ABBREVIATIONS AND SYMBOLS

ABA	Abscisic acid
ABRE	Abscisic acid-responsive-element
ASR	Abscisic acid stress ripening
α	Alpha
ACC	Aminocyclopropane-1-carboxylic acid
ACO	Aminocyclopropane-1-carboxylic acid oxidase
ACS	Aminocyclopropane-1-carboxylic acid synthase
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
arg1	Arginine biosynthesis gene
APX	Ascorbate peroxidase
Avr	Avirulence
β	Beta
bHLH	Basic helix-loop-helix
BLSD	Black leaf streak disease
BLAST	Basic Local Alignment Search Tool
BLASTx	BLAST algorithm to compare the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.
bp	Base pairs
bZIP	Basic-domain leucine zipper
C4H	Cinnamate 4-mono-oxygenase/cinnamate 4-hydroxylase
Ca^{2+}	Calcium (II) ions
CAT	Catalase
CAV	Culture collection of Altus Viljoen
$Ca(NO_3)_2.H_20$	Calcium nitrate
°C	Degrees Celsius
CBF	C-repeat binding factor
CDPK	Calcium-dependant protein kinase
cDNA	complementary Deoxyribonucleic Acid
cDNA-AFLP	cDNA-amplified fragment length polymorphism

clc	Chloride channel
chsV	Chitinase class V
COR	Cold-responsive
CRISP	Cysteine-rich secretory protein-1 precursor
CRT	C-repeats
Ct	Cycle number at which the fluorescence signal crosses a fixed threshold
CTAB	Cetyl trimethyl ammonium bromide
cv	Cultivar
CWDE	Cell wall degrading enzymes
DAG	Diacylglycerol
Dpi	Days post inoculation
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DON	Deoxynivalenol
DRE	Dehydration responsive elements
DREB	Dehydration responsive element binding
ERD	Early-dehydration inducible
ERF1	Ethylene response factor 1
EST	Expressed sequence tags
ET	Ethylene
ETI	Effector-triggered immunity
E-value	Expect value
FABI	Forestry and Agricultural Biotechnology Institute
fga1	G protein α subunit
fgb1	G protein b subunit
FHIA	Fundación Hondurereña de Investigacón Agrícola
f. sp.	Formae speciales
Foc	*Fusarium oxysporum* f. sp. *cubense*
Foc STR4	*Fusarium oxysporum* f. sp. *cubense* ‘subtropical’ race 4
Foc TR4	*Fusarium oxysporum* f. sp. *cubense* ‘tropical’ race 4
Fol	*Fusarium oxysporum* f.sp. *lycopersici*
fost12	*Fusarium oxysporum* serine/threonine protein kinase homolog

fow1	Mitochondrial protein
fow2	$Zn(II)_2Cys_6$-type transcription regulator
fmk1	*Fusarium* mitogen-activated protein kinase
frp	F-box protein required for pathogenicity
g	Gram
G6DH	Glucose-6-phosphate 1-dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate
g/L	Gram per litre
GMO	Genetically modified organism
h	Hour
Hin1	Harpin inducing protein 1
HL	Human lysozyme
Hpi	Hours post inoculation
H_2O	Water
H_2O_2	Hydrogen peroxide
HR	Hypersensitive Response
hrs	Hours
ICE	MYC-like basic helix–loop–helix transcriptional activator
IDH	Isocitrate dehydrogenase
INIBAP	International network for the improvement of banana and plantain
IP_3	1,4,5-trisphosphate
JA	Jasmonic acid
KIN	Cold inducible
lcc	Laccase
LEA	Late embryogenesis abundant proteins
LRR	Leucine-rich repeats
LTI	Low-temperature induced
M	Molarity
MAPK	Mitogen-activated protein kinases
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MeJA	Methyl jasmonate

MFS	Major facilitator superfamily
min	Minutes
mg	Milligrams
MgCl2	Magnesium chloride
ml	Millilitre
mM	Millimolar
MM	Minimal medium
mRNA	messenger Ribonucleic Acid
MT	Metallothionein
N_2	Nitrogen
NAD^+	Nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
NDR1	Non-race-specific disease resistance gene
ηg	Nanogram
ηm	Nanometre
NR	Non-redundant
PA	Phosphatidic acid
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
pflp	Plant ferredoxin-like protein
pl1	Pectate lyase
pg1	Endo-polygalacturonase
pgx4	Exo-polygalacturonase
pH	Log hydrogen ion concentration
PIP2	Phosphotidylinositol 4,5-bisphosphate
PLA2	Phospolipase A2
PLC	Phosphoinositide phospholipase C
PLD	Phospolipase D
PR	Pathogenesis-related
PRR	Pattern recognition receptors
PTI	Pathogen-associated molecular patterns triggered immunity

qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
QTL	Quantitative trait locus
R	Resistance
R_2	Correlation coefficient
RD	Responsive to desiccation
rhoI	GTPase activating protein
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNA	ribosomal Ribonucleic Acid
RT	Reverse transcriptase
SA	Salicylic acid
siRNA	Small interfering RNA
six	Secreted in the xylem
SNAC2	Stress-responsive NAC 2
snf	Sucrose non-fermenting
SOD	Superoxide dismutase
spp	Species
ste12	Serine/threonine protein kinase
TEF	Elongation factor 1α
TDF	Transcript derived fragments
tlp	Thaumatin-like protein
TUB	ß-tubulin
µg	Micrgram
µl	Microlitre
µM	Micromolar
VCG	Vegetative Compatibility Group
wai	Weeks after infection
WDS	Water deficit stress
Zn finger	Zinc finger
%	Percentage
Σ	Sum of

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CHAPTER 1

THE EFFECT OF COLD STRESS ON PLANT DISEASE DEVELOPMENT, WITH SPECIAL REFERENCE TO FUSARIUM WILT OF BANANA

INTRODUCTION

The environment has a significant impact on crop productivity. Abiotic stresses, such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity affect growth and yield of agricultural crops, and can account for more than 50% of total yield losses (Wang *et al.*, 2003). During unfavourable conditions, which affect normal growth, plants are predisposed to infection by plant pathogens (Agrios, 2005). Predisposition involves non-genetic factors that increase the vulnerability of plants to diseases (Schoeneweiss, 1975). Temperature is considered one of the most important predisposing factors (Colhoun, 1973), and has phenotypic, physiological and molecular effects on all plant species.

Banana (*Musa* spp. L.) is one of the most important food crops, with production worldwide of around 86 million tons (FAOSTAT, 2010). With two billion people suffering of hunger, the banana is a vital staple food for 400 million people in the tropics (Ammar-Khodja, 2000; Sundaram, 2010). Banana is versatile and can be planted in backyards or in large plantations, and can serve as a cash crop for poor farmers (Robinson, 1996). Cooking bananas have a starchy content and can be cooked, fried or roasted and served as a primary food source with a high concentration of vitamins A, B1, B2 and C (Sharrock and Lusty, 2000). Sweet dessert bananas are consumed as a flavoured fruit by many people around the world and are exported to various countries. Other uses for banana include flour, juice, chips and beer (Sharrock, 1997; Ammar-Khodja, 2000).

Production of bananas is often affected by biotic and abiotic stresses (Wairegi *et al.*, 2010). Fusarium wilt is known as a significant biotic stress, and resulted in one of the most devastating plant disease epidemics in agricultural history, because of its destruction of almost 40,000 ha of Gros Michel plantations in Central America in the 1960's (Ploetz and Pegg, 2000). To rescue the export banana industry, Gros Michel bananas were replaced by Cavendish cultivars. However, Cavendish bananas have succumbed to a variant of the Fusarium wilt pathogen in the subtropics with increased disease severity following cold predisposition (Ploetz, 2006).

Banana, a tropical plant, can be severely affected by low temperature. Low temperatures damage leaves that cause a decrease in yield, and also predisposes the plant to biotic constraints such as Fusarium wilt (Ploetz, 2006). Understanding the impact of environmental conditions on disease incidence is important to prevent serious economic problems. In this review, the response of plants to cold stress will be discussed, particularly where it contributes as a predisposing factor to plant diseases. A brief background of banana as a major staple food will be provided, and how the crop is affected by abiotic stresses. The role of cold stress in Fusarium wilt development will then be discussed, and how transgenic approaches can be used to increase resistance in banana to Fusarium wilt and factors such as cold stress.

COLD STRESS IN PLANTS

Plants are distributed geographically around the world according to their temperature threshold (Margesin *et al.*, 2007). In some instances, plants are able to increase their tolerance to freezing temperatures to survive in marginal environments; a process called cold acclimation (Ruelland *et al.*, 2009). Tropical plants, like banana, cucumber, mango, tomato and maize, however, are unlikely to acclimatize to freezing temperatures and are, therefore, more sensitive than other plants when exposed to cold temperatures (Lyons, 1973). Cold stress of plants can be defined as low temperatures that affect the plant's metabolism, growth and development, thereby resulting in a plant not functioning optimally (Rabbani *et al.*, 2003). The effect of cold stress on plants is influenced by the plant species, intensity and duration of cold temperatures, developmental stage of the plant and the part of the plant subjected to the low temperatures (Winfield *et al.*, 2010). In the following section, the phenotypic, physiological, biochemical and molecular responses to cold stress will be discussed.

Phenotypic effect of low temperature

Phenotypic symptoms of cold stress differ between plants, but the most common symptoms include wilting, chlorosis, necrosis, restriction in leaf expansion and reduced growth and development (Lyons, 1973; Wilkinson *et al.*, 2001; Mahajan and Tuteja, 2005). Wilting is caused by reduced water uptake through the roots while

stomata are continuously open (Wilkinson *et al.*, 2001; Bloom *et al.*, 2004), as well as a reduction in water flow through the phloem vessels (Strand *et al.*, 1999; Stitt and Hurry, 2002). Chilling-tolerant plants have a greater ability to close their stomatal openings which reduces water loss and wilting (Wilkinson *et al.*, 2001; Bloom *et al.*, 2004). Chlorosis (yellowing of the leaves) is a result of inhibition of photosynthesis. Low temperatures lead to photodamage as the utilization of ATP and NADPH by the Calvin cycle is reduced as well as changes in conformation of D1 in photosystem II (Campbell *et al.*, 2007). As ice crystals form in the intercellular spaces, water-soaked lesions appear that progress to necrotic lesions. The leaf expansion and reduction in the growth of plants at lower temperature is due to the decline in cell cycle rate (Rymen *et al.*, 2007).

Banana leaves subjected to cold stress turn yellow in colour, which reduces the photosynthesis ability (Fig. 1.1). There is also a decline in growth rate leading to extended length of the growth cycle. Frost damage initiates water-soaked lesions on the leaves which turn brown and eventually die. Leaf bleaching occurs as a result of repeated cold and radiation cycles, as found in subtropical winter conditions (Israeli and Lahav, 2000). Low temperature further extends the time between flowering and harvest, fruit filling and significantly lowers the yield.

Physiological and biochemical responses of plants to lower temperatures

The plant cell membrane is at the forefront of freezing injury. Cold conditions disrupt the membrane integrity of the plant which causes leakage and leads to cellular dehydration. Upon exposure to cooler temperatures, the plant increases the unsaturated fatty acid content (Nishida and Murata, 1996) and this leads to more fluidity of the membrane accompanied by higher freezing tolerance (De Palma *et al.*, 2008; Kargiotidou *et al.*, 2008). After banana fruit (cvs. Gros Michel and Namwa) was dipped in hot water at 42^{o}C for 15 min and transferred to cold storage conditions at 4^{o}C, blackening of the fruit was reduced with an increase in unsaturated fatty acids (Promyou *et al.*, 2008). The increase in unsaturated fatty acids may contribute to changes in the viscosity of the plasma membrane, thereby protecting the banana fruit against the cold damage.

The ultra-structural changes in plants during chilling injury include degeneration of the chloroplast, enlargement of the thylakoid, formation of peripheral reticulum and a reduction in the number of starch granules (Kratsch and Wise, 2000). During cold acclimation, winter oil-seed rape plants showed an increase in pectin content in the cell structure (Solecka *et al.*, 2008). With the increase in pectin esterase, there is an increase in cell wall stiffness and cell wall rigidity to minimize intracellular freezing (Pelloux *et al.*, 2007; Solecka *et al.*, 2008).

Plants can also protect themselves from freezing conditions by accumulating osmoprotectants to osmotically significant levels without disrupting plant metabolism (Chinnusamy *et al.*, 2007; Margesin *et al.*, 2007). These solutes include proline, sucrose, polyols, trehalose and quaternary ammonium compounds such as glycine betaine and other amines, with proline and glycine betaine being the most studied. There are two possible roles for osmoprotectants. It firstly raises the osmotic potential, thereby restricting the movement of water to intercellular spaces and, secondly, it protects the macromolecular structures and/or membranes against low temperatures (Holmberg and Bülow, 1998).

Accumulation of proline is a widespread phenomenon in plants following abiotic stress (Kishor *et al.*, 2005). Overexpression of *CBF3*, a cold-induced transcription factor, increased the proline levels between six and 15 times in transgenic rice (*Oryza sativa* L.) and further increased the tolerance to drought, high salinity and low temperature stresses (Ito *et al.*, 2006). Mutation in the *esk1* gene in *Arabidopsis* sp. (L.) Heynh. also increased the concentration of free proline 30-fold and lead to higher freeze-tolerant plants after cold acclimatization at 4°C for two days (Xin and Browse, 1998). In the case of banana, Jiezhong *et al.* (1999) showed that banana cultivars differ in response to cold stress. Banana cultivar (cv.) Xiangjiao (*Musa* sp., AAA) had higher levels of free and soluble proline compared to cv. Dajiao (*Musa* sp., ABB) (Jiezhong *et al.*, 1999). However, cv. Dajiao showed a higher increased rate of free and soluble proline levels compared to cv. Xiangjiao after the plants were transferred to a temperature of 1^{o}C (Jiezhong *et al.*, 1999).

Another osmoprotectant, glycine betaine, plays a major role in cell protection after plant cells have been exposed to cold temperature (Ashraf and Foolad, 2007; Chen and Murata, 2008). Glycine betaine protects plants against cold damage by the increase of unsaturated fatty acids in the thylakoid membrane as well as protecting protein complexes such as ATPase and violaxanthin de-epoxidase (Wang *et al.*, 2008a). An increase in cold tolerance can be achieved through exogenous application of glycine betaine or through genetic modification by incorporating and expressing glycine betaine in plants. By applying glycine betaine and chitosan exogenously at 5 mol/L and 0.3% respectively, increased cold tolerance in banana seedlings was reported (Li *et al.*, 2007; Li *et al.*, 2008). The authors indicated that betaine and chitosan enhanced the cold resistance of banana seedlings by increasing superoxide dismutase (SOD), preventing oxidation of the cell membrane and increasing malondialdehyde (Li *et al.*, 2007). Transformation of sweet potato with a gene overexpressing betaine aldehyde dehydrogenase led to increased tolerance to cold, salt and oxidative stress (Fan *et al.*, 2012).

Molecular responses of plants to lower temperatures

Visual symptoms of cold stress can only be detected after a few hours to a few days but in reality, plants respond to cold stress within minutes. Early responsive genes, which are induced within minutes to a few hours after the cold treatment, are usually involved in signalling responses and their increase in expression is only temporal (Thomashow, 1999). In comparison, genes induced later in the cold response often maintain their expression levels and translate to functional proteins such as late embryogenesis abundant proteins (LEA)-proteins, membrane stabilizing proteins and osmoprotectant synthesis-related proteins. Different classes of genes are activated in response to cold that are also linked with other abiotic stresses, namely cold-responsive (*COR*), low-temperature induced (*LTI*), cold inducible (*KIN*), responsive to desiccation (*RD*) and early-dehydration inducible (*ERD*) genes (Thomashow, 1999). As thousands of genes are up- or down-regulated due to cold stress, only the major groups of genes and proteins will be discussed (Fig. 1.2).

Genes and proteins involved in repair and protection against cell damage

Reactive oxygen species (ROS) form during stress conditions and act as signalling molecules in biotic and abiotic stress pathways in plants. Since ROS cause damage of cellular components and DNA by oxidation (Gechev *et al.*, 2006; Suzuki and Mittler, 2006), it is necessary for the plant to detoxify ROS to protect itself. Several enzymes such as SOD, ascorbate peroxidase (APX), catalase (CAT), monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase are formed to prevent oxidation (Iba, 2002). During the early response of *Arabidopsis* to low temperature, *CAT2* was up-regulated to catalyse the toxic hydrogen peroxide (H_2O_2) (Du *et al.*, 2008). Figueroa-Yanez *et al.* (2012) found an up-regulation of putative *MaCAT2* in the peel of banana fruit during a response to low temperature (10^{o}C). They hypothesized that *CAT* is involved in signal transduction in banana at low temperatures (Figueroa-Yanez *et al.*, 2012).

