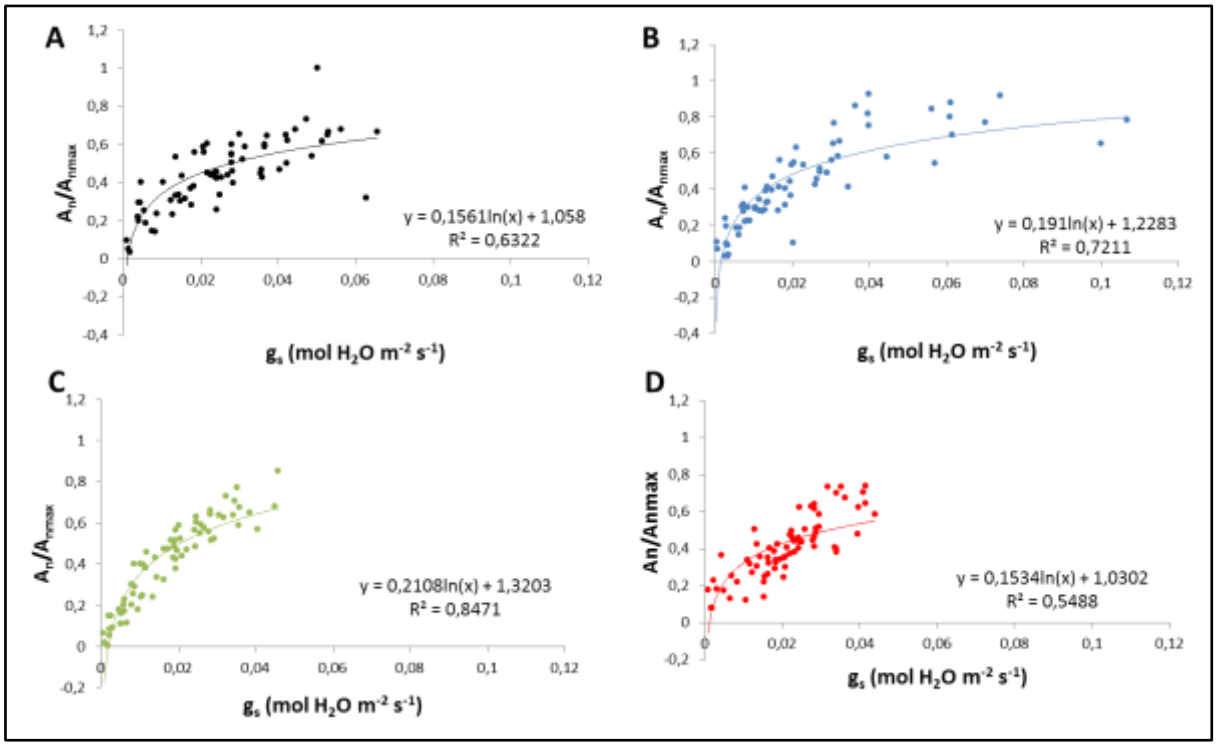

Figure B6.5 Relationship between the A_n/A_{nmax} ratio versus stomatal conductance (g_s) of *V. vinifera* cv. Red Globe (A) Wild type, (B) Red Globe Rs-AFP1 line 4, (C) Red Globe Hc-AFP1 and (D) Red Globe Rs-AFP1 line 1 undergoing an active drying experiment in a greenhouse. A_n is the actual CO_2 assimilation and A_{nmax} is the maximum value of CO_2 assimilation.

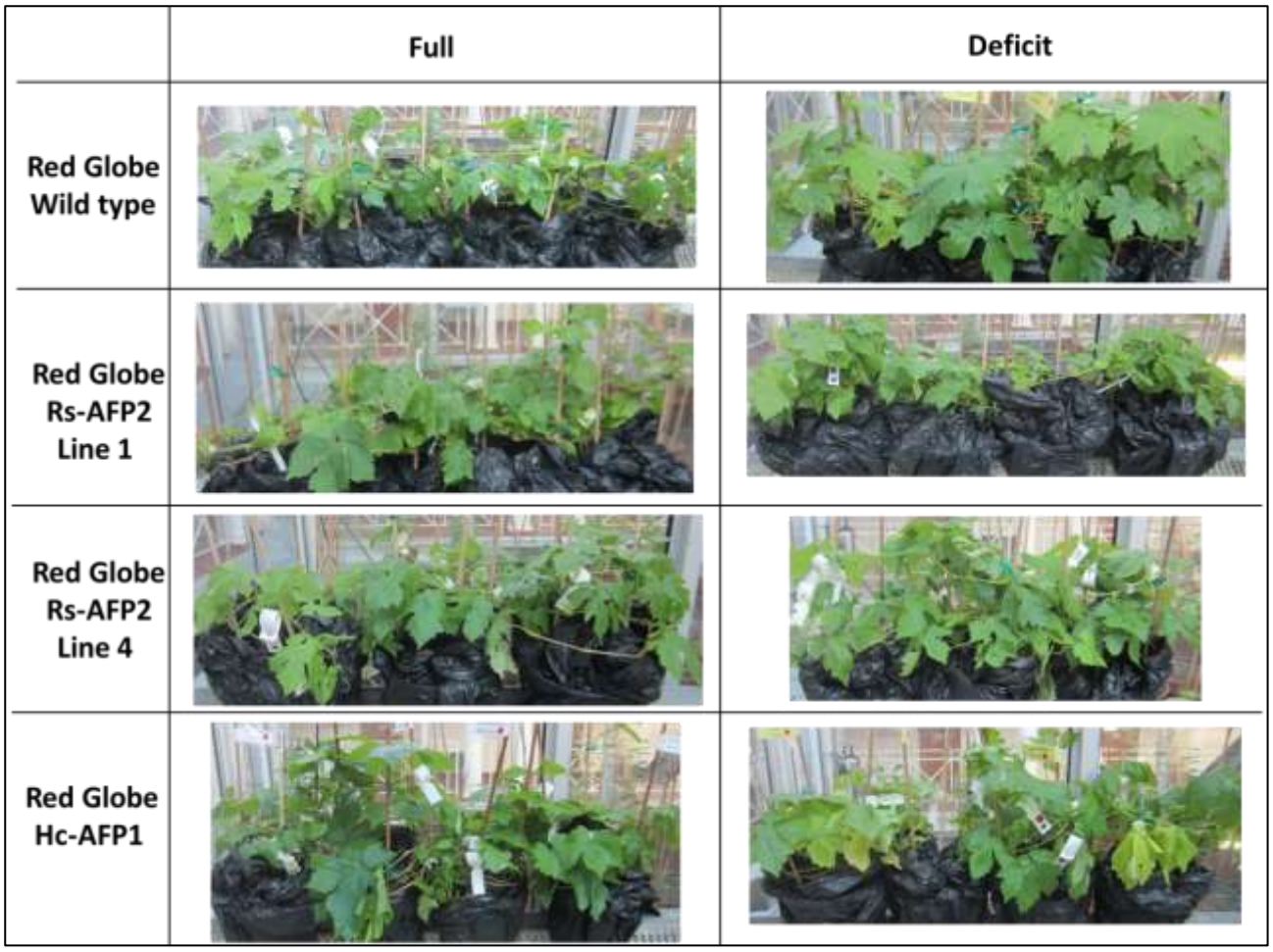

Figure B6.6 Pictures of Red Globe wild type and transgenic plants exposed to well-watered and active drying treatments in greenhouse conditions at the end of the active drying experiment.