The cold stress tolerance of young banana plants (*Musa* sp. AAA Group cv. Williams) has previously been enhanced by means of exogenous salicylic acid (SA). Pretreatment of leaves and roots with SA one day before cold conditions (5^{o}C) increased SOD, CAT and APX, and inhibited H_2O_2 content in banana plants which aid in protecting the cells against ROS during cold stress (Kang *et al.*, 2003). Dipping banana fruit (*Musa* sp., AAA Group cv. Brazil) in hot water (52^{o}C) for 3 min resulted in an increase in cold stress tolerance at 7^{o}C (Wang *et al.*, 2008b). The increased tolerance was explained by the lower level of H_2O_2 and increased levels in CAT and APX content which plays a major role in preventing ROS damage (Wang *et al.*, 2008b). Furthermore, the catalase activity differs between banana genomic groups. The catalase activity in Aiba Dajiao (*Musa* sp. ABB Group) was higher compared to Dazhong Gaoba (*Musa* sp. AAA Group), which may explain its increased cold tolerance in the field (Wang and Liang, 1994). It is therefore believed that the regulation of SOD, CAT and APX levels in transgenic plants may allow for the generation of cold tolerant banana plants by protecting the plant against harmful ROS induced during cold stress.

Functional genes expressed under cold stress may also protect and repair plant cells damaged by the denaturation of proteins. These include heat shock proteins that may act as molecular chaperones and aid the folding of proteins (Vinocur and Altman, 2005). Heat shock proteins are up-regulated in several plant species after cold stress, including sunflower (Fernandez *et al.*, 2008), potato (Bagnaresi *et al.*, 2008) and field pennycress (Sharma *et al.*, 2007), but also following abiotic stresses such as salt (Baisakh *et al.*, 2006) and heat stress (Sung *et al.*, 2003). In the study by Santos *et al.* (2005), 27 different heat shock expressed sequence tags (ESTs) were identified in banana leaves after cold and heat temperature stress. These transcripts code for proteins that are located in the cytoplasm and chloroplast and protect other proteins against temperature damage (Santos *et al.*, 2005). Carpentier *et al.* (2007) found that heat shock proteins, like HSP60, HSP70 and HSP90, were regulated with different expression profiles in drought tolerant banana plants after osmotic stress. In the case of HSP60 and HSP70, both were up-regulated in high sucrose conditions (osmotic stress). Furthermore, Mbwazirume (*Musa* sp., AAA Group highland banana) showed higher levels of HSP60 than Cachaco (*Musa* sp., ABB Group cooking banana) with and without osmotic stress.

Proteins that serve as protection factors of macromolecules

Cold acclimation is associated with the up-regulation of LEA proteins in plants (Hong-Bo *et al.*, 2005; Hundertmark and Hincha, 2008). It is hypothesized that LEA perform several functions including stabilizing proteins and membranes under stress conditions by preventing aggregation of proteins (Tunnacliffe and Wise, 2007). They consist of a large group of mainly hydrophilic proteins, for example Cor15 and Cas15. These proteins are also up-regulated in response to other abiotic stress conditions like dehydration or application of abscisic acid (ABA) (Espelund *et al.*, 1992). In wheat, LEA proteins were up-regulated after one day of low temperature and reached a plateau after three to seven days (Kobayashi *et al.*, 2004). Furthermore, the level of expression of LEA proteins could be correlated with the cold tolerance of the wheat plants (Kobayashi *et al.*, 2004). *LEA* transcripts have recently been identified in banana after the release of the banana genome (D'Hont *et al.*, 2012). A keyword BLAST search from the annotated genome revealed that the genome contained 17

putative *LEA* transcripts including *LEA5* and *LEA14-A*. However, the role of these transcripts has not been investigated.

Proteins and genes involved in cellular metabolic processes

When plants are exposed to cold stress there is a dramatic increase in amylase activity (Kaplan *et al.*, 2006). β-amylase leads to the breakdown of starch, which results in increased accumulation of soluble sugars. With increased β-amylase, the maltose concentration is higher and it is hypothesized that this leads to the protection of the photosynthetic electron transport chain and proteins in chloroplast stroma during lower temperatures (Kaplan *et al.*, 2006). The increase of soluble sucrose has been demonstrated to enhance tolerance to low temperatures in the flavedo (coloured outer peel layer) of grapefruit (Maul *et al.*, 2008). Several other metabolic enzymes involved in the accumulation of soluble sugars in response to cold stress have been identified in plants, for example sucrose phosphate synthase (Guy *et al.*, 1992) and galactinol synthase (Taji *et al.*, 2002). Sucrose phosphate synthase led to the increase of sucrose, glucose, and fructose in spinach after cold stress (Guy *et al.*, 1992) whereas galactinol synthase led to the increase of galactinol and raffinose in *Arabidopsis thaliana* after cold stress (Taji *et al.*, 2002). The accumulation of high levels of soluble sugars helps the plant to enhance its cold tolerance (Kaplan *et al.*, 2006).

Respiration- and photosynthesis-related proteins and genes

Cold-tolerant plants react differently to low temperatures when compared to cold-sensitive plants regarding photosynthesis and respiration processes. A study by Yamori *et al.* (2009) showed that 11 cold-tolerant herbaceous plants maintained homeostasis of both photosynthesis and respiration better than the cold-sensitive plants after low temperature. With a decrease in temperature, proteins involved in energy metabolism such as photosynthesis proteins, chlorophyll-binding proteins and Rubisco are down-regulated (Hewezi *et al.*, 2006). Chilling-sensitive plants do not adjust to cold stress, whereas plants tolerant to cold stress increase soluble sugars as well as photosynthesis enzymes, which results in recovery from cold stress (Hewezi *et*

al., 2006). Therefore, plants with toleranance to cold stress may be generated by increasing the expression of soluble sugars and photosynthesis genes that will aid in the recovery after cold temperatures.

Water and ion movement genes and proteins

Aquaporins regulate the movement of water across the membrane by forming water-specific pores (Maurel *et al.*, 2008). As water freezes at temperatures below the freezing point, the water potential decreases and water moves to the intercellular spaces between plant cells. This results in water deficiency developing within the cell (Thomashow, 1999). With a need for water and ion regulation as well as protection against dehydration, several mechanisms such as aquaporin regulation in a plant must be controlled in response to freezing stress. Shortly after the onset of cold temperature, the majority of the plasma membrane aquaporins are down-regulated in *Arabidopsis* (tolerant to low temperature), and this plays a role in maintaining homeostasis and hydraulic conductivity (Jang *et al.*, 2004).

Dehydration responsive element binding (DREB) regulons

DREB regulons are transcription factors that play a prominent role in cold acclimation at the onset of cold stress, commonly found in monocotyledons as well as dicotyledons (Nakashima and Yamaguchi-Shinozaki, 2006). They belong to the APETALA2/ethylene-responsive element binding protein family of transcription factors (Nakashima and Yamaguchi-Shinozaki, 2006). Several genes, for example *COR* genes expressed in the cold stress response, contain dehydration responsive elements (DRE) or C-repeats (CRT) in the promoter region (Mahajan and Tuteja, 2005). These genes are regulated by a family of transcription factors namely C-repeat binding factor (*CBF*)/DRE-binding protein (DREB1) that bind specifically to the DRE/CRT sequence without the involvement of ABA (Nakashima *et al.*, 2009). DREB1A/*CBF3*, DREB1B/*CBF1*, DREB1C/*CBF2* are induced under cold stress but not by drought, whereas DREB2A/*CBF4* plays an important role in drought and salt stress. They do not only induce gene expression but are also involved in suppression of certain genes like photosynthesis-related genes (Nakashima and Yamaguchi-

Shinozaki, 2006). Therefore, transgenic plants with enhanced expression of the *CBF* regulon under stress conditions will not only have an increased tolerance towards cold stress, but to other abiotic stresses as well (Kasuga *et al.*, 2004; Qin *et al.*, 2004; Ito *et al.*, 2006).

The three *CBFs* have different functions in cold acclimation, as *CBF1* and *CBF3* are induced earlier than *CBF2* (Novillo *et al.*, 2007) and *CBF2* negatively regulates *CBF1* and *CBF3* expression. In a study by Vogel *et al.* (2005), the role of *CBF2* in *A. thaliana* was investigated, and it was found that 514 genes were regulated by *CBF2* of which 302 genes were up-regulated and 212 genes down-regulated. The transcripts that were up-regulated include a *LEA14*, *COR15b*, *sucrose synthase I*, *flavin-containing monooxygenase* and the *MYB* family transcription factor. Down-regulated transcripts include those coding for pathogenesis-related protein 1 (*PR-1*), *plant defensin protein* and a *putative leucine-rich repeat protein kinase*. The *CBF* transcription factors are regulated by different factors. The expression of *CBF3* is induced by MYC-like basic helix–loop–helix transcriptional activator (*ICE1*) (Chinnusamy *et al.*, 2003). In contrast, the *hos1* gene is a negative regulator of *CBF*. The transcription factor, *ICE2* plays an important role in the expression of *CBF1* (Fursova *et al.*, 2009). Furthermore, the extent of expression of the *CBF* genes correlates with frost tolerance in wheat (Vágújfalvi *et al.*, 2005). Using oligonucleotide microarrays, Davey *et al.* (2009) identified two transcripts in banana that showed high homology to the *DREB* transcription factor family after drought stress. The function of *DREB* transcription factors in banana under cold stress is still unknown.

Other transcription factors

An essential family of transcription factors duting cold acclimation, according to the study by Lee *et al.* (2005), is the zinc finger transcription factors. Constitutive expression of a cold-inducible zinc finger protein in *Arabidopsis*, *SCOF-1*, resulted in initiation of the expression of *COR* genes and lead to cold tolerance (Kim *et al.*, 2001). *ZAT12* (zinc finger transcription factor) regulated the induction and suppression of genes involved with low temperature responses (Vogel *et al.*, 2005).

This transcription factor is also involved in cold tolerance as overexpression led to an increase in cold tolerance (Vogel *et al.*, 2005). Another group of transcription factors in plants is the *MYB*/*MYC* transcription factors, which are bHLH (basic helix-loop-helix) stress inducible transcription factors involved in cold stress signalling and other processes. They play an active role in signalling during the late stages of the cold stress response (Nakashima and Yamaguchi-Shinozaki, 2006). Temperature stress (cold and heat) of the Fusarium resistant leaves of banana variety Calcutta 4 led to the expression of the *MYB* zinc finger and heat-shock transcription factors (Santos *et al.*, 2005). Transcription factors identified in banana plants exposed to drought included nine NAC-domain transcription factors, four basic-domain leucine zippers (bZIPs), eight bHLHs and seven MYB-domain transcription factors (Davey *et al.*, 2009).

Mitogen activated protein kinase (MAPK) cascade

MAPK is the mediator in several transduction signal pathways in plants after abiotic and biotic stresses (Teige *et al.*, 2004). These protein kinase cascades are highly conserved in eukaryotes and consist of three subsequently acting protein kinases, a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAP kinase (MAPK) (Kumar *et al.*, 2008). MAPKKK acts as primary signal receivers which act on phosphorylation and activates MAPKK (Viswanathan and Zhu, 2002). Thereafter, MAPKK phoshorylates MAPK, where MAPK enters the nucleus to regulate appropriate transcription factors such as the expression of the *CBF* regulon, which activates cold stress genes. In *Arabidopsis*, MAPKK2 was induced under cold and salt stress conditions, which changed the expression of genes required in signal transduction, cellular defence, stress metabolism and regulation of transcription (Teige *et al.*, 2004). MAPK have been reported from banana (GenBank: ABF69963.1), but the precise physiological function is still unknown.

Cytosolic calcium influx

As the temperature drops, calcium permeable channels act as sensors for low temperature and are responsible for Ca^{2+} influx (Smallwood and Bowles, 2002). A cytosolic Ca^{2+} rise takes place within minutes after a cold shock (Sung *et al.*, 2003).

This increase is ubiquitous in chilling-sensitive and cold-tolerant plants, as well as in all cell types (Knight, 2002). In addition, there is a linear relationship between the degree of cooling temperature and calcium influx concentration, with higher calcium influx during more intense cold temperatures (Knight, 2002). Intracellular Ca^{2+} is sensed by the calcium sensor family of proteins, including calcium dependent protein kinases, calmodulin, calmodulin-like proteins and calcineurin B-like proteins (Solanke and Sharma, 2008). It is strongly hypothesized that these sensory proteins then interact with their respective interacting partners and target the major stress responsive genes or the transcription factors controlling these genes, but their precise role is still unclear (Penfield, 2008). In *Arabidopsis*, cytosolic free calcium concentration is at its highest during daybreak and correlated with *CBF* transcription factors which are highly expressed (Fowler *et al.*, 2005). The products of these stress genes ultimately lead to plant adaptation and help the plant to survive and surpass the unfavourable conditions. In banana, hot water dipping of the fruit for 3 min at 52°C lead to an increase in Ca^{2+}-ATPase as well as increased tolerance to cold stress (7°C) (Wang *et al.*, 2008a). For this reason, Wang *et al.* (2008a) suggested that banana utilize calcium signalling to respond to cold temperatures.

Phosphoinositide phospholipase C (PLC) turnover-related proteins

Phosphoinositols are essential in stress-related signalling in plants (Shinozaki *et al.*, 2003) (Fig. 1.3). During cold stress, 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) is formed after PLC catalyzes the hydrolysis of phosphotidylinositol 4,5-bisphosphate (PIP_2) (Mahajan and Tuteja, 2005). IP_3 is involved in inducing Ca^{2+} levels, whereas DAG activates protein kinase C. After *Arabidopsis* cell suspensions were exposed to low temperature, a significant increase in phospholipase C and phospholipase D occurred (Ruelland *et al.*, 2002). Inositol polyphosphate kinases' role in abiotic stress in plants is still unknown (Yang *et al.*, 2008).

Abscisic acid (ABA)

Cold stress is regulated by ABA-dependent and independent signalling pathways. With a slight decrease in temperature, genes involved in ABA signalling such as *COR*

genes, cytosolic Ca^{2+} concentration and stress-responsive NAC 2 (*SNAC2*) transcription factors are induced in cold sensitive and cold tolerant plants (Penfield, 2008; Usadel *et al.*, 2008). Some of the low temperature responsive genes contain an ABA-responsive-element (ABRE) within their promoter sequence and is up-regulated by application of exogenous ABA (Thomashow, 1999). Furthermore, exogenous application of ABA enhances freezing tolerance. Foliar application of ABA in the field increased cold tolerance in grapevine (Zhang and Dami, 2012) whereas application to roots increased cold tolerance in winter wheat (Zabotin *et al.*, 2009). Signals in the response to ABA in cold acclimation can be regulated by secondary messengers such as H_2O_2 and Ca^{2+} (Chinnusamy *et al.*, 2006). The precise role of ABA in cold signalling is still being investigated (Penfield, 2008).

ELEVATED LEVELS OF DISEASE INCIDENCE AND DEVELOPMENT DUE TO TEMPERATURE

Low temperature is a predisposing factor for several fungal diseases. In male-sterile sorghum plants, non-fertilized ovaries are severely affected by the ergot fungus *Claviceps africana* Freder., Mantle & De Milliano (Bhuiyan *et al.*, 2009) when cooler temperatures prevail seven to nine days before blooming. Ergot development is increased at temperatures around 10°C (Garcia, 2004). High temperatures enable wheat plants to be resistant to stripe rust caused by *Puccinia striiformis* Westend, whereas low temperatures resulted in susceptibility (Line and Chen, 1995). There are also differences between the susceptibility of cultivars at low temperatures. The winter wheat cv. AGSECO 7853 is more vulnerable to *Stagonospora nodorum* (Berk.) E. Castell. & Germano under cooler temperatures, while cv. Heyne was unaffected by the change in temperature (Kim and Bockus, 2003). Furthermore, diseases of soybean seed caused by fungal pathogens like *Fusarium graminearum* Schwabe and *Alternaria alternata* (Fr.) Keissl. increased after frost damage at -4.5°C (Osorio and McGee, 1992).

Low temperature influences disease tolerance to bacterial infections. In the case of ice-nucleation active bacteria, frost damage is a predisposing factor for infection. Examples include stem necrosis caused by *Springomonas* spp. and *Xanthomonas* spp.

in willows (Nejad, 2005) or Salix dieback caused by *Springomonas* spp. (Cambours *et al.*, 2005) and bacterial leaf streak on barley caused by *Xanthomonas campestris* pv. *translucens* (Kim *et al.*, 1987). The study by Vigouroux and Bussi (1998) showed that the incidence of bacterial canker, caused by *Pseudomonas syringae,* was higher in peach trees that were pruned in the winter compared to spring.

Cold stress does not only play a role as a predisposing factor for the infection of plants by pathogens, but also influences the rate of symptom and disease development. Symptoms of barley yellow dwarf virus in maize *(Zea mays* L.) are more severe under low temperatures between 18-25^{o}C than 25-30^{o}C (Brown *et al.*, 1984). Disease development of Rhizoctonia root rot on winter wheat, caused by *Rhizoctonia solani* Kühn, was enhanced in natural soils by low temperatures between 6-19^{o}C compared to higher temperatures of between 16-27^{o}C (Smiler and Uddin, 1993). An increase in rainfall associated with lower temperatures also led to the increased development of Fusarium head blight and higher levels of deoxynivalenol (DON) production in winter wheat when compared to higher average daily temperature and lower rainfall (Tamburic-Ilincic *et al.*, 2007).

FACTORS THAT GIVE RISE TO INCREASED DISEASE INCIDENCE WITH A CHANGE IN TEMPERATURE

Cold stress, an important abiotic factor, impacts the growth and yield of agricultural crops. Temperature has a significant effect on plant-pathogen interactions by influencing the disease incidence and severity. Cold stress can further predispose plants to infection (Colhoun, 1973). Low temperatures not only affect the host, but it also has a significant effect on the pathogen or vector itself. The manner in which temperature influences the defence response will be discussed below.