Chapter 7

General discussion and conclusions

GENERAL DISCUSSION AND CONCLUSIONS

7.1 The scope of the study

Grapevine is highly susceptible to pathogens and pests due to, amongst others, domestication, vegetative propagation practices and human selection over centuries for quality rather than resistance traits. Currently grapevine production practices require the frequent use of preventative spraying programs, negatively impacting environments and adding to production costs (Wilson and Tisdell 2000; Aktar et al. 2009; Carvalho 2017). Finding alternative strategies to limit the use of fungicides and improve the resistance of grapevine is imperative. The study of plants' natural highly sophisticated defence mechanisms are therefore important to devise rational protection strategies (Estruch et al. 1997; Broglie et al. 2016). A vital component of the innate immunity of plants is plant defensin peptides. These peptides are small, cysteine rich antimicrobial peptides that form part of a large family of antimicrobial peptides and are widely distributed throughout the plant kingdom (Broekaert et al. 1995). Plant defensins have been studied in numerous plant species (Thomma et al. 2002) and have become known for their potent antifungal activities from peptide isolation and *in vitro* characterisation studies. Several other biological activities, such as heavy metal tolerance, ion channel blocking, α-amylase and protease inhibitors and modulators of growth and development have also been linked to defensins (Tregear et al. 2002; Allen et al. 2008; Stotz et al. 2009; Amien et al. 2010). A review of the literature confirms that defensin peptides have been extensively studied in terms of their structure, antimicrobial *in vitro* functions/activities, their modes of action, and potential applications, but much less information exists of their broader potential impacts on plant growth and development and particularly non-defence related stress-mitigating functions of these peptides within their host plants.

The goal of this study was therefore to study plant defensins for their *in planta* functions; the approach was to perform functional characterisation studies of plant defensins overexpressed in grapevine. In grapevine, 79 defensin-like genes (DEFL) were identified in the reference genome (Giacomelli et al. 2012), however, only four grapevine plant defensins have been isolated and characterised to date (De Beer and Vivier 2008; Du Plessis 2012; Giacomelli et al. 2012; Nanni et al. 2014). These promising, but limiting data for defensins in grapevine, specifically with regards to their involvement in growth and/or abiotic and biotic stress phenomena, as shown with other defensins, prompted this study. Moreover, very little comprehensive phenotyping has occurred on plants manipulated to express defensins and the materials available to this study, such as several transgenic grapevine lines overexpressing different defensin peptides, provided an important resource to explore potential defensin phenotypes in grapevine.

7.2 Characterisation of the plant resources available to this study:

Uncharacterised transgenic populations of two *V. vinifera* cultivars (Sultana and Red Globe), overexpressing four different defensin peptides (*Heliophilia coronopifolia* antifungal peptides 1 and 4 (Hc-AFP1, Hc-AFP4) and *Raphanus sativa* antifungal peptide 2 (Rs-AFP2), as well as a previously characterised population of Vvi-AMP1 overexpressed in Sultana, and the relevant controls, were available to this study. Genotypical screens were conducted on the primary (uncharacterised) transgenic populations to assess transgene integration and expression before plants were selected for growth, as well as biotic and abiotic stress phenotypical analysis in the transgenic populations. These analyses revealed unique transgenic lines for every transgenic population with the majority of these lines expressing the transgene (these became part of the panel of lines used for the growth and stress phenotype analyses). The transgenic *V. vinifera* (cv. Sultana) Hc-AFP4 and *V. vinifera* (cv. Red Globe) Hc-AFP4 lines, however did not show any transgene expression, even though the presence of the transgene was confirmed. The reasons for this apparent transgene silencing were however not further investigated and the transgenic Hc-AFP4 populations were not used for any further *in planta* analysis. The characterisation also did not include any analysis on the protein level in this study, so the actual protein levels or stability of the peptides within the plants were not studied further and could be pursued further following the extensive phenotypical screens conducted on the populations in this study.

7.3 Major findings from the phenotypical characterisation studies, including a critical evaluation, to contextualise the results obtained

In silico analysis of grapevine plant defensins provided insight into the native expression patterns of DEFL genes in the grapevine host under normal and stressed conditions.