Resistance genes (*R* genes) interact with avirulence (effector) genes from the pathogen and are involved in the gene for gene interaction. The R proteins are regulated by temperature, which influences the susceptibility of plants at different temperature regimes. *Puccinia striiformis* f. sp. *tritici* Erikss., causal agent of wheat stripe rust was hampered by the resistance gene, *Yr36* which was induced at relative

high temperatures (25-35°C) compared to low temperatures (15°C) (Fu *et al.*, 2009). Induction or suppression of *R* genes by temperature is dependent on the specific R gene. For example, the *R* gene in rice against *Xanthomonas oryzae* pv. *oryzae*, *Xa7,* was more effective at high temperatures, whereas genes *Xa3*, *Xa4*, and *Xa5* at lower temperatures (Webb *et al.*, 2010). *RPP4* involved in the *Hyaloperonospora parasitica* (Pers.) Constant./*A. thaliana* interaction, was up-regulated by low temperatures (4°C) and activated the basal defence response which had an impact on the disease tolerance (Huang *et al.*, 2010). Thus, the expression of *R* genes, which results in disease resistance in a compatible interaction, is regulated by temperature.

Basal resistance is a non-specific resistance mechanism against pathogens through pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). PTI triggers a variety of downstream responses, including a calcium burst, activation of MAPK cascades, production of ROS and the expression of numerous defence-related genes. As with *R* genes, basal level resistance is regulated by temperature. In a study by Wang *et al.* (2009), they reported that the basal resistance to *Pseudomonas syringae* was suppressed at high temperature in tomato. Low temperature in *Arabidopsis* plants induced a NAC transcription factor which resulted in the up-regulation of *PR* genes and decreased susceptibility to *P. syringae* (Seo *et al.*, 2010). Therefore, a change in temperature resulted in the adjustment in the expression of defence related genes, important in host and pathogen interactions.

Upon recognition of a pathogen by a host plant, signalling cascades are activated and defence related genes are induced. The major hormones involved in defence are SA, JA, ET and ABA. These hormones are also regulated by temperature, as discussed earlier, but also essential for disease resistance. Rice plants became more susceptible to the rice blast pathogen *Magnaporthe grisea* (T.T. Hebert) M.E. Barr at low temperatures that reduce the expression levels of whole plant-specific resistance by the induction of ABA (Kim and Mogi, 1986). At high temperatures enhanced ABA levels led to a reduction in the effectiveness of *SNC1* and *RPS4 R* genes in *Arabidopsis* (Mang *et al.*, 2012). Application of ABA on tomato fruit led to an increase in tolerance to cold stress as well as infection by opportunistic pathogens (Ding *et al.*, 2002). As there is an interaction between temperature stress and

signalling defence pathways, any change in temperature will have an effect on the disease susceptible or tolerance of the plant.

PR genes are known to be elicited upon infection or wounding and are also influenced by a change in temperature (Sels *et al.*, 2008). Soybean seeds showed increased levels of tolerance towards *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., at higher temperatures with the up-regulation of *PR10* (Upchurch and Ramirez, 2011). Koeda *et al.* (2012) showed that the pepper cv. Sy-2 (*Capsicum chinense* Jacq.) showed induction of *PR* upon cold stress with increased tolerance towards *Xanthomonas campestris* pv. *vesicatoria*. As *PR* genes are important in plant defence, down regulation of *PR* genes at low temperatures can result in loss of tolerance.

Small interfering RNAs (siRNAs) play a vital part in plant defence by silencing targeted genes required by the pathogen for infection. The siRNAs are integrated into an active RISC complex and induce cleavage of the target mRNA, thereby preventing translation. Tobacco plants showed a increase in disease symptoms caused by Cymbidium ringspot virus as those plants showed suppression of siRNA-mediated RNA at low temperatures (Szittya *et al.*, 2003). siRNA-mediated RNA silencing inhibition at low temperature has led to the increase in susceptibility of plants to virus infection.

Chaperones like heat shock proteins are induced upon temperature stress and play a role in protection of the plant cells. *Hsp70* which is induced upon heat or cold stress is also important in virulence of *P. syringae* to *Arabidopsis* (Jelenska *et al.*, 2010). *HopI1*, a virulence effector, binds to *Hsp70* which was induced with heat stress, and resulted in an increase in disease severity (Jelenska *et al.*, 2010). Therefore, genes up-regulated due to temperature stress like heat shock proteins can be utilized by the pathogen to increase disease severity.

Temperature can increase or decrease plant susceptibility towards a specific pathogen. In the case where plants showed an increase in tolerance towards a pathogen at low temperature by activation of *R* or basal resistance genes, it resulted in low disease incidence in the winter. However, with the onset of spring, the pathogen population

had time to build up and *R* or basal resistance genes were suppressed at the warmer temperatures, which resulted in a significant increase in disease incidence at the start of spring (Moyer *et al.*, 2010). Disease susceptibility differs between seasons in many different plant/pathogen interactions. *Alternaria alternata* which causes early leaf senescence on cotton, only showed symptoms in the beginning of spring due to the fact that cotton increased its tolerance to *A. alternata* in cold temperature (Moyer *et al.*, 2010). Fusarium wilt of banana is evident in the beginning of spring and little disease incidence reports occur in winter, however the role of temperature still needs to be elucidated.

The change in temperature also has an effect on plant pathogens. Growth and reproduction is influenced by temperature. Oomycete pathogens produce zoospores for infection which is triggered by a decrease in temperature (Walker and van West, 2007). A decrease in temperature results in an increased chance for host infection, as more zoospores are produced. Furthermore, pathogens have different optimum growth temperatures. Therefore, a change in temperature may increase the ability of the pathogen to colonise host tissue if the temperature range is closer to it optimum temperature.

The virulence of pathogens is also affected by temperature. Fang *et al.* (2011) showed that *Fusarium oxysporum* Schltdl. is more virulent at 27^{o}C compared to 17^{o}C on strawberry plants. Additionally, the expression of virulence factors is also regulated by temperature (Leimeister-Wachter *et al.*, 1992; Kimes *et al.*, 2012). In the plant pathogen *P. syringae*, a decrease in temperature increased virulence gene expression and increased toxin production (Ullrich *et al.*, 1995). Thus, temperature influences the pathogen, thereby increasing or reducing the disease severity on susceptible plants.

BANANA, A TROPICAL PLANT

Origin and cultivation

The banana plant is a giant monocotyledonous perennial herb that contains 80% water (Ammar-Khodja, 2000). The plant consists of roots, a rhizome, pseudostem and

leaves. From the rhizome, vegetative buds are formed which can become fully developed banana plants (Cronauer and Krikorian, 1986). The apical growing point first produces young leaves that progress through the pseudostem and finally differentiate into an inflorescence meristem with the male bud and female flowers (Cronauer and Krikorian, 1986). Fruit develops from the female flower and is produced all year round.

Most cultivated bananas evolved from two wild diploid species, *Musa acuminata* Colla (A genome) and *M. balbisiana* Colla (B genome) (Stover and Simmonds, 1987). Inter- and intra-specific crosses over many years resulted in diploid and triploid hybrids; some that are parthenocarpic and unable to produce seed (Jones, 2000). Genetic diversity in the seedless, edible bananas thereafter arose through the occurrence of somatic mutations. The edible triploid bananas were selected, domesticated and distributed as a food crop around the world. Today, the world export market is based mainly on Cavendish varieties, which comprise 70% of bananas planted worldwide (Jones, 2000). A lack of genetic diversity makes Cavendish bananas particularly vulnerable to pests and diseases and results in significant economical losses each year. The problem is further exacerbated where bananas are planted in monoculture.

Bananas are usually cultivated in temperature zones of between 19 and 33^{o}C, in a tropical humid climate. The main banana production countries are India (32 million metric tons), China (9.8 million metric tons), Philippines (9.1 million metric tons) and Ecuador (7.9 million metric tons) (FAOSTAT, 2010). The crop is also cultivated under subtropical conditions, but plant physiology and development, as well as their tolerance to diseases, are affected during cold winter temperatures. Subtropical banana-producing countries include Australia, the Canary Islands, and South Africa.

Banana is produced in South Africa under a subtropical climate which significantly increases the length of the cropping cycle. South Africa has a total of five banana growing areas: Onderberg, Levubu, Letaba (Tzaneen), Kiepersol (Hazyview) and KwaZulu-Natal, which comprise about 8000 hectares (Table 1.1). South Africa is the 28th highest banana producing country in the world, and tenth in Africa with almost

400 thousand tonnes annually (FAOSTAT, 2010). The majority of the fruit is sold on the fresh fruit market. Currently, no fruit is exported to other countries.

Abiotic stresses of bananas

Abiotic stresses that negatively influence banana include low temperature (Robinson and Human, 1988; Robinson, 1996), frost (Linbing *et al.*, 2003), wind damage (Eckstein *et al.*, 1996), hail (Israeli and Lahav, 2000), heat (Robinson, 1996), drought (Abele and Pillay, 2007; Turner *et al.*, 2007), flooding (Israeli and Lahav, 2000) and nutrition deprivation/toxicity (Turner, 1994; Rufyikiri *et al.*, 2000). Abiotic stresses usually occur in combinations (Fig. 1.4). High temperature leads to a higher evaporation rate in dryer soil and results in plants being exposed to higher concentrations of salt. The plant must then face heat, drought and salinity stress together (Mittler, 2006).

Banana, a tropical plant, is very sensitive to low temperatures (Lyons, 1973). With the onset of chilling temperatures, the leaves turn yellow with a reduction in photosynthesis and hence also chlorophyll (Fig. 1.1). The growth of the plant ceases at approximately 14°C, with irreversible damage occurring below freezing point (Robinson, 1996). Freeze damage causes water-soaked leaves which turn brown and eventually die. With the death of the leaves, fruit become susceptible to sunburn. An increase in radiation enhances damage. In 1999, 150 000 ha of banana plantations were destroyed in China by frost damage (Linbing *et al.*, 2003).

Repeated cold and radiation cycles, as found in subtropical winter conditions, lead to leaf bleaching (Israeli and Lahav, 2000). The cooler winter temperatures cause growth cycle extension, resulting in smaller banana bunches and shorter fingers (Robinson and Human, 1988), as well as a decrease in root growth (Robinson and Alberts, 1989) and in transpiration rate (Robinson and Alberts, 1989). Symptoms associated with low temperatures include ‘choking’, ‘choke throat’, ‘November dump’ (May flowering) as well as under-peel discoloration (Robinson, 1996) (Fig. 1.5). ‘Choking’ refers to the shortening of the length between the petioles from the pseudostem, ‘choke throat’ is the failure of the flowering stalk or fruit bunch to emerge from the pseudostem and

'November dump' results in abnormal flowering of bananas grown in the subtropics of the Southern hemisphere (Robinson, 1996). Low temperature further extends the time between flowering and harvest, fruit filling and significantly lowers the yield. Banana fruit shows underpeel discoloration due to oxidation of phenolic compounds from the latex in vascular tissue (Fig. 1.6).

Tolerance to cold temperatures differs between banana cultivars and genotypes. Genotypes with the B genome (*M. balbisiana*) are less sensitive to cold temperatures than genotypes with the A genotype (*M. acuminata*) (Israeli and Lahav, 2000). Within Cavendish cultivars, plants with smaller stature are less prone to cold damage. Higher tolerance to low temperature and light was also reported in dwarf off-type tissue-cultured banana plants compared to normal tissue-cultured plants (Damasco *et al.*, 1997).

Biotic stresses of bananas

Bananas, especially cultivated varieties, are susceptible to a wide range of pathogens and pests that, if left uncontrolled, could wipe out the crop within the next few years (Pearce, 2003). Production of bananas has been severely affected by diseases such as banana bacterial wilt (Biruma *et al.*, 2007; Tripathi *et al.*, 2009), banana bunchy top virus (Smith *et al.*, 1998a; Hooks *et al.*, 2008), and pests such as nematodes (Queneherve *et al.*, 2009) and the banana weevil borer (Gold *et al.*, 2004). The two major fungal pathogens of banana are *Fusarium oxysporum* f. sp. *cubense* (E.F. Sm.) W.C. Snyder & H.N. Hansen (*Foc*), a root pathogen causing Fusarium wilt, and *Mycosphaerella fijiensis* Morelet, a foliar pathogen causing black Sigatoka (Jones, 2000). Since Fusarium wilt is considered one of the most serious threats internationally, to both export banana production and subsistence farmers (Ploetz, 2005), the remainder of this review will focus on this disease.

History of Fusarium wilt

The centre of origin of the Fusarium wilt pathogen is believed to be Southeast Asia (Stover, 1962), which is also the centre of origin for banana. The disease, however,

was first report from Australia in 1874, when Bancroft discovered diseased cv. Sugar banana plants in Brisbane (southern Queensland) (Moore *et al.*, 1999). Fusarium wilt became notorious when almost 40 000 ha of cv. Gros Michel export plantations were destroyed by *Foc* race 1 in Central America (Ploetz and Pegg, 2000), resulting in a loss of an estimated $400 million of revenue before 1960 (Ploetz, 2005). Since the banana export industry at the time relied exclusively on cv. Gros Michel as export fruit, it had to be replaced with resistant Cavendish banana cultivars. However, Cavendish bananas later proved to be susceptible to *Foc* race 4, first in the subtropics and later in the tropics (Ploetz and Pegg, 2000).

Disease symptoms

Fusarium wilt is a lethal disease affecting mostly the vascular tissue of banana plants. The spores of *Foc* germinate to form germinating tubes upon exposure to banana root exudates (Ploetz and Pegg, 2000). The hyphae enter the feeder roots and spread to the vascular system where microconidia block the xylem tissue and result in severe wilting from the older to the younger leaves until the banana plant eventually collapses and dies (Fig. 1.7)(Beckman, 1990). A cross-section through the pseudostem reveals a brown discoloration of the vascular system (Ploetz and Pegg, 2000). Another well known symptom of Fusarium wilt is the longitudinal splitting of the pseudostem base (Ploetz and Pegg, 2000). Once introduced into a banana field, *Foc* stays dormant as chlamydospores in the soil for up to 30 years. Susceptible cultivars can, therefore, not be replanted to the same soil (Stover, 1962).

Pathogenic races and VCG's in Foc

Four races of *Foc* are recognized based on cultivar susceptibility. *Foc* race 1 was responsible for the epidemic in 1960 on cv. Gros Michel and further affects cvs. Silk, Pome, Pisang Awak and Maqueño. *Foc* race 2 affects cooking bananas including cv. Bluggoe and AAAA hybrids, and *Foc* race 4 causes disease on race 1 and race 2 susceptible cultivars, Cavendish cultivars, and cv. Pisang Mas (Ploetz, 2005). Of the four, *Foc* race 4 is the most important as 80% of the world's bananas, including the export Cavendish bananas, are susceptible to this race (Ploetz, 2005). *Foc* race 4 is

further subdivided into 'tropical' and 'subtropical' race 4, where *Foc* 'tropical' race 4 causes disease to Cavendish bananas in the tropical banana growing countries, where it is currently limited to Southeast Asia and the northern part of Australia (Molina *et al.*, 2008). *Foc* 'subtropical' race 4 causes disease to Cavendish bananas in subtropical countries like South Africa, Australia, Taiwan and the Canary Islands, usually after predisposition to cooler temperatures in winter. *Foc* is further sub-divided into VCGs that are smaller genetic sub-groupings determined by the ability of certain strains of the pathogen to form heterokaryons with other strains in a process of asexual recognitioning (Puhalla, 1985). *Foc* is divided into 24 VCGs where VCG 01213 and 01216 comprise of *Foc* 'tropical' race 4 and VCG 0120, 0129, 01211, 0122 and 01215 include *Foc* 'subtropical' race 4 isolates (Ploetz and Pegg, 2000).

FACTORS INFLUENCING THE DEVELOPMENT OF FUSARIUM WILT OF BANANA

Fusarium oxysporum strains live as saprophytes in the soil, plant debris, as well as in roots of non-host plants such as weeds (Ploetz, 1998). Pathogenic strains of the fungus cause disease to plants once they come into contact with a susceptible host, thereby making them opportunistic pathogens. The ability of *F. oxysporum* to cause disease to hosts is often enhanced by environmental stresses such a drought, cold stress or waterlogging/flooding (Stover and Malo, 1972; Shivas *et al.*, 1995; Aguilar *et al.*, 2000). Other abiotic conditions influencing the resistance of plants to Fusarium wilt include pH, wind damage, clay mineral composition and type of propagation material used (Rishbeth, 1955; Stotzky and Martin, 1963; Stover and Malo, 1972). A biotic factor that affects Fusarium wilt development includes the microbial composition of soils (Smith *et al.*, 1998b).

Temperature stress

Seasonal variation, particularly the cooler winter temperatures in subtropical banana-producing countries not only affects plant physiology, but also the crop's susceptibility to Fusarium wilt. This has been demonstrated by the dramatic increase in the incidence of Fusarium wilt of Cavendish bananas, caused by *Foc* 'subtropical' race 4 (VCG 0120), soon after winter (with the onset of spring) in the subtropics

(Viljoen, 2002). VCG 0120 also occurs in the tropics where it causes disease to Gros Michel, Bluggoe and other banana varieties, but not to Cavendish bananas, unless they are severely stressed (Ploetz, 2006). The observation that disease incidence is usually low in late summer and early autumn, and that it becomes severe in spring and early summer, suggests that cold temperatures predispose plants to infection (Ploetz, 2006).