In order to have a framework of the native expression patterns of DEFL genes of the host, an *in silico* analysis was conducted. The Corvina gene atlas data was used to evaluate the expression of DEFL genes (Fasoli et al. 2012); under normal non-stressed conditions, DEFL genes were expressed in all grapevine tissues at various developmental stages. This was in accordance with what we know about plant defensin expression as these peptides are normally constitutively expressed (to participate in the innate defence system of plants) and this expression can be induced upon infection or mechanical wounding (Broekaert et al. 1995). Furthermore the expression of the grapevine DEFL genes were evaluated in response to various stresses that included biotic stresses, such as *B. cinerea* infection and *Planococcus ficus* (mealybug) infestation, as well as abiotic stresses, namely leaf dehydration and local heat stress. Several of the DEFL, Snakin and Vvi-AMPs genes were upregulated in response to a challenge with mealybugs, whereas only the DEFL and Snakin genes were upregulated in response to the *B. cinerea* infection. Furthermore, several of the DEFL, Snakin and Vvi-AMPs genes were upregulated in response to locally applied heat stress and leaf dehydration, with Vvi-AMP1 showing the strongest

upregulation. Interestingly, the grapevine Snakin genes showed expression in all tissues tested under non stress conditions as well as all the stressed conditions evaluated. Not much is known about grapevine Snakins and none have been isolated to date. Some Snakin peptides have been isolated form potato and tomato and characterized and shown to have a broad range of antimicrobial activity towards several fungal pathogens as well Gram-positive and Gram-negative bacteria (De Souza Cândido et al. 2014). From the information available on these peptides, the constitutive expression patterns as well as the increased expression of these peptides to the various biotic and abiotic stresses evaluated, the grapevine Snakin gene family show promising potential and should be further characterized.

Our *in* silico analysis was conducted with experiments conducted on the NimbleGen microarray © 2011 Roche (NimbleGen, Inc, Madison, WI, USA) based on the V1 gene prediction by CRIBI of the 12x grapevine genome assembly by the French-Italian consortium (Jaillon et al. 2007). One of the major limitations of this *in silico* analysis was the mapping of the DEFL genes to the V1 transcriptome, as not all of the genes delivered a satisfying hit and others mapped to the same or multiple VIT identities. As a result the expression of some genes had to be evaluated as a group and the individual expression patterns of these genes could not be determined. It also did not include data form RNA sequence analysis. This excluded several valuable datasets generated in the recent few years from our analysis. The *in silico* analysis was conducted at the start of the study to give direction to the stress phenotyping and was useful to contextualize the expression patterns of the native grapevine DEFL genes in different grapevine organs expressed under normal plant growth and development and non-stressed conditions (Fasoli et al. 2012). There is thus definitely scope for a more comprehensive analysis of expression patterns of DEFLs, using all the available data from RNA sequencing as well. Our limited analysis however directed our phenotyping experiments to include induced stresses from fungi, mealybug infestation as well as water stress.

The overexpression of the plant defensins lead to multiple phenotypes in grapevine, supporting data that defensins have roles in growth and several stress responses *in planta*

Guided by the results of the initial *in silico* analysis, the *in vivo* functions of plant defensin peptides in grapevine were evaluated by analysis of the transgenic grapevines, overexpressing plant defensin peptides Hc-AFP1, Rs-AFP2 and Vvi-AMP1 in terms of growth, as well as biotic and abiotic stress. The main findings of these analyses is summarised in Figure 7.1. The contrast between the two genetic backgrounds (comparing the inherent differences between WT Sultana and Red Globe), as well as the phenotypes obtained for each peptide in either of the genetic backgrounds of the transgenic populations are shown (except for VviAMP1, which was only available for analysis in Sultana).

Two general observations became evident from these phenotypical analyses: The first was that the genotypical background (Sultana versus Red Globe) had a strong effect on several of the phenotypes observed. First, it was evident that the transgenic populations of *V. vinifera* cv. Red Globe were more impacted in terms of growth than the Sultana populations (also refer to Figure 4.11 in Chapter 4 for a more comprehensive summary). Another example was seen in the water stress analysis where the Rs-AFP2 peptide lead to improved intrinsic WUE in the Sultana background, but with an opposite reaction in the Red Globe population. Second, some of the phenotypes were peptide-specific, whereas in most other instances all peptides caused the same type of response to a particular stress, but with varying strength of the response, or some differences in mechanism (particularly the responses against the powdery mildew pathogen).The main findings with regards to the different phenotypes observed will be discussed below and summarised in Figure 7.1.