Other abiotic stresses

Dwarf Cavendish, susceptible to *Foc* race 4 but resistant to *Foc* race 1, succumbed to *Foc* race 1 under waterlogged conditions (Stover and Malo, 1972). In Western Australia, increased disease severity was observed in Williams to *Foc* race 4 in waterlogged and drought conditions (Shivas *et al.*, 1995). According to Aguilar *et al.* (2000), hypoxia in waterlogged soils decreases the peroxidase activity that leads to susceptibility of Williams to Fusarium wilt. In contrast, Stotzky and Martin (1963) stated that flooding has a minimal effect on Fusarium wilt incidence, but indicated that the clay mineral composition of the soil, rather, has an effect on disease development. A low pH in the soil, wind damage and salinity favours development of Fusarium wilt in bananas (Rishbeth, 1955; Simmonds, 1959; Stover and Malo, 1972).

Propagation material

Fusarium wilt is affected by the type of plant material used in the establishment of a banana plantation. In a study by Smith *et al.* (1998b), tissue-cultured plants were found to be more susceptible than suckers and bits. They hypothesized that suckers and bits are protected from *Foc* infection by antagonistic organisms or that conventional propagation materials are less affected by environmental conditions after planting (Smith *et al.*, 1998b). Suckers connected to an infected mother plant should, theoretically, always be infected with *Foc*, but seldom show symptoms (Jeger *et al.*, 1996). Such symptomless suckers, however, can disseminate *Foc* when transplanted to new fields and the disease then develops at a later stage.

Soil microbial composition

Several studies investigated the role of disease suppressive soils on Fusarium wilt development (Peng *et al.*, 1999; Getha *et al.*, 2005; Nel *et al.*, 2006). Peng *et al.* (1999) found that the suppressive nature of the soil is due to the activity of antagonistic micro-organisms. Although biocontrol looks promising, field testing is lacking and effective biological control of Fusarium wilt of banana is not a realistic option for disease management as yet (Belgrove *et al.*, 2011). A few reports have been published where Fusarium wilt was enhanced by altering the nutrition of the plant. In a study by Domínguez-Hernández *et al.* (2008), it was concluded that an increase in potassium fertilisers with greater clay-sized particle content increased Fusarium wilt development. They hypothesized that the increase in clay-sized particles affects soil physicochemical properties by enhancing soil aggregation and accessibility of Fe for chlamydospore germination (Domínguez-Hernández *et al.*, 2008). Pittaway *et al.* (1999) also reported an increase in infection of banana roots with *Foc* after application of chicken manure. According to Rishbeth (1955), the application of nitrogenous fertilizer reduced resistance of banana plants to Fusarium wilt.

APPROACHES TO IMPROVE COLD TOLERANCE IN PLANTS

Developing cultivated bananas with resistance to cold stress is a major challenge. As cold tolerance can not be achieved with chemical control, other methods have to be investigated. The most affordable and environmentally friendly way to combat cold temperatures would be the planting of cold tolerant plants. The development of such bananas can be achieved by means of conventional and unconventional improvement.

Conventional breeding

Conventional breeding can be used to improve plants for characteristics like cold tolerance or disease and pest resistance. Although classical breeding is a long and arduous process, the outcomes can be improved by screening the progeny with molecular markers obtained by genomic tools such as gene expression profiling or quantitative trait loci (QTLs). In conventional banana breeding, the process is

hampered by long growth cycles, banana streak virus sequences incorporated into the B genome of *Musa* and the parthenocarpic nature of several commercial bananas (Crouch *et al.*, 1998). In 1989, Goldfinger (FHIA-01), a hybrid between Dwarf Prata and SH-3142 was released with increased tolerance to *Foc* race 1, *Foc* 'subtropical' race 4, black leaf streak disease as well as cold tolerance (Rowe and Rosales, 1993). This hybrid has not been accepted by the market due to its acidic taste (Robinson and Galán Saúco, 2010). Conventional breeding remains a viable strategy for banana improvement, as genetically modified bananas have not been released as a commercial product after 17 years of experimentation.

Genetic engineering

Genetic transformation of bananas has focused on disease resistance with no reports of improving the cold tolerance of banana plants. Examples of increased resistance against *Foc* 'tropical' race 4 include incorporation of *pflp* (plant ferredoxin-like protein) in *Musa acuminata* cv. Pei Chiao and *M. acuminata* cv. Gros Michel (Yip *et al.*, 2011), *HL* (human lysozyme) in cv. *Taijiao* (AAA) (Pei *et al.*, 2005) and *tlp* (thaumatin-like protein) in cv. Pisang Nangka (AAB) (Mahdavi *et al.*, 2012). Increased resistance against *Foc* race 1 has been achieved by the introduction of *Bcl-2* (negative regulated apoptosis) in cv. Lady Finger (Paul *et al.*, 2011) and pROKla-*Eg* (β-1,3-glucanase) in cv. Rastali (AAB) (Maziah *et al.*, 2007), and increased resistance against *Foc* race 2 by the introduction of MSI-99 (magainin analogue) in cv. Rastali (AAB) (Chakrabarti *et al.*, 2003). Unfortunately, most of these studies focus on the incorporation of foreign genes into the banana genome which is disapproved by the public sector. Therefore, it is important to discover defence- and cold stress-related genes in bananas for production of cisgenic plants that would be more acceptable to consumers.

Genetic modification against cold temperature in crops was initially conducted by transforming vulnerable plants with a single gene (Bhatnagar-Mathur *et al.*, 2008). Cold tolerance genes used in plant transgenic studies mainly translate to enzymes associated with membrane fluidity, such as LEA-proteins, membrane stabilizing proteins and osmoprotectant synthesis-related proteins, which protect the cell against

freezing conditions (Table 1.2). This has lead to numerous reports of enhanced cold tolerance (Kaye *et al.*, 1998), including a few incidences of insignificant increase in cold tolerance. Transgenic tobacco plants over-expressing the *fad7* gene had increased amounts of trienoic fatty acids, hexadecatrienoic and linolenic acids which resulted in better cold tolerance (Kodama *et al.*, 1994) (Table 1.2). Also, the over-expression of superoxide dismutase and ascorbate peroxidase to detoxify ROS species in sweet potato resulted in improved tolerance to chilling stress (Lim *et al.*, 2007).

As resistance to cold is a polygenic trait, it is necessary to introduce multiple genes, signalling molecules and/or transcription factors in cold-sensitive plants. A lack of knowledge of candidate genes, together with their regulatory processes in cold tolerance, is hampering the manipulation of plants (Sreenivasulu *et al.*, 2007). Plants transformed with cold tolerant-related genes under constitutive expression can show reduced growth under normal conditions. In a study by Kasuga *et al.* (2004), the over-expression of the *DREB1A* transcription factor under the control of stress-inducible *rd29A* promoter in tobacco not only led to slight growth retardation, but also enhanced tolerance to cold and drought. In comparison, Ito *et al.* (2006) reported stunted growth of rice plants with constitutive expression of *DREB1*. It is, therefore, essential to transform plants with stress inducible promoters to minimize growth retardation.

Signalling pathways for resistance to biotic and abiotic stresses may occasionally overlap. Transformation of plants with transcription factors not only leads to plants with enhanced cold tolerance but also tolerance to other abiotic stresses, such as salt and drought (Kasuga *et al.*, 2004; Qin *et al.*, 2004; Ito *et al.*, 2006). In a study by Seong *et al.* (2007), a zinc finger transcription factor, *CaPIF1*, was identified that increased tolerance to both cold stress and the bacterial pathogen *P. syringae* when over expressed in tomato. Similarly, an AP2/ERF type transcription factor, *GmERF3,* conferred enhanced tolerance towards salt, drought and infection by *Ralstonia solanacearum, A. alternata*, and the tobacco mosaic virus, while its cold sensitivity was unaffected (Zhang *et al.*, 2009).

The development of transgenic plants with resistance against abiotic stresses still faces some challenges. The gene of interest needs correct post-translational modification for correct functioning, specific location, sufficient precursor, optimal conditions like pH and sufficient expression (Holmberg and Bülow, 1998). As most studies on genetic modification for resistance to abiotic stresses involved *Arabidopsis* and tobacco, gene function in non-model crops still has to be demonstrated. One of the major hurdles involves field testing of GMO plants under different environmental conditions (Wang *et al.*, 2003). Several scientists have appealed for less strict regulation of cisgenic than transgenic plants under GMO regulation (Schouten *et al.*, 2006; Rommens *et al.*, 2007; Jacobsen and Schouten, 2008).

CONCLUSION

A complex interaction exits between plants and the environment. Cold stress, which is fundamentally an abiotic stress condition, not only has phenotypic effects on the plant itself, but causes a cascade of events in the plant on a molecular level. Upon recognition of low temperature, signalling pathways are activated in which genes are enhanced or suppressed to protect the plant cells against cold temperatures. The plant regulates cold-related genes to acclimatize and overcome cold conditions.

Low temperature is a limiting factor on the yield of banana in the subtropics. Frost damage destroys the functional leaves of the banana plant which reduces the photosynthetic capacity, leading to reduction in yield (Robinson, 1996). Most of the studies concerning cold stress, however, have been reported on the fruit (Caamal-Velázquez *et al.*, 2007; Promyou *et al.*, 2008; Wang *et al.*, 2008b). Cold stress, such as the winter regime in subtropical areas, not only decreases the yield, but predisposes the plant to Fusarium wilt, a major fungal disease on bananas. With the onset of spring, disease incidence is significantly increased. The increase in disease can be linked either to an increased virulence of the pathogen, *Foc* 'subtropical' race 4, or to an increase in the susceptibility of Cavendish banana plants, due to the effects of cold temperature.

To investigate the possible increase in virulence of *Foc* 'subtropical' race 4, pathogenicity factors present in *Foc* race 4 should first be identified. To date, only one study has focused on pathogenicity genes in *Foc* race 4 (Meldrum *et al.*, 2012). They found that secreted in the xylem (*six*) genes, namely *six7* and *six8*, are uniquely present in *Foc* STR4 and *Foc* TR4, whereas *six1* is found in *Foc* race 1, 2 and 4 (Meldrum *et al.*, 2012). It would be interesting to determine whether fungal virulence factors change under different environmental conditions, especially under cold temperatures. Furthermore, studies to compare *Foc* 'tropical' race 4 and *Foc* 'subtropical' race 4 may broaden our knowledge on the infection of these pathogens and will aid in combating Fusarium wilt.

Little is known about the effect of cold temperatures on the defence response of Cavendish bananas. Although the defence response in GCTCV-218, a somaclonal Cavendish mutant tolerant to *Foc* race 4 (Van den Berg *et al.*, 2007), has been elucidated against *Foc* STR4, and the transcriptomes of other Cavendish mutants characterized after infection with *Foc* TR4 (Li *et al.*, 2012; Wang *et al.*, 2012), the effect of cold temperature during infection is lacking. Some questions can be raised like: A. Is there a delay in defence responses in Cavendish banana during cold temperatures which gives *Foc* 'subtropical' race 4 the opportunity to invade the roots and cause disease, B. What are the differences in the transcriptomes of Cavendish bananas with infection, with and without cold temperatures, C. Are plant defence responses suppressed in the plant during cold acclimatizing and D. Will tolerance to *Foc* 'subtropical' race 4 be increased if tolerance to cold temperatures in Cavendish plants is increased?

One hypothesis why Cavendish banana is more susceptible to *Foc* 'subtropical' race 4 after cold temperatures is that the expression of defence-related genes is suppressed. For instance, the *CBF2* transcription factor induced in *Arabidopsis* following exposure to low temperatures led to the down-regulation of *PR-1* and the genes coding for plant defensin and leucine rich repeat protein kinase (Vogel *et al.*, 2005). These genes are all involved in the general plant defence response (Van Verk *et al.*, 2009). Down regulation of plant defensin which also has antimicrobial activity (Broekaert *et al.*, 1995) will increase the susceptibility of plants towards pathogens.

Lowering the expression of resistance genes with a leucine rich repeat protein kinase domain will increase the vulnerability to pathogens (Dangl and Jones, 2001; Tameling and Takken, 2008).

In-depth gene expression studies are required to compare infection of Cavendish plants by *Foc* 'subtropical' race 4 at different temperatures. Identification of these transcripts will increase our knowledge of the cold stress response of banana as well as the defence response towards Fusarium wilt. These defence transcripts will be an ideal base for transformation studies to generate a resistant Fusarium wilt banana plant with cold resistance. Banana is an ideal crop for genetic transformation as Cavendish bananas are parthenocarpic, therefore escape of genes through pollen does not occur (Dickman, 2004). Since defence/cold stress is a polygenic trait and involves complex interactions, it is necessary to pyramid genes such as signalling molecules, transcription factors and/or master switches into Cavendish bananas to obtain a tolerant or resistant plant. Once developed, such a plant would be of considerable value for the production of bananas in subtropical banana growing countries.

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TABLES AND FIGURES

Table 1.1. Banana production in South Africa.

	2005	**2006**	**2007**	**2008**	**2009**	**2010**
Area Harvested (ha[a])	6962	6900	7000	7950	7500	7950
Yield (Hg/ha[b])	505045	498100	493927	494517	494407	494681
Production (tonnes)	351612	343689	345749	393141	370805	393271

[a] Hectare

[b] Hectogram per hectare

Table 1.2. Expression of cold tolerance genes in transgenic plants.

GENE	GENE ACTION	TRANSGENIC PLANT	PERFORMANCE OF TRANSGENIC PLANTS	COMMENT	REFERENCE
FUNCTIONAL PROTEINS					
fad7	Chloroplast ω-3 desaturase gene	Tobacco	Enhances cold tolerance	The transgenic plants showed that increased amounts of trienoic fatty acids could alleviate the cold damage	Kodama *et al.* (1994)
fad7	Chloroplast ω-3 desaturase gene	Tobacco	Tolerance to prolonged exposure to cold temperatures.	The *fad7* gene is under the control of a cold inducible promoter. The proportion of trienoic fatty acids in leaves was higher than in wild-type leaves after long-term exposure to cold.	Khodakovskaya *et al.* (2006)
desC	Acyl-lipid Δ 9-desaturase	Tobacco	Tolerance to cold temperature	Increased proportion of polyunsaturated fatty acids in membrane lipids	Popov *et al.* (2005)
CuZnSOD +APX	CuZn superoxide dismutase and ascorbate peroxidase	Sweetpotato	Improves tolerance to chilling stress	These results suggest that the transgenic plants had developed a tolerance to the oxidative stress mediated by chilling exposure.	Lim *et al.* (2007)

GENE	GENE ACTION	TRANSGENIC PLANT	PERFORMANCE OF TRANSGENIC PLANTS	COMMENT	REFERENCE
GST/GPX	Glutathione-S-transferase with glutathione peroxidase activity	Tobacco	Improves tolerance to chilling stress	Reduced oxidative damage	Roxas *et al.* (2000)
CaHSP26	Chloroplast (CP)-localized small heat shock protein	Tobacco	Improves tolerance to chilling stress	Protection of PSII and PSI during chilling stress under low irradiance	Guo *et al.* (2007)
Wcor15	Cor/Lea gene family	Tobacco	Improves level of freezing tolerance	Transport and abundant accumulation of the COR15 protein in the stromal compartment of the chloroplasts.	Shimamura *et al.* (2006)
Dhn24	SK3-type dehydrin (late embryogenesis abundant (LEA) proteins)	Cucumber	Increases chilling and freezing tolerance of seedlings	*Dhn24* expression was organ-type dependent with the highest expression observed in roots.	Yin *et al.* (2006)
BjDHN3	SK2-type dehydrin genes (late embryogenesis abundant (LEA) proteins)	Tobacco	Increases tolerance to cold and salt stress.	Enhanced the stress tolerance by suppressing the electrolyte leakage level and malondialdehyde content in transgenic tobacco.	Xu *et al.* (2008)

GENE	GENE ACTION	TRANSGENIC PLANT	PERFORMANCE OF TRANSGENIC PLANTS	COMMENT	REFERENCE
P5CS	Pyrroline 5-carboxylate synthase (proline synthesis)	Larch	More resistant to cold, salt, and freezing stresses	There was an approximately 30-fold increase in proline level in transgenic tissue compared to non-transformed controls.	Gleeson *et al.* (2005)
betA *betB*	Glycine betaine	Tobacco	Improved tolerance to photoinhibition under low temperature conditions as well as salt stress tolerance	Improve protecting of photosynthesis apparatus	Holmström *et al.* (2000)
PgTIP1	Aquaporin	*Arabidopsis*	Lower cold acclimation ability compared to the wild-type	Salt-stress tolerance as well as tolerance to water stress	Peng *et al.* (2007)
PIP2;5	Aquaporin	*Arabidopsis*/ Tobacco	Enhances germination under cold stress and showed enhanced water flow	Delay in germination and growth of *Arabidopsis* and tobacco plants under drought stress. The expression of one aquaporin isoform influences the expression levels of other aquaporins under stress conditions.	Jang *et al.* (2007)
REGULATORY AND SIGNALLING TRANSDUCTION					
OsDREB1A / *OsDREB1B*	*DREB* transcription factor	Rice	Tolerance to drought, high-salt and low-temperature stresses	Growth retardation under normal growth conditions	Ito *et al.* (2006)