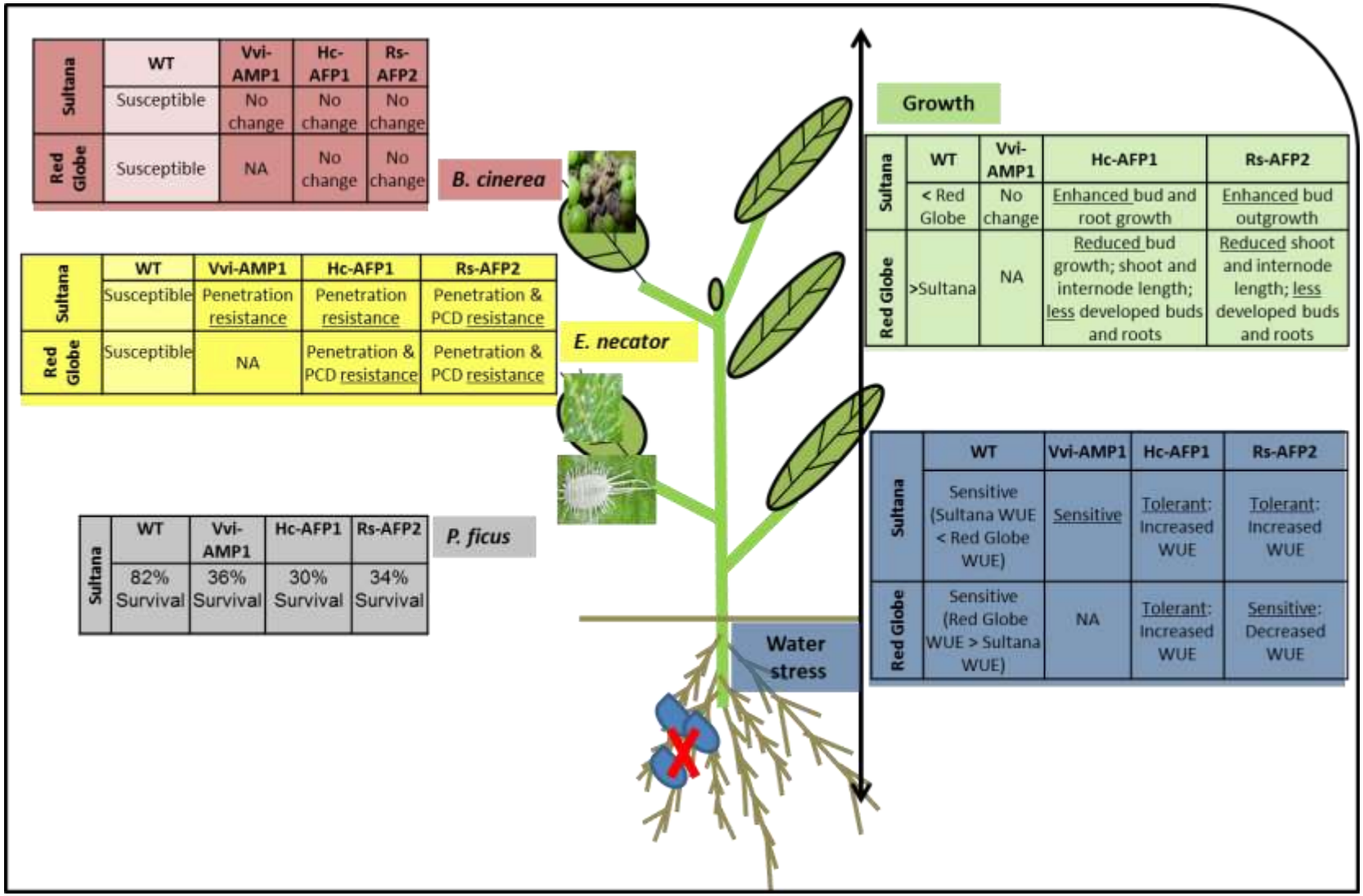

Figure 7.1. Summary of the phenotypic characterization of the Sultana and Red Globe transgenic populations expressing Hc-AFP1, Rs-AFP2 and Vvi-AMP1 peptides under non-stressed and stressed (biotic and abiotic) conditions and in comparison with the respective untransformed controls.