GENE	GENE ACTION	TRANSGENIC PLANT	PERFORMANCE OF TRANSGENIC PLANTS	COMMENT	REFERENCE
ZmDREB1 A	*DREB* transcription factor	*Arabidopsis*	Higher tolerance to drought and freezing stresses.	Transgenic plants have dwarf phenotype. Functional similarity to DREB1s/CBFs in Arabidopsis and maize	Qin *et al.* (2004)
DREB1A	*DREB* transcription factor	Tobacco	Higher tolerance to cold and drought	The stress-inducible rd29A promoter minimized the negative effects on the plant growth	Kasuga *et al.* (2004)
OsCOIN	Zinc finger proteins (a RING finger protein)	Rice	Increased tolerance to chilling, salt and drought	OsCOIN is expressed in all rice organs with enhanced proline level in cells.	Liu *et al.* (2007)
SCOF-1	C2H2-type zinc finger protein	*Arabidopsis*/ tobacco	Enhanced cold tolerance	Induced cold-regulated (COR) gene expression by ABRE via protein interaction	Kim *et al.* (2001)
OsMYB3R-2	*Myb* transcription factor	*Arabidopsis*	Increases tolerance to freezing, drought, and salt stress	Induce stress-responsive genes	Dai *et al.* (2007)
Osmyb4	*Myb* transcription factor	Apple	Enhance tolerance to cold and drought stress and modified metabolite accumulation.	Transgenic plant has a dwarf phenotype that was proportional to the Myb4 expression level	Pasquali *et al.* (2008)
NPK1	Mitogen-activated protein kinase kinase kinase	Maize	Enhances freezing tolerance	Expressed at low-level	Shou *et al.* (2004)

GENE	GENE ACTION	TRANSGENIC PLANT	PERFORMANCE OF TRANSGENIC PLANTS	COMMENT	REFERENCE
AtNDPK2	Mitogen-activated protein kinase kinase kinase	*Arabidopsis*	Tolerance to multiple environmental stresses such as cold, salt, and H_2O_2	Tolerance possibly through activation of MAPK cascade.	Moon *et al.* (2003)
InsP 5-ptase	Inositol polyphosphate 5-phosphatase	*Arabidopsis*		Decrease in the peak Ca2+ in the transgenic seedlings compared with the control	Perera *et al.* (2008)
AtIpk2b	Inositol phosphates	Tobacco	Increased tolerance to osmotic, drought, freezing temperature and oxidative stress	Increased expression of various stress responsive genes. *AtIpk2b* involved in one or more signal transduction pathway(s)	Yang *et al.* (2008)
CDPK13/ CRTintP1	Calcium-dependent protein kinase 13 and calreticulin interacting protein 1	Rice	Reduced cold damage in transgenic plants as well as enhanced levels of seedling survival at low temperature.	Accumulation of calreticulin and CRTintP1 was not detected in non-transformed cold-sensitive rice varieties	Komatsu *et al.* (2007)

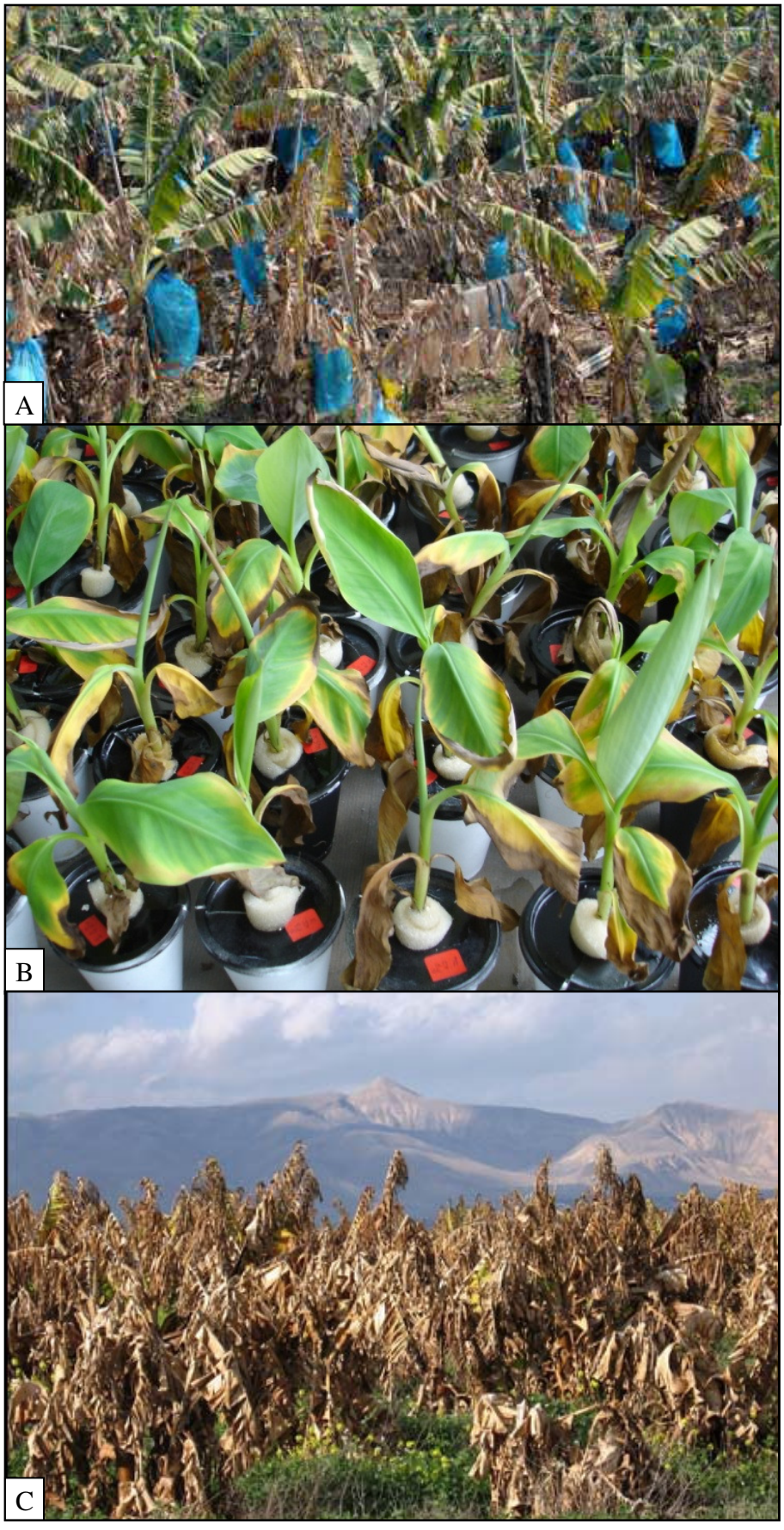

Figure 1.1. Phenotypic symptoms of cold stress on banana. A. Banana plantation in Pan Yu Nan Sha Island showing symptoms of cold damage. Freeze damage caused water-soaked lesions on the leaves which turned brown and died (Chan, 2009). B. Symptoms of cold temperature on young banana plants. Plants subjected to cold temperatures showed extensive yellowing and necrosis on the leaf edges and stunted growth. C. A banana field in the West Bank city of Al-Ouja was completely destroyed by frost damage (Westervelt, 2008).

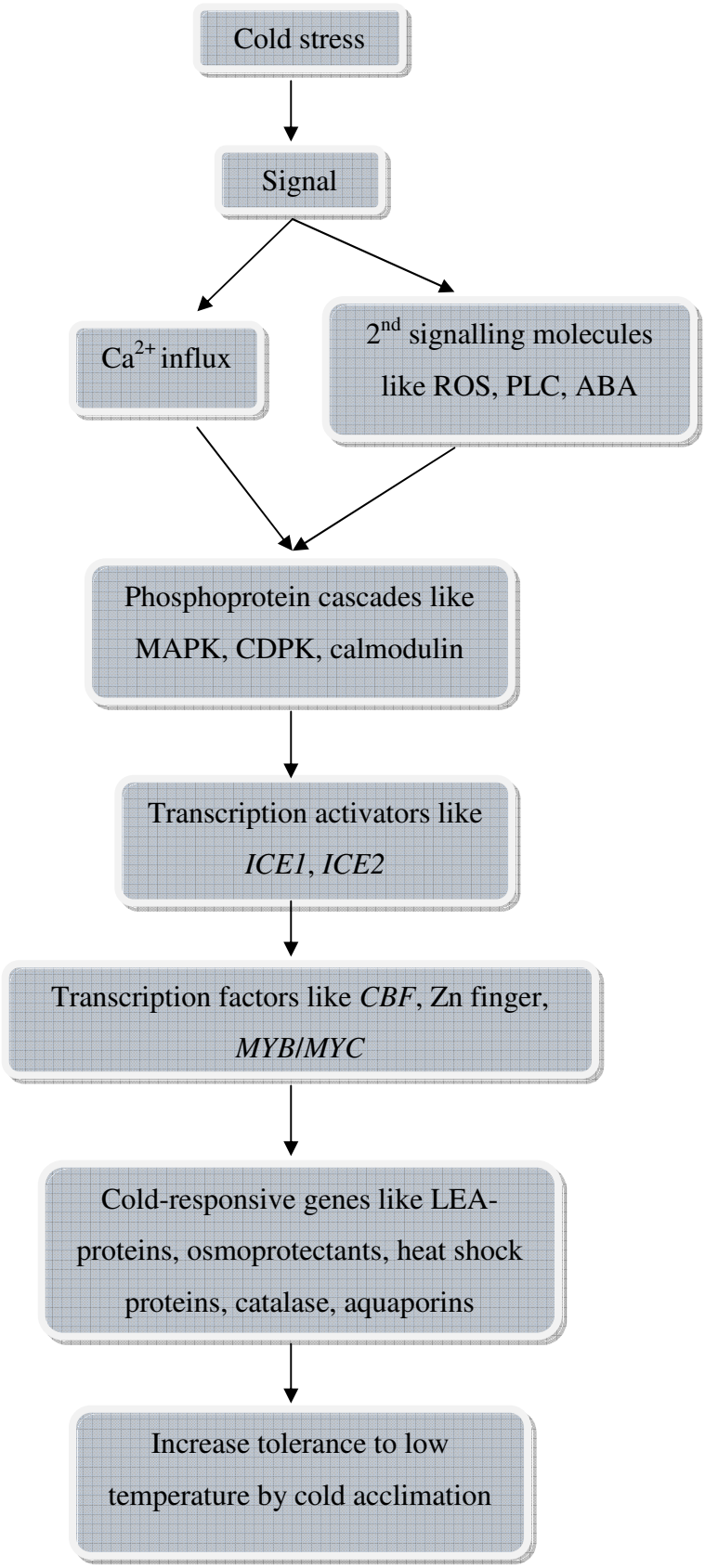

Figure 1.2. Schematic representation of the cold stress response in plants (adapted from Xiong *et al.* (2002)). The cold stress is sensed by receptors and a signal is induced by cytosolic calcium influx (Ca^{2+} infux) as well as other signalling molecules like reactive oxygen species (ROS), phosphoinositide phospholipase C (PLC) and abscisic acid (ABA). The signalling molecules activate the phosphoprotein cascades for example mitogen activated protein kinase (MAPK), calcium dependent protein kinase (CDPK) and calmodulin. By this activation, transcription activators like MYC-like basic helix–loop–helix transcriptional activator 1 (*ICE1*) and *ICE2* are induced and activates transcription factors like C-repeat binding factor (*CBF*), zinc finger (Zn finger) and *MYB*/*MYC*. These transcription factors regulate the expression of cold-responsive genes like late embryogenesis abundant proteins (LEA)-proteins, osmoprotectants, heat shock proteins, catalase and aquaporins. The up- or down-regulation of these genes increases the tolerance to low temperature in the plant by a process called cold acclimation.

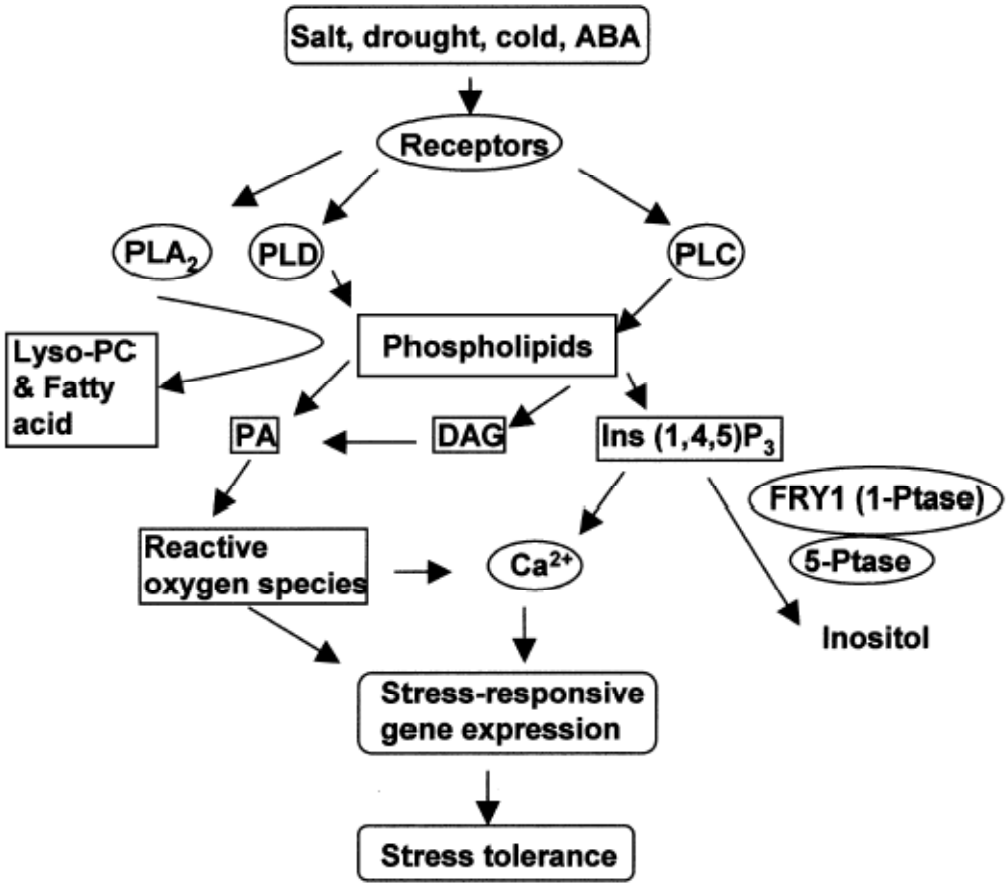

Figure 1.3. Phospholipid signalling during abiotic stress in plants (Zhu, 2002). Abiotic stresses are sensed by receptors. Phospholipase A_2 (PLA_2), phospholipase D (PLD) and phospholipase C (PLC) activate phospholipases to induce phosphatidic acid (PA), diacyglycerol (DAG) and 1,4,5-trisphosphate (IP_3), which are important in stress signalling. The levels of IP_3 are regulated by 1-phosphatase (FRY1) and 5-phosphatase.

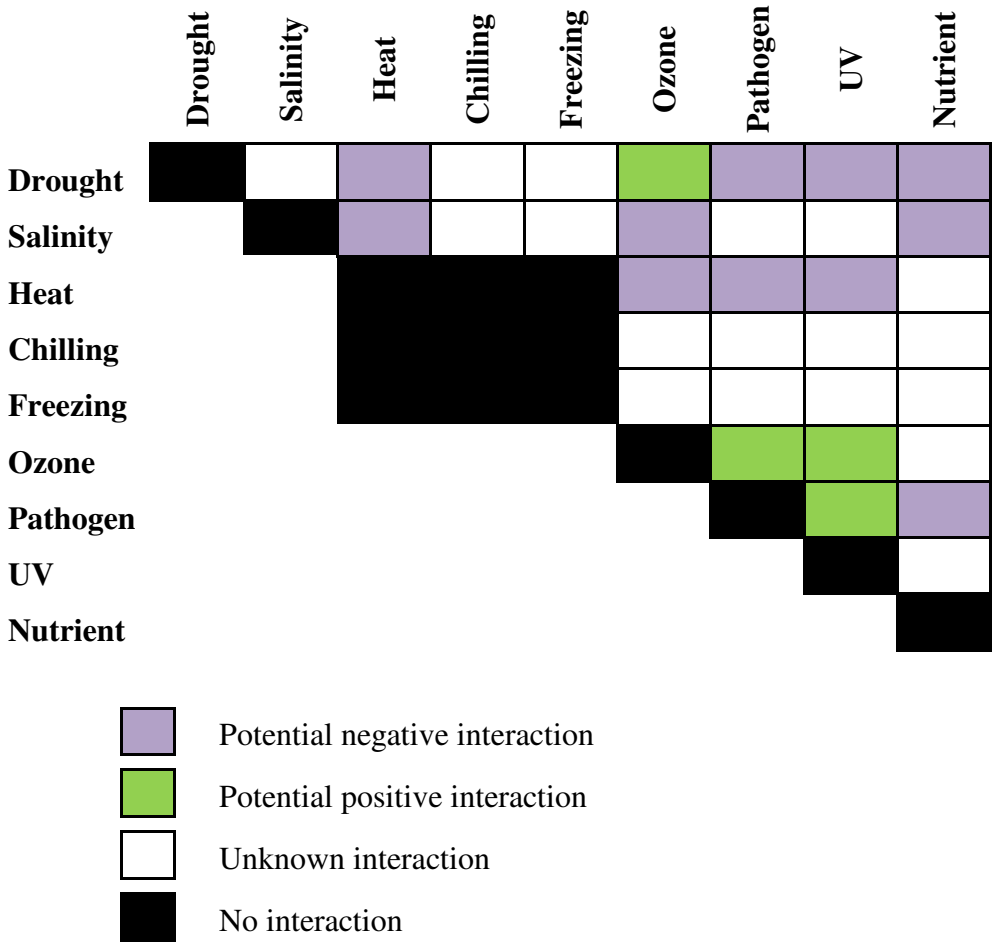

Figure 1.4. Interaction between different types of abiotic stresses in plants (Mittler, 2006). A potential negative reaction causes increased damage or stress (purple block), while a potential positive reaction decreases damage or stress (green block). No interactions are indicated by the black blocks, while unknown interactions are shown with white blocks.

Figure 1.5. Symptoms associated with low temperatures. A. Cold temperature leads to November dump where bunches are malformed and small (Daniells, 2005). B. Choke throat caused by cold temperature limits the movement of the inflorescense in the pseudostem (Lagerwall, 2005).

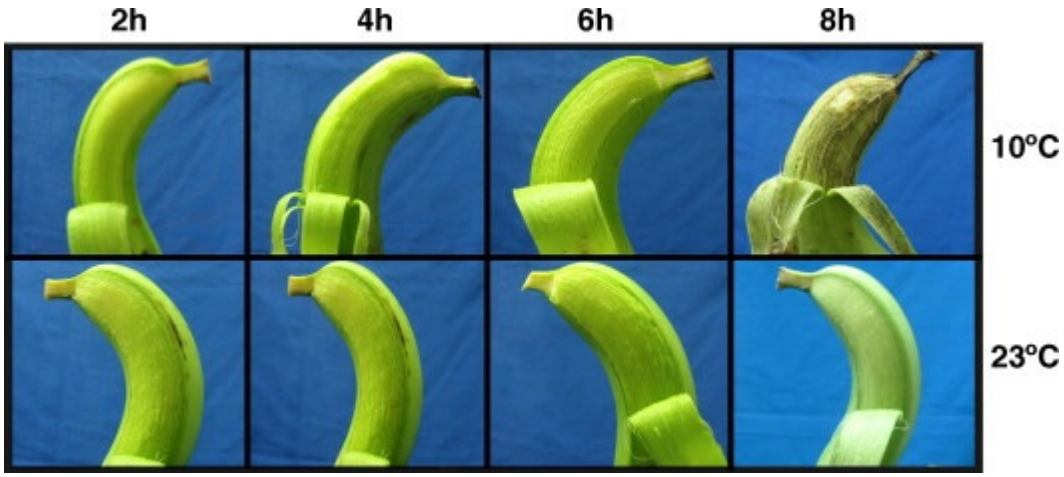

Figure 1.6. Underpeel discolouration of the banana fruit caused by low temperatures (Caamal-Velázquez *et al.*, 2007).

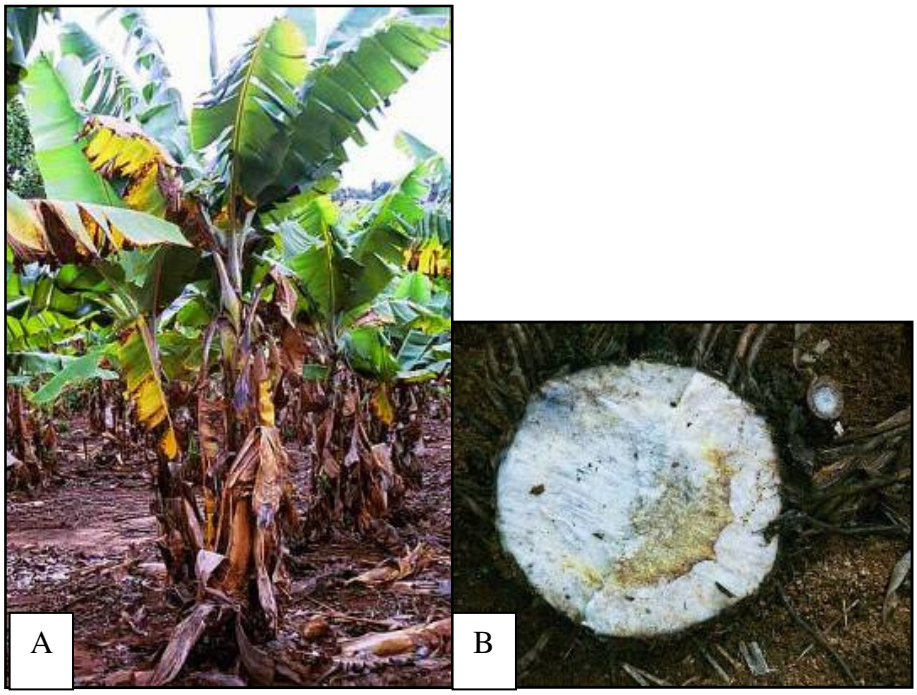

Figure 1.7. Visual disease symptoms in bananas caused by *Fusarium oxysporum* f. sp. *cubense*. A. External symptoms include severe wilting and yellowing from the older to the younger leaves. B. A cross-section through the pseudostem reveals a brown discoloration of the vascular system. Photos courtesy of Prof Altus Viljoen.

CHAPTER 2

PATHOGENICITY ASSOCIATED GENES IN *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* RACE 4

Sutherland R, Viljoen A, Myburg AA, Van den Berg N. (2013) Pathogenicity associated genes in *Fusarium oxysporum* f. sp. *cubense* race 4. South African Journal of Science 109 (5/6)

ABSTRACT

Fusarium oxysporum f. sp. *cubense* (*Foc*) is a fungus that infects banana roots and causes a destructive plant disease called Fusarium wilt. *Foc* consists of three pathogenic races (*Foc* races 1, 2 and 4) based on their selective impairment of banana cultivars. *Foc* race 4 is economically important as it comprises strains that infect Cavendish bananas, the most widely planted variety in the world, in both the tropics (*Foc* TR4) and subtropics (*Foc* STR4). The aim of this study was to investigate genes potentially involved in fungal pathogenicity by comparing transcript derived cDNA fragments (TDFs) from *Foc* STR4 and *Foc* TR4 to those from non-pathogenic *F. oxysporum* using cDNA-AFLP analysis. This resulted in the identification of 229 unique gene fragments which included the putative pathogenicity-related TDFs encoding chitinase class V (*chsV*), GTPase activating protein, Major Facilitator Superfamily (MFS) multidrug transporter and serine/threonine protein kinase (*ste12*) genes. Quantitative analysis of transcript abundance showed a significant increase in expression of *chsV*, MFS multidrug transporter and *ste12* genes in *Foc* STR4 and *Foc* TR4 when compared to the non-pathogenic *F. oxysporum*. These genes play a role in escaping host defence responses and in cell wall degradation. In addition, pathogenicity-related genes from other *formae speciales* of *F. oxysporum*, such as the sucrose non-fermenting, cytochrome P450 and F-box protein required for pathogenicity genes, were significantly up-regulated in *Foc* STR4 and *Foc* TR4 but not in *F. oxysporum* isolates non-pathogenic to banana. This study provides the first *in vitro* comparative analysis of TDFs expressed in pathogenic *Foc* race 4 isolates compared to non-pathogenic *F. oxysporum* isolates from banana.

INTRODUCTION

The vascular wilt fungus *Fusarium oxysporum* is a soil-borne facultative parasite that causes disease in more than 100 plant species, including important agricultural crops (Michielse and Rep, 2009). The fungus is a morphospecies that is divided into specialized groups (i.e. *formae speciales*) according to the hosts they attack, and subdivided into races according to the susceptibility of specific host cultivars (Gordon and Martyn, 1997). Host specificity is believed to have evolved independently in *F. oxysporum*, and does not necessarily reflect phylogenetic relatedness among pathogenic members of the individual hosts (Gordweon and Martyn, 1997). In *F. oxysporum*, host specificity has been attributed to mutations in avirulence genes and lateral chromosome transfer that overcome defence responses in the host plant (Ma *et al.*, 2010; Rep and Kistler, 2010).

Fungal pathogenicity genes are responsible for events such as spore attachment and germination, infection and colonization of the host, and are divided into categories such as formation of infection structures, cell wall degradation, toxin biosynthesis and signalling (Idnurm and Howlett, 2001; Werner *et al.*, 2007; Möbius and Hertweck, 2009). Certain pathogenicity genes also encode proteins that are involved in the suppression or disruption of host defence mechanisms (Chi *et al.*, 2009; De Wit *et al.*, 2009). In *F. oxysporum*, genes that encode cell wall degrading enzymes (CWDE), such as endo-polygalacturonase (*pg1*), exo-polygalacturonase (*pgx4*), pectate lyase (*pl1),* xylanase and a plant defence detoxifying enzyme like tomatinase, have been identified in *F. oxysporum* f. sp. *lycopersici* (*Fol*) (Di Pietro and Roncero, 1998; Huertas-González *et al.*, 1999; Roldán-Arjona *et al.*, 1999; Ruiz-Roldán *et al.*, 1999; García-Maceira *et al.*, 2000). Pathogenicity is also influenced by the expression of CWDE which are regulated by sucrose non-fermenting (*snf*) gene in *F. oxysporum* strain O-685 and the F-box protein required for pathogenicity (*frp1*) gene in *Fol* (Di Pietro *et al.*, 2001; Ospina-Giraldo *et al.*, 2003; Duyvesteijn *et al.*, 2005). Signalling genes expressed during pathogenesis have also been identified in *Fol* (e.g. *Fusarium* mitogen-activated protein kinase (*fmk1*)) (Di Pietro *et al.*, 2001) and *F. oxysporum* f. sp. *cucumerinum* (e.g. G protein α subunit (*fga1)* and G protein b subunit (*fgb1)*) (Jain *et al.*, 2002; Jain *et al.*, 2003). Several transcription factors that regulate pathogenicity

genes during infection have been discovered in *F. oxysporum*, such as serine/threonine protein kinases (*ste12*) (Rispail and Di Pietro, 2009), a $Zn(II)_2Cys_6$-type transcription regulator (*fow2*) (Imazaki *et al.*, 2007) and *F. oxysporum ste12* homolog (*fost12*) (Garcia-Sanchez *et al.*, 2010).

Strains of *F. oxysporum* pathogenic to bananas are known as *F. oxysporum* f. sp. *cubense* (*Foc*). Three races of *Foc* are recognized based on their ability to cause disease in a set of different banana cultivars, with *Foc* race 1 affecting Gros Michel, Silk and Pome bananas and *Foc* race 2 affecting Bluggoe and other cooking bananas (Ploetz and Pegg, 2000). *Foc* race 4 affects Cavendish bananas, which make up 80% of the world's banana export, as well as *Foc* race 1 and 2 susceptible bananas (Ploetz, 2005). *Foc* race 4 is further subdivided into 'tropical' and 'subtropical' strains. Those belonging to *Foc* 'tropical' race 4 (TR4) are limited to tropical Asia and northern Australia, while *Foc* 'subtropical' race 4 (STR4) strains are mostly associated with Cavendish bananas in subtropical countries like South Africa, Australia, Taiwan and the Canary Islands. *Foc* TR4 is more virulent than *Foc* STR4, and can infect Cavendish bananas under stressed and non-stressed conditions, whereas *Foc* STR4 infects bananas typically after the host has been exposed to stressful environments (Ploetz and Pegg, 2000).

Despite the economic importance of *Foc* (Ploetz, 2005), the mechanisms of pathogenesis to banana are still poorly understood. Additionally, non-pathogenic strains of *F. oxysporum* are known to infect and colonize the cambium tissue of banana roots, but do not enter the xylem to cause Fusarium wilt. Occasionally the non-pathogens even protect the banana plant from damage caused by *Foc* (Thangavelu and Jayanthi, 2009; Belgrove *et al.*, 2011) and nematodes (Athman *et al.*, 2006). It is not known why non-pathogenic strains of *F. oxysporum* are unable to cause disease to banana. Therefore, the objective of this study was to identify gene transcripts that are present in *Foc* TR4 and *Foc* STR4 but absent in non-pathogenic *F. oxysporum* using cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis. In addition, quantitative reverse transcriptase PCR (qRT-PCR) was employed to study the transcript abundance of eight previously described pathogenicity genes from other *formae speciales* of *F. oxysporum* (Tomura *et al.*,

1994; Di Pietro *et al.*, 2001; Namiki *et al.*, 2001; Inoue *et al.*, 2002; Ospina-Giraldo *et al.*, 2003; Duyvesteijn *et al.*, 2005; Imazaki *et al.*, 2007; Cañero and Roncero, 2008b).

MATERIALS AND METHODS

Fungal isolates and culture conditions

Twenty seven *F. oxysporum* isolates were selected for this study. These included isolates of *Foc* STR4 from South Africa, Australia and the Canary Islands, isolates of *Foc* TR4 from Malaysia, Indonesia and Northern Australia, and non-pathogenic *F. oxysporum* isolates obtained from Cavendish banana roots in South Africa (Table 2.1). The non-pathogenic *F. oxysporum* isolates were shown to be non-pathogenic as no internal disease symptoms developed after inoculating banana roots with a spore suspension ($1x10^5$ spores/ml) in a hydroponic system (Athman *et al.*, 2006; Nel *et al.*, 2006; Van den Berg *et al.*, 2007; Belgrove *et al.*, 2011). All isolates are maintained in 15% glycerol at -80°C at the Department of Plant Pathology, Stellenbosch University.

RNA extraction

RNA was extracted from fungal mycelia grown *in vitro* rather than *in planta*, as insufficient genes of fungal origin were previously detected in the roots of tissue-cultured banana plants 14 days after inoculation with *Foc* race 4 ($1x10^5$ spore/ml). The *F. oxysporum* isolates were first grown on half strength potato dextrose agar (PDA) (19.5 g/L PDA and 10 g/L agar) for five days at ± 25°C, and transferred to liquid minimal medium (MM) without a carbon source to enhance the transcript abundance of pathogenicity genes (Trail *et al.*, 2003). After culturing the isolates in MM on a rotary shaker set at 90 rpm for five days at 25°C, the medium was filtered through sterile cheesecloth. The mycelial mass was scraped and frozen in liquid nitrogen, ground to a fine powder with a basic analytical mill (IKA A111, United Scientific (Pty) Ltd., San Diego, USA), and stored at -80°C until RNA was extracted.

RNA of each isolate was extracted from mycelia using Qiazol (Qiagen, Valencia, USA), quantified with a NanoDrop ND-1000 spectrophotometer (Nanodrop

Technologies, Inc., Montchanin, USA) and assessed by formaldehyde agarose gel electrophoresis (1.2%). RNA from three isolates collected from the same country/location were pooled (Table 2.1), *DNaseI*-treated (Fermentas) and column-purified with an RNeasy mini kit (Qiagen). Messenger RNA (mRNA) was isolated using the Oligotex mRNA mini kit (Qiagen). Double stranded cDNA was synthesized with the cDNA Synthesis System (Roche Diagnostics, Mannheim, Germany) using oligo dT_{15} primers and contamination was assessed by performing a PCR with the intron flanking primers EF1 and EF2 (O'Donnell *et al.*, 1998).

cDNA-AFLP analysis

Transcript expression levels of putative pathogenicity genes in *F. oxysporum* were assessed by cDNA-AFLP analysis. The AFLP® Expression Analysis Kit (LICOR) was employed according to the manufacturer's instructions to determine differential gene expression patterns. Briefly, cDNA was digested with the restriction enzymes *Taq*I and *Mse*I, followed by ligating adapters using T4 DNA ligase. Pre-selective amplification was performed with *Taq*I+0/*Mse*I+0 primers, and 31 different *Taq*I+2/*Mse*I+2 primer combinations were used during selective amplification (Table 2.2). Band intensities of differentially expressed fragments on cDNA-AFLP gels were visually assessed and divided into four groups, namely no transcripts detected (-), low level of transcript abundance (+), moderate level of transcript abundance (++) and high level of transcript abundance (+++). Band intensities corresponded to the original expression level.

Isolation of polymorphic fragments and sequence data analysis

After polyacrylamide gels were resolved on the LICOR analyzer and scanned with the Odyssey® infrared imaging system (LICOR), unique bands were identified using Quantity One 1-D analysis software (Bio-Rad Laboratories Inc., Hercules, CA, USA). Bands were excised and cloned into a vector with the InsTAclone™ PCR cloning kit (Fermentas) and sequenced in both directions. Vector sequences were manually removed from the raw sequences by Chromas 1.45 (www.technelysium.com.au/chromas.html), while BioEdit Sequence Alignment

Editor 7.0.5.3 software (Hall, 1999) was utilized to create a consensus sequence for each individual fragment. The consensus sequences were compared to *Fusarium* genome sequences at the Broad Institute (http://www.broad.mit.edu/annotation/genome/fusarium_group/Blast.html) and to the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification. The transcript derived fragments (TDFs) obtained with cDNA-AFLPs were further characterized using Desktop cDNA Annotation System (dCAS, NIAID, Bethesda, MD, USA) (Guo *et al.*, 2009). Functional groups were defined according to the MIPS (Ruepp *et al.*, 2004) and GO (Ashburner *et al.*, 2000) databases.