Growth phenotypes: The growth phenotypes observed were mild and no severe stunting or abnormalities were observed, although some slower growth and root inhibition was observed *in vitro*. Rs-AFP2 has been shown to impact root growth in *Arabidopsis,* as well as this study (Allen et al. 2008; Parisi et al. 2018) and this inhibition is likely due to intrinsic features of the peptide itself. Slower growth of the aerial parts was also observed for several of the peptides, but particularly for the Rs-AFP2 and Hc-AFP1 peptides in the transgenic *V. vinifera* cv. Red Globe populations with delayed auxiliary outgrowth; less developed buds and shorted shoot length. It is possible that these observed growth alterations could have been the result of a higher metabolic load on the plants due to the overexpression of the peptides at all times and in all organs, leading to mild fitness cost due to upregulated resistance mechanisms. It is well known that resistance genes can reduce the growth performance of a plant in order to enhance the resistance (Zeller et al. 2013; Li et al. 2018) and this has also been reported for some plant defensins, namely the tomato defensin, Def2 and the *Nicotiana alata* defensin 1 (NaD1) (Anderson et al. 2009; Stotz et al. 2009). The transgenic populations also all had lower photosynthetic CO_2 assimilation rates under non-stressed conditions, which could indicate that the plants have indeed prioritised defence, with some small fitness costs. This aspect could be examined in further work to assess the populations' metabolic state in terms of activated defence systems and a more broader calculation of potential fitness cost. The plants analysed were all in a juvenile growth stage and therefore analysis on reproductive organs were not conducted, but should form part of a more comprehensive analysis of mature plants, particularly since defensins have been shown previously to impact reproductive organs and processes (Amien et al. 2010; Nanni et al. 2014).

Antifungal phenotypes:

Against *Botrytis*: The populations were infected by two strains of the necrotrophic *Botrytis cinerea*, as well as the powdery mildew pathogen *Erysiphe necator*. The transgenic lines showed some initial suppression of *Botrytis* during the early infection periods, but ultimately, the disease symptoms on the transgenic and control plants were similar as the infections spread and developed. The *in planta* susceptibility phenotypes to *Botrytis* is in contrast to the strong antifungal activity of the three tested plant defensin peptides towards the necrotrophic fungus *B. cinerea in vitro* (De Beer and Vivier 2011; Barkhuizen 2013). It is possible that the peptide quantities in the transgenic populations were not high enough as the reported IC_{50} values towards *B. cinerea* of Rs-AFP2, Vvi-AMP1 and Hc-AFP1 were 2 µg/mL, 13 µg/mL and above 25 µg/mL, respectively. This aspect however needs further study since we did not quantify and evaluate the expressed peptide levels and stability *in vitro*. Moreover, the infection assays were conducted under conditions that would favour the pathogen (high humidity and very high spore loads inoculated at several spots per leaf) and the peptide levels could possibly not be high enough to control the pathogen under these conditions at such localised infections spots. Defensins are known to be secreted to plant surfaces, typically occurring in the apoplast (Osborn et al. 1995; Terras et al. 1995; Broekaert et al.

1995; Lay and Anderson 2005; Oomen et al. 2011), but it is possible that the peptide levels at the specific infection spots were not high enough to effectively inhibit all *Botrytis* spores and hyphae. Most defence responses towards *Botrytis* involve defence responses on the local level (creating physical barriers to limit entry) and subsequent defence signalling. Interestingly, the *in silico* analysis did not show any response of Vvi-AMP1 towards *B. cinerea* infection (only some DEFL and Snakin genes responded), indicating that these peptides might not form part of the natural *in planta* defence response towards *B. cinerea* in grapevine.