Quantitative analysis of transcript abundance

The transcript abundance of six putative pathogenicity genes identified by cDNA-AFLP in the current study, and eight known pathogenicity genes of *F. oxysporum* (Table 2.3), was assessed using a LightCycler 480 instrument (Roche Diagnostics). Five reference genes were also evaluated in the study (Table 2.3) and included the elongation factor 1α (*TEF*), ß-tubulin (*TUB*), isocitrate dehydrogenase (*IDH*), glucose-6-phosphate 1-dehydrogenase (*G6DH*) and glyceraldehyde 3-phosphate (*GAPDH*) genes. Primers for the putative and known pathogenicity and reference genes were designed using Primer3 (Whitehead Institute, MIT, Cambridge, MA, USA) and Netprimer (Premier Biosoft, Palo Alto, CA, USA) (Table 2.3) and synthesized by Operon Biotechnologies GmbH (Cologne, Germany).

RT-qPCR reactions were performed in 10-µl volumes containing cDNA template (1:10 dilution) and 1 µM of each of the forward and reverse primers and 5 µl DNA Master^{PLUS} SYBR Green mix (Roche Diagnostics). The protocol included 10 min at 95^{o}C followed by 55 cycles of 10 s at 95^{o}C, 10 s at 57^{o}C and 10 s at 72^{o}C. The amplification process was completed by a melting cycle from 55^{o}C to 95^{o}C to assess specificity. The fluorescence reading was recorded at 72^{o}C at the end of the elongation cycles. The PCR products were analysed by electrophoresis on a 2% agarose gel to verify that a single product of the expected size was produced. All reactions were performed in triplicate with three independent biological replicates as well as a negative control (no template) for all genes. A standard curve was generated

by preparing a dilution series (1:10, 1:100 and 1:1000) for each pathogenicity and reference gene. Gene expression stability (M-value) and pairwise variation (V-values) were determined using Genorm (Vandesompele *et al.*, 2002). Ct values were imported into qbase^{PLUS} (Biogazelle, Ghent, Belgium) for further analysis. The difference in Ct values was determined statistically by One-way ANOVA, followed by Tukey's post-hoc analysis where a p value of < 0.05 was considered as statistically significant.

RESULTS

cDNA-AFLP analysis

cDNA expression patterns of approximately 3150 transcripts were examined with 31 different TaqI+2/MseI +2 primer combinations. For each primer combination, 63-138 TDFs were visualized and varied from 100 to 700 bp with approximately 8% of the TDFs showing differential expression. cDNA-AFLP analysis allowed the identification and isolation of 229 differentially expressed TDFs of between 103 and 546 bp in size (Table 2.4). The TDFs were classified into functional categories, including hypothetical proteins from *Fusarium* (90) and other fungal species (six), energy metabolism (13), transport (13), cell division and growth (11), protein turnover (eight), cell signalling (nine), lipid/fatty acid metabolism (five), transcription and translation factors (six), and those with no significant homology (68) (Figure 2.1). BLAST analysis with an rRNA operon showed that only one TDF (0.4%) had homology to rRNA. Several TDFs represented genes with numerous functions, including pathogenicity. These TDFs included the putative chitinase class V (*chsV*) (TDF107), GTPase activating protein (*rhoI*) (TDF223), Major Facilitator Superfamily (MFS) multidrug transporter (TDF9), laccase (*lcc*) (TDF168), Ca^{2+} ATPase (TDF24) and serine/threonine protein kinase (*ste12*) (TDF214) genes (Table 2.4). The TDFs corresponding to *chsV*, *rhoI*, *lcc*, Ca^{2+} ATPase, and *ste12* showed low intensity levels in *Foc* STR4 and *Foc* TR4 as compared to non-pathogenic *F. oxysporum* where transcripts were not visually detected. The TDF representing the MFS multidrug transporter gene displayed moderate intensity in *Foc* STR4 and *Foc* TR4 compared to no detectable levels in non-pathogenic *F. oxysporum* (Figure 2.2A).

Several different transcript abundance patterns were detected during cDNA-AFLP gel analysis (Table 2.4). In the first pattern, high transcript abundance was detected in *Foc* STR4 with no transcripts detected in *Foc* TR4 or non-pathogenic *F. oxysporum*. Examples included TDFs corresponding to galactokinase (TDF57) and O-acetylhomoserine (TDF215). The second transcript abundance pattern showed an increase in transcripts in *Foc* TR4 with no transcripts detected in *Foc* STR4 or non-pathogenic *F. oxysporum.* TDFs that exhibited this pattern were 60S ribosomal protein L2 (TDF12), meiosis induction protein (TDF13), L-aminoadipate semialdehyde dehydrogenase large subunit (TDF64), fatty acid synthase subunit alpha reductase (TDF105), small G-protein Gsp1p (TDF174) and glutamine-dependent NAD+ synthetase (TDF190). In the third pattern, transcripts were detected in *Foc* STR4 and *Foc* TR4 with no detection in non-pathogenic *F. oxysporum*. Examples included Ca^{2+} ATPase (TDF24), *chsV* (TDF107), FAD-dependent oxidoreductase (TDF42), *ste12* (TDF214), GTPase activating protein (TDF223) and *lcc* (TDF168). Other transcript abundance patterns included transcript presence in *Foc* STR4 and the non-pathogenic strains, with no transcripts detected in *Foc* TR4. These transcripts included eukaryotic translation initiation factor 2 subunit gamma (TDF115) and ATP-cone (TDF204). Another pattern displayed low levels of transcript abundance in *Foc* STR4 with high transcript abundance in *Foc* TR4 and non-pathogenic *F. oxysporum*.

Quantitative verification of cDNA-AFLP

Five reference genes (Table 2.3) were evaluated for stable expression levels. The average pairwise variation (V-value) calculated for *IDH, G6DH* and *GAPDH* was 0.113, with *TEF* and *TUB* showing less stable expression levels (V=0.225). As a result, the reference genes *IDH, G6DH* and *GAPDH* were used to normalize the data as suggested by Vandesompele *et al.* (2002).

The relative transcript abundance of six genes measured by cDNA-AFLP analysis; encoding a MFS multidrug transporter (TDF9), a L-aminoadipate-semialdehyde dehydrogenase large subunit (TDF64), an aspartyl-tRNA synthetase (TDF52), a *chsV* (TDF107), a *ste12* (TDF214) and *rhoI* (TDF223); was compared to results obtained by qRT-PCR (Figure 2.2). Both cDNA-AFLP and qRT-PCR analyses showed an

increased abundance of the MFS multidrug transporter gene in *Foc* STR4 and *Foc* TR4 when compared to non-pathogenic *F. oxysporum* (Figure 2.2A). When the abundance levels of L-aminoadipate-semialdehyde dehydrogenase large subunit were compared, the cDNA-AFLP analysis demonstrated that the transcript was present in *Foc* TR4 compared to the absence of the transcript in *Foc* STR4 and the non-pathogenic *F. oxysporum*. The qRT-PCR data showed similar levels of transcript abundance in *Foc* TR4, *Foc* STR4 and the non-pathogenic *F. oxysporum* (Figure 2.2B). cDNA-AFLP analyses showed an increased abundance of transcripts of aspartyl-tRNA synthetase in *Foc* STR4 when compared to *Foc* TR4 and the non-pathogenic *F. oxysporum*, while qRT-PCR revealed similar transcripts levels between the different isolates (Figure 2.2C). An increase in transcript abundance of *chsV* was found in *Foc* STR4 and *Foc* TR4 when compared to the non-pathogenic *F. oxysporum* using both cDNA-AFLP analysis and qRT-PCR (Figure 2.2D). Transcript abundance profiles were similar for *ste12* in cDNA-AFLP and qRT-PCR, which showed an increase in *Foc* STR4 and *Foc* TR4 compared to the non-pathogenic *F. oxysporum* (Figure 2.2E). In the case of *rhoI*, cDNA-AFLP analysis showed an increase in the number of transcripts in *Foc* race 4 compared to the non-pathogenic *F. oxysporum* (Figure 2.2F). However, qRT-PCR showed an increase in quantity of transcripts in *Foc* STR4 compared to *Foc* TR4 and the non-pathogenic *F. oxysporum*. Thus, the transcript abundance patterns measured with qRT-PCR were similar to that measured for the corresponding TDFs analyzed during the cDNA-AFLP analysis.

Transcript abundance of known pathogenicity genes in *Foc* STR4, *Foc* TR4 and non-pathogenic *F. oxysporum* with qRT-PCR

Foc STR4 and *Foc* TR4 expressed the pathogenicity genes *snf* (Figure 2.3A), *frp1* (Figure 2.3B) and *cyp55* (Figure 2.3C) at significantly higher levels when compared to non-pathogenic *F. oxysporum*. In the case of *snf*, *Foc* STR4 had a 2.6-fold increase in transcript abundance compared to the non-pathogenic *F. oxysporum*. The transcript abundance levels of *frp1* was reduced in non-pathogenic *F. oxysporum* isolates when compared to pathogenic *Foc* STR4 and *Foc* TR4 isolates, by 3.6- and 2.5-fold, respectively. *Snf* and *frp1* are involved in the degradation of plant cell walls (Ospina-Giraldo *et al.*, 2003; Jonkers *et al.*, 2009). *Cyp55* had a 1.6-fold increase in expression

in *Foc* TR4 compared to *Foc* STR4, but this increase was not statistically significant. *Cyp55* is a nitric oxide reductase involved in the nitrogen response pathway, which is fundamental for pathogenicity.

Fmk1 is responsible for maintaining fungal cell wall architecture and signalling whereas *clc* controls laccase activity. *Fmk1* (Figure 2.3D) was expressed significantly more in *Foc* STR4 than in either *Foc* TR4 or non-pathogenic *F. oxysporum*. *Fmk1* expression was 2.9-fold higher in *Foc* STR4 when compared to the non-pathogenic *F. oxysporum* (Figure 2.3D). In addition, there was a significant 2.1-fold increase in transcript abundance of *fmk1* in *Foc* STR4 compared to *Foc* TR4. The chloride channel (*clc*) gene also had a significant increased expression in *Foc* STR4 than in the non-pathogenic *F. oxysporum* (Figure 2.3E). In contrast, the fungal gene involved in regulating pathogenicity-related transcription, *fow2* (Figure 2.3F), was expressed significantly more in *Foc* TR4 than it was in the non-pathogenic *F. oxysporum*, but not significantly more than in *Foc* STR4. There were no significant differences observed in the transcript abundance profiles of arginine biosynthesis gene (*arg1*) (Figure 2.3G) and mitochondrial protein (*fow1*) (Figure 2.3H).

DISCUSSION

The transcriptomes of *Foc* STR4, *Foc* TR4 and non-pathogenic *F. oxysporum* isolates on MM medium (without carbon source) were visually detected with cDNA-AFLP. More than 3000 TDFs were detected of which 8% showed differential expression patterns. A total of 3% of these TDFs were putatively involved in pathogenicity. Several fungal gene transcripts that have previously been associated with pathogenicity in other fungal organisms have been identified for the first time in the banana pathogen *Foc*. These include *chsV*, *rhoI*, MFS multidrug transporter and *stel2*. In addition, genes that resulted in disease of crops other than banana that were more abundantly expressed in *Foc* STR4 and *Foc* TR4 than in non-pathogenic *F. oxysporum* include *snf*, *frp1* and *cyp55.*

ChsV and *rhoI* have previously been associated with pathogenicity in *Fol* on tomato (Madrid *et al.*, 2003; Martínez-Rocha *et al.*, 2008). *ChsV* restricts toxic substances

produced by the plant for its defence against pathogens (Madrid *et al.*, 2003), and *rho1* plays a role in preventing the host plant from recognising the pathogen (Martínez-Rocha *et al.*, 2008). Both genes, therefore, protect the pathogen against the host's defence response. Since *chsV* and *rho1* showed an increased transcript abundance in *Foc* STR4 and *Foc* TR4 compared to the non-pathogen, we hypothesize that *Foc* expresses these genes when infecting the xylem vessels of Cavendish bananas to avoid the plant's defence responses.

The MFS multidrug transporter showed a five-fold increase in transcript abundance in pathogenic *Foc* when compared to the non-pathogen. This family of transporters regulates the movement of sugars, Krebs-cycle metabolites, phosphorylated glycolytic intermediates, amino acids, peptides, osmolites, iron-siderophores, nucleosides and organic and inorganic anions and cations (Stergiopoulos *et al.*, 2002). In addition, MFS transporters have been linked to fungal pathogenicity by avoiding toxic compounds produced by the pathogen, or by protection against plant defence compounds (Del Sorbo *et al.*, 2000). MFS transporter gene in the ascomycete, *Verticillium dahlia*, a vascular pathogen, is essential for pathogenicity on lettuce plants (Maruthachalam *et al.*, 2011). With the significant increase in transcript abundance of a MFS multidrug transporter, *Foc* STR4 and *Foc* TR4 may possibly protect itself from toxic substances produced by the plant during defence.

The transcription factor *ste12* is important during fungal infection of plant roots where it regulates genes involved in the MAPK cascade (Rispail and Di Pietro, 2009, 2010). In a study by Garcia-Sanchez *et al.* (2010), a *ste12*-like gene, *fost12*, showed an increased expression after 12-24 h of infection of bean plants by *F. oxysporum* f. sp. *phaseoli*. A significant increase in transcript abundance of *ste12* in *Foc* STR4 and *Foc* TR4 can activate the MAPK signalling pathways, thereby increasing CWDE during the infection process. A second transcription factor, *fow2* (Imazaki *et al.*, 2007), a $Zn(II)_2Cys_6$-type transcription regulator involved in pathogenicity in *F. oxysporum* f. sp. *melonis*, showed a significant increase in *Foc* TR4 compared to the non-pathogen, but with no significant difference between *Foc* STR4 and the non-pathogen. Since *Foc* TR4 is a more virulent pathogen than *Foc* STR4, *fow2* may assist in the more

rapid invasion of root tissue or may be differentially regulated in *Foc* STR4 and *Foc* TR4.

Two well-studied pathogenicity genes previously isolated from *F. oxysporum,* that regulate the abundance of CWDE are *snf* and *frp1* (Ospina-Giraldo *et al.*, 2003; Duyvesteijn *et al.*, 2005; Jonkers *et al.*, 2009). Both *snf* and *frp1* showed a significant increase in *Foc* STR4 and *Foc* TR4 as opposed to the non-pathogen, which suggests that these genes are important for the Fusarium wilt pathogen to enter the host xylem tissue. As an endophyte, the non-pathogenic *F. oxysporum* isolates are usually restricted to the root cortex, and do not enter the xylem vessels (MacHardy and Beckman, 1981). In contrast, *Foc* STR4 and *Foc* TR4 both have to degrade the xylem cell walls to enter the vascular tissue.

Pathogenicity and cell wall degradation are affected by the enhanced expression of MAP kinases in several fungi, for example *Fol* (Di Pietro *et al.*, 2001), *Fusarium graminearum* (Jenczmionka and Schäfer, 2005), *Magnaporthe grisea* (Xu and Hamer, 1996) and *Ustilago maydis* (Mayorga and Gold, 1999). In *Fol, fmk1* also aids in root attachment, penetration, invasive growth and increased CWDE activity (Di Pietro *et al.*, 2001). The significant increase in *fmk1* in *Foc* STR4 and non-significant increase in *Foc* TR4 compared to non-pathogenic *F. oxysporum* may explain pathogenesis in the banana Fusarium wilt pathogen by accelerating invasive growth, as in other *Fusarium* species (Di Pietro *et al.*, 2001; Zhang *et al.*, 2011). Pathogenic *Foc* isolates are able to colonize both the cortex and the xylem tissue, resulting in severe discoloration of the corm and blocking of the vascular bundles. In contrast, the non-pathogenic strains are restricted to the root cortex, resulting in no symptom development. The reason that *fmk1* did not show a significant increase in transcript abundance in *Foc* TR4 is not certain, but one possible explanation could be that *fmk1* transcripts amplified during pathogenicity at earlier time points, were not sampled in this study. Genes expressed during the early time points are either translated into proteins or the RNA is degraded as the half-life of RNA is short and therefore cannot be detected at later time points.

Cyp55 in *Foc* race 4 was more abundant compared to that in non-pathogenic *F. oxysporum.* This gene plays a role in the ability to regulate the nitrogen response pathway, which is essential for pathogenicity (Lopez-Berges *et al.*, 2010). *Cyp55*, a cytochrome P450 gene involved in the reduction of nitric oxide in *F. oxysporum*, was first characterized by Kizawa *et al*. (1991). *Cyp55* gene of *F. oxysporum* f. sp. *vasinfectum* was also previously reported to be highly expressed in cotton plants following root inoculation (McFadden *et al.*, 2006).