Another possibility is that the stability and activity of the peptides were affected by the conditions in the cells under attack. In order for *B. cinerea* to establish infection it modulates its host by the secretion of lytic enzymes to cross the epithelial wall and establish a primary lesion. In order for the fungus to consume the infected plant cells, it secretes cell wall degrading enzymes, toxins and oxalic acid (Armijo et al. 2016). This acidification of *Botrytis* at the infection sites could possibly inactivate the plant defensins, since these peptides have optimal pH ranges of between 4.0 and 10.0 (De Oliveira Carvalho and Gomes 2011). Furthermore, the degradation of the plant cells at the infection site would lead to the release of cations that are known to antagonise the biological activity of plant defensin peptides (Broekaert et al. 1995; Thevissen et al. 1999; Aerts et al. 2008; De Oliveira Carvalho and Gomes 2011; Vriens et al. 2016). Previous work in our own group has shown that the Hc-AFP1 peptide is strongly antagonised by divalent cations, with significant impacts on peptide stability and function (Barkhuizen 2013).

Furthermore, some of these peptides, specifically Rs-AFP2, is known to cause reactive oxygen species (ROS) and subsequent programmed cell death as part of their antifungal mode of action (Aerts et al. 2007; Cools et al. 2017; Parisi et al. 2018). This mode of action would however be favouring the pathogen as the necrotrophic fungus will thrive on the dead tissue generated by this programmed cell death, further explaining the susceptible phenotypes observed (Heath 2000).

Against *Erysiphe necator*: In contrast to the generic susceptibility towards the necrotrophic *Botrytis*, all the transgenic lines showed enhanced resistance towards the biotrophic powdery mildew fungus. Microscopic analysis of leaves being infected, showed that the fungal infection structures on transgenic lines were directly affected, showing signs of tip swelling and other abnormalities that are characteristic features of peptide action and ultimately leading to unsuccessful or poor penetration. Using the classification system of Feechan et al., (2011), it was confirmed that the transgenic lines were increased in resistance against the pathogen. This resistance was achieved through increased penetration resistance whereas some lines also displayed programmed cell death (PCD) associated resistance. This overall enhanced penetration resistance observed for all the plant lines lead to a significant reduction of the fungal feeding structures and limited colonisation of the tissue, development of secondary hyphae and dramatic reductions in spore load at the end of the incubation period. This penetration resistance forms part of the pathogen-

associated molecular patterns (PAMP)-triggered immunity (PTI) of plants. A gene associated with penetration resistance has been identified in grapevine, namely *V. vinifera* penetration 1 (VviPEN1) (Feechan et al. 2013; Qiu et al. 2015). This gene will be a useful target for future studies for a deeper evaluation of the observed enhanced penetration resistance of these lines since it was also observed for one non-expressing line and it would help determine if the enhanced penetration resistance observed for all these transgenic lines is a result of the integration of the defensin transgene within the genome. Furthermore, some plant lines presented slight increases in PCD, especially the transgenic plants that contained the Rs-AFP2 construct. This resistance mechanism is associated with the effector triggered immunity (ETI) and resistance genes that that have only been identified and described in non-*vinifera* species. As mentioned above we know that PCD is part of the mechanism of action of the Rs-AFP2 peptide suggesting that this mechanism is associated with the presence of the defensin peptide (Thevissen et al. 1999; Cools et al. 2017; Parisi et al. 2018). Rs-AFP2 is one of the most well-studied defensins, also in terms of mode-of action, whereas the other peptides that form part of this study have not been subjected to the same level of study. The similarity in phenotypes observed, provide hints that the other peptides might also use ROS production as part of their mode-of actions, an aspect that could form part of further study on these peptides.

Anti-insect phenotypes: The transgenic grapevine plant lines also showed promising results towards the soft scale insect *Planococcus ficus.* This is the first report of an *in planta* anti-insect activity of plant defensins Hc-AFP1, Rs-AFP2 and Vv-AMP1 and also the first report of plant defensins conferring enhanced resistance towards insects in grapevine.

Anti-insect activity has been reported as a biological function of several plant defensins and is achieved by their ability to inhibit the α-amylase activity of the insect gut, through the inhibition of proteases or protein translation, or by blocking ion channels (Mendez et al. 1990; Bloch and Richardson 1991; Kushmerick et al. 1998; Wijaya et al. 2000; Pelegrini et al. 2008; Carvalho and Gomes 2009). The phenotyping results provide scope for future studies to evaluate the mechanism of this observed anti-insect activity. The soft scale insects that are not only responsible for early leaf loss and weakened vines, but also for spread of the Grapevine leaf role virus 3 (GvRL3) (Walton and Pringle 2004; Daane et al. 2012; Almeida et al. 2013) and possibly other viruses.

Water-stress phenotypes: Guided by the results of our *in silico* analysis, as well as previous *in silico* and transcriptomic analyses (Do et al. 2004; Du Plessis 2012) plant lines were subjected to an active drying experiment in order to establish a more direct link of the role between plant defensins and abiotic stress. Some of these plant lines showed promising results for drought stress in grapevine as the Sultana Hc-AFP1 and Rs-AFP2 transgenic plant lines, as well as the Red Globe Hc-AFP1 transgenic plant lines all demonstrated increased intrinsic water use efficiency (WUE) in the active drying experiment. This response was most likely the result of rapid stomatal

closure observed for these plant lines, as stomatal conductance is the earliest response to drought stress even at mild drought stress conditions (Flexas and Medrano 2002; Medrano et al. 2002; Zúñiga et al. 2018). In contrast, the Red Globe Rs-AFP2 plant line showed a decrease in intrinsic WUE. This could possibly be linked to the reduced growth parameters demonstrated for this plant line, especially root growth. The reduced root growth could have reduced the efficiency of the plant to utilise the available soil water. Future studies will include further evaluation on how these peptides induce the stomatal conductance response observed in grapevine and will also focus on confirming the increased intrinsic water use efficiency (WUE) results by calculating the WUE where the biomass of the plants are also brought into the evaluation (this was not possible in the initial screening of the plant lines). Furthermore, future study will also focus on confirming the possible link between the decreased intrinsic WUE and reduced growth parameters of the Red Globe Rs-AFP2 plant line.

The increased intrinsic water use efficiency (WUE) results holds great potential for drought stress improvement and water management in grapevine. More importantly, these results established a direct link between plant defensins and abiotic stress in terms of drought stress. This is the first direct link that has been made between plant defensins as previous reports only included transcriptomic analysis. Overall these results indicate that plant defensin peptides have multiple *in planta* functions in grapevines and plants and contribute to several stress protection strategies of plants.

7.4 Conclusions and future perspectives

In conclusion, this study satisfied the aims set out in the beginning of the project and delivered a comprehensive characterisation of grapevine plants overexpressing several defensins. The overarching aim to evaluate possible *in vivo* functions of plant defensins could confirm growth impacts, antifungal activities, anti-insect activity and a role in water stress management. To our knowledge, the research described in this thesis demonstrated the first experimental link that these peptides might have functional roles in abiotic stress. Furthermore, the results generated further confirmed that plant defensin peptides are multifunctional peptides that have roles in growth and development as well as biotic and abiotic stress.

Taken together the results of this study contribute to the current understanding of how plant defensins function *in vivo* and makes an important contribution to defensin research in general. The novel results also provided vital insight into the *in vivo* functions of grapevine plant defensin peptides that will be instrumental for future studies. For example, a deeper evaluation of the underlying mechanisms involved of how exactly these plant defensin peptides exert or contribute to these established *in vivo* functions can now proceed. The characterised transgenic populations provide an important resource for these studies towards the characterization and elucidation of these underlying mechanisms. Defensin peptides, as part of the innate defence systems of plants

warrant research attention and in grapevine there is still significant scope to study and understand the peptides further.

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