Laccases serve as virulence factors in fungal pathogens by playing a role in pigmentation, appressorium formation and protection against toxic phytoalexins (Mayer and Staples, 2002). qRT-PCR analysis in this study revealed a significant increase in *clc* transcripts in *Foc* STR4 compared to the non-pathogen. In *Fol*, mutations of *lcc1*, *lcc3* and *lcc5* had no effect on the pathogenicity to tomato plants (Cañero and Roncero, 2008a). As six *lcc* genes have been identified in *F. oxysporum*, Cañero and Roncero (2008a) suggested that a mutation in one of them may not necessarily prevent laccase activity, as the other isozymes fulfil their role. *Clc* mutants, however, showed a decrease in laccase activity with a reduction in virulence to tomato seedlings (Cañero and Roncero, 2008b). Increased *clc* expression and the role of laccases and choride transport in the banana Fusarium wilt pathogen may be important pathogenicity determinants.

The cDNA-AFLP technique was useful in differentiating the transcript abundance of genes present in *Foc* race 4 and non-pathogenic *F. oxysporum*. However, DNA sequence differences could result in the absence/presence of a TDF not necessarily implicating differential expression. To minimize these SNP polymorphisms, nine isolates from different geographic regions were combined for each of the *Foc* STR4, *Foc* TR4 and non-pathogenic *F. oxysporum* fungal samples. Most of the gene expression patterns measured by cDNA-AFLPs were confirmed by qRT-PCR analyses. However, next-generation DNA and RNA sequencing could provide significantly better results for identifying pathogenicity genes in *Foc*, both in STR4 and TR4, especially once the full genome sequence of the Fusarium wilt fungus becomes available. Comparison of the *Foc* genome with that of other *forma speciales* of *F. oxysporum* will elucidate the ability of *Foc* to infect banana roots. Virulence

factors can be studied when the genomes of *Foc* TR4, a more virulent pathogen, are compared to *Foc* STR4. Furthermore, the function of putative pathogenicity genes during infection should be investigated by gene knockout studies and RNAi silencing. Knockout mutants would help to identify additional genes required for pathogenicity in *Foc* race 4.

An in-depth understanding of pathogenicity in *Foc* is required if novel approaches to disease management are to be developed. We have identified several transcripts in *Foc* race 4 that are more abundant in the pathogenic strains compared to the non-pathogens. Many of these TDFs have been shown to play a role in host infection and colonization by other Fusarium *spp*. These TDFs encode for CWDE and proteins involved in avoiding toxic substances produced during plant defence. To establish function, knockout mutants of genes underlying these transcripts need to be generated, and the role of genes such as *chsV, rhoI*, MFS multidrug transporter, *ste12, snf, frp1, cyp55* and *fmk1* needs further investigation. With the rapid advancement in molecular techniques in recent years, new strategies for increasing plant resistance against specific Fusarium wilt pathogens can be generated by exploiting the molecular and cellular basis of pathogenicity.

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TABLES AND FIGURES

Table 2.1. *Fusarium oxysporum* isolates used for cDNA-AFLP and quantitative reverse transcriptase PCR analysis.

CAV number[a]	Strain	VCG	Host	Region	Origin	Grouped
CAV 045	*Foc* STR4[b]	120	Williams	Port Edward	South Africa	S1[e]
CAV 092	*Foc* STR4	120	Grand Naine	Kiepersol	South Africa	S1
CAV 105	*Foc* STR4	120	Cavendish	Kiepersol	South Africa	S1
CAV 179	*Foc* STR4	120	Not available	Wamuran, Queensland	Australia	S2
CAV 1116	*Foc* STR4	120	Cavendish	Wamuran, QLD	Australia	S2
CAV 1180	*Foc* STR4	120	Cavendish	Byron Bay, NSW	Australia	S2
CAV 291	*Foc* STR4	120	Cavendish	Canary Islands	Canary Islands	S3
CAV 292	*Foc* STR4	120	Dwarf Cavendish	Las Galletas	Canary Islands	S3
CAV 981	*Foc* STR4	0120/15	Grand Naine	Canary Island	Canary Island	S3
CAV 858	*Foc* TR4[c]	1216	Cavendish	Malaysia	Malaysia	T1
CAV 865	*Foc* TR4	1216	Cavendish	Malaysia	Malaysia	T1
CAV 870	*Foc* TR4	1216	Cavendish	Malaysia	Malaysia	T1
CAV 302	*Foc* TR4	1213	Williams	Southeast Sumatra	Indonesia	T2
CAV 604	*Foc* TR4	1216	Grand Naine	Indonesia	Indonesia	T2
CAV 811	*Foc* TR4	1213	Cavendish	Indonesia	Indonesia	T2
CAV 789	*Foc* TR4	01213/16	Cavendish	Middle point, Northern Territory	Australia	T3
CAV 1065	*Foc* TR4	01213/16	Grand Naine	Lambell's lagoon, NT	Australia	T3
CAV 1072	*Foc* TR4	01213/16	Cavendish	Darwin, NT	Australia	T3
CAV 255	*F. o*[d]		Soil, *Musa sp.*	Kiepersol	South Africa	N1
CAV 241	*F. o*		Soil, *Musa sp.*	Kiepersol	South Africa	N1
CAV 282	*F. o*		Soil, *Musa sp.*	Kiepersol	South Africa	N1
CAV 552	*F. o*		Roots, *Musa sp.*	Kiepersol	South Africa	N2
CAV 553	*F. o*		Roots, *Musa sp.*	Kiepersol	South Africa	N2
CAV 560	*F. o*		Roots, *Musa sp.*	Kiepersol	South Africa	N2
CAV 744	*F. o*		Roots, *Musa sp.*	Tzaneen	South Africa	N3
CAV 745	*F. o*		Roots, *Musa sp.*	Tzaneen	South Africa	N3
CAV 750	*F. o*		Roots, *Musa sp.*	Tzaneen	South Africa	N3

[a] Number of the isolate in the culture collection of Altus Viljoen
[b] *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4
[c] *F. oxysporum* f. sp. *cubense* 'tropical' race 4
[d] Non-pathogenic *F. oxysporum*
[e] Isolates with the same designation were grouped for DNA and RNA extraction

Table 2.2. Primers used for the selective amplification of cDNA-AFLP fragments in *Fusarium oxysporum*.

Primers	
Labelled *Taq*I primer +2	*Mse*I primer +2
T[a]-GA	M[b]-TG
T-TG	M-AC
T-GT	M-TC
T-TC	M-GT
T-AC	M-AC
T-AG	M-AG
T-TC	M-TC
T-AG	M-AC
T-GT	M-GA
T-TG	M-TG
T-CA	M-TG
T-GA	M-CT
T-AG	M-GT
T-TC	M-CA
T-CT	M-TC
T-CT	M-AG
T-GT	M-AG
T-AG	M-TC
T-CA	M-GA
T-TC	M-CT
T-AC	M-GT
T-GA	M-GT
T-TC	M-AC
T-GT	M-TG
T-AC	M-AG
T-AC	M-CA
T-TC	M-AC
T-TC	M-CT
T-TG	M-GA
T-CA	M-CA
T-GA	M-AC

[a]T=*Taq*I primer: 5' CTCGTAGACTGCGTAC 3'

[b]M=*Mse*I primer: 5' GATGAGTCCTGAGTAA 3'

Table 2.3. Primer sequences of genes from *Fusarium spp.* used in quantitative reverse transcriptase PCR analysis.

Primer ID	NCBI accession number	Gene identity	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Product size	Reference
TDF9	-	Major facilitator superfamily multidrug transporter	CATGGGCCTCGTGAATATGT	CCTGGATGCCTTGTCAAGTT	97	
TDF52	-	Aspartyl-tRNA synthetase	CGAAGACGATGAAGGGTGAT	GCTTACCCCTCAACTGCAAC	96	
TDF64	-	L-aminoadipate-semialdehyde dehydrogenase large subunit	CGAACACCAAGAGTGGATCA	ACCATGACAGCTCCGATCTC	87	
TDF107	-	Putative chitinase class V	TGCAATTCCTTGAGGCTCTT	TCACCAGCAAAGTGCTTGAC	145	
TDF214	-	Serine/threonine protein kinase	ACCTTGGCTCACTCGAAGAA	TACTTGAGGGTGGGGTTGAG	99	
TDF223	-	GTPase-binding protein gene	CTGCCAAGGTCTCCCTATCA	GGCTTCTGACTGGTCTTTCG	96	
snf1	AF420488.1	*Fusarium oxysporum* protein kinase SNF1 gene	GGTCGGTATCTTGCCTTCAA	GGGAGGTTCGTCGTTGATAA	115	(Ospina-Giraldo *et al.*, 2003)
frp1	AY673970.1	*Fusarium oxysporum* f. sp. *lycopersici* Frp1 gene	CCTCCAAATCGTGGCATACT	CCCGCATAGATGTTGGAAGT	143	(Duyvesteijn *et al.*, 2005)
cyp55	D14517.1	*Fusarium oxysporum* cyp55A1 gene for cytochrome P450nor	TTATCGCATCCAACCAGTCA	GCAAGATGCTCAGCGATACA	142	(Tomura *et al.*, 1994)
fmk1	AF286533.1	*Fusarium oxysporum* f. sp. *lycopersici* mitogen-activated protein kinase gene	GGAGCTGATGGAGACGGATA	CGGAGGGTCTGGTAGATGAA	90	(Di Pietro *et al.*, 2001)
clc	EU030436.1	*Fusarium oxysporum* f. sp. *lycopersici* CLC voltage-gated chloride channel gene	ACCATATCCGTGGTGGTCAT	AATTCGCTGACAGCTTTGGT	101	(Cañero and Roncero, 2008b)

Primer ID	NCBI accession number	Gene identity	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Product size	Reference
fow2	AB266616.1	*Fusarium oxysporum* FOW2 gene for $Zn(II)_2Cys_6$ transcription factor	ATGCCACCCTGTTTGAGAAG	GAGGAGCCATCGTCGAGTAG	148	(Imazaki *et al.*, 2007)
arg1	AB045736.1	*Fusarium oxysporum* ARG1 gene for argininosuccinate lyase	GCATGGTCTGCTTGAAGTGA	GACGCTCGTTTGCAGTATGA	145	(Namiki *et al.*, 2001)
fow1	AB078975.1	*Fusarium oxysporum* plasmid pWB60Sl FOW1 gene for putative mitochondrial carrier protein	CGAGATCACCAAGCACAAGA	CGTTGACACCCTTGTTGATG	116	(Inoue *et al.*, 2002)
TEF	AF008486.1	Elongation factor	TCGTCGTCATCGGCCACGTC	CGATGACGGTGACATAGTAG	243	(O'Donnell *et al.*, 1998)
TUB	AF008529.1	ß-tubulin gene	CCCCGAGGACTTACGATGTC	CGCTTGAAGAGCTCCTGGAT	68	
IDH	XM_385909.1	*Gibberella zeae* PH-1 isocitrate dehydrogenase	AGTCCGTCGCTTCTCTCAAG	AAGCTGATGCTGGCGTAAAT	133	
G6DH	XM_381455.1	*Gibberella zeae* PH-1 glucose-6-phosphate 1-dehydrogenase	ATATTCCCCGAAACGAGCTT	ATGCTGAGACCAGGCAACTT	88	
GAPDH	XM_386433.1	*Gibberella zeae* PH-1 glyceraldehyde 3-phosphate	CCAGATCAAGCAGGTCATCA	GTTGGTGTTGCCGTTAAGGT	106	

Table 2.4. Putative identities of selected genes identified in *Fusarium oxysporum* using cDNA-AFLP analysis based on BLASTx results obtained from the Broad Institute Database.

Transcript derived fragment	Most similar homologous protein	Species of homologous protein	Accession number	E-value	GO number	Transcript abundance patterns[a]		
						Foc STR4	*Foc* TR4	NP
TDF8	Hypothetical protein similar to glycosyl transferase	*Fusarium verticillioides*	FVEG_12066	$4.0e^{-5}$	GO: 0016757	+++	+++	++
TDF9	MFS multidrug transporter	*Aspergillus fumigatus*	EDP53405.1	$2.0e^{-10}$	GO: 0015238	++	++	-
TDF12	60S ribosomal protein L2	*F. oxysporum*	FOXG_01889	$3.0e^{-47}$	GO: 0003735	-	+	-
TDF13	Hypothetical protein similar to meiosis induction protein kinase Ime2	*F. verticillioides*	FVEG_11244	$7.0e^{-41}$	GO: 0004672	-	+	-
TDF15	Heat shock protein 60, mitochondrial precursor	*F. oxysporum*	FOXG_07996	$7.0e^{-18}$	GO: 0005524	+++	++	+++
TDF24	Hypothetical protein similar to Ca2+ ATPase	*F. oxysporum*	FOXG_10713	$2.0e^{-16}$	GO: 0005388	+	+	-
TDF25	Hypothetical protein similar to coatomer subunit delta	*F. verticillioides*	FVEG_07091	$2.0e^{-13}$	GO: 0042802	+++	-	-
TDF32	Vacuolar protease A precursor	*F. oxysporum*	FOXG_12714	$3.0e^{-12}$	GO: 0004175	+++	+++	+
TDF39	Hypothetical protein similar to RING-8 protein	*F. oxysporum*	FOXG_00847	$1.0e^{-4}$	GO: 0005515	++	+++	+++
TDF42	Hypothetical protein similar to FAD-dependent oxidoreductase	*F. graminearum*	FGSG_09373	$1.0e^{-13}$	GO: 0016491	+	+	-

Transcript derived fragment	Most similar homologous protein	Species of homologous protein	Accession number	E-value	GO number	Transcript abundance patterns[a]		
						Foc STR4	*Foc* TR4	NP
TDF52	Aspartyl-tRNA synthetase	*F. graminearum*	FGSG_09373	0.0	GO: 0004815	++	-	-
TDF53	Ubiquitin carboxyl-terminal hydrolase 6	*F. oxysporum*	FOXG_03651	$2.0e^{-97}$	GO: 0004843	++	++	+
TDF55	Hypothetical protein similar to protein kinase	*F. oxysporum*	FOXG_03168	$3.0e^{-17}$	GO: 0004672	+++	+	-
TDF56	Origin recognition complex subunit 1	*F. oxysporum*	FOXG_00048	$4.0e^{-17}$	GO: 0003677	++	+++	++
TDF57	Hypothetical protein similar to galactokinase	*F. oxysporum*	FOXG_11551	$3.0e^{-31}$	GO: 0005353	+++	-	-
TDF59	Hypothetical protein similar to hexose transporter	*F. oxysporum*	FOXG_12267	$1.0e^{-7}$	GO: 0005353	++	+	-
TDF64	L-aminoadipate-semialdehyde dehydrogenase large subunit	*F. oxysporum*	FOXG_11115	$5.0e^{-39}$	GO: 0004043	-	+	-
TDF70	Hypothetical protein similar to transporter protein smf2	*F. verticillioides*	FVEG_03655	$2.0e^{-15}$	GO: 0005384	++	++	-
TDF80	ATP-dependent helicase NAM7	*F. oxysporum*	FOXG_05494	$2.0e^{-35}$	G0: 0003682	++	++	-
TDF89	ER lumen protein retaining receptor 1	*F. oxysporum*	FOXG_11078	$8.0e^{-30}$	GO: 0005046	++	+	+
TDF105	Fatty acid synthase subunit alpha reductase	*F. verticillioides*	FVEG_04241	$3.0e^{-15}$	GO: 0004315	-	+	-
TDF107	Hypothetical protein similar to class V chitinase	*F. graminearum*	FGSG_02354	$4.0e^{-17}$	GO: 0004568	+	+	-

Transcript derived fragment	Most similar homologous protein	Species of homologous protein	Accession number	E-value	GO number	Transcript abundance patterns[a]		
						Foc STR4	*Foc* TR4	NP
TDF108	Urease	*F. oxysporum*	FOXG_01071	$3.0e^{-68}$	GO: 0004497	++	++	-
TDF115	Eukaryotic translation initiation factor 2 subunit gamma	*F. oxysporum*	FOXG_01983	$1.0e^{-21}$	GO: 0003743	+	-	+
TDF138	Protein SEY1	*F. verticillioides*	FVEG_00725	$2.0e^{-20}$	GO: 0005525	+	+	-
TDF140	Serine/threonine-protein kinase hal4	*F. graminearum*	FGSG_06939	$1.0e^{-8}$	GO: 0004674	+	+	-
TDF141	Protein SEY1	*F. verticillioides*	FVEG_00725	$6.0e^{-25}$	GO: 0005525	++	-	++
TDF144	Glutamine synthetase	*F. graminearum*	FGSG_10264	$3.0e^{-4}$	GO: 0006541	+++	+++	-
TDF147	Hypothetical protein similar to BET3 family protein	*F. verticillioides*	FVEG_04550	$5.0e^{-36}$	GO: 0006888	+	+	-
TDF151	Hypothetical protein similar to HAD-superfamily hydrolase subfamily IIB	*F. oxysporum*	FOXG_16804	$3.0e^{-37}$	GO: 0016787	+	-	-
TDF154	Frequency clock protein	*F. verticillioides*	FVEG_04686	$3.0e^{-23}$	GO: 0097167	+	+	-
TDF156	Hypothetical protein similar to coenzyme A transferase	*F. graminearum*	FGSG_02146	$1.0e^{-30}$	GO: 0008260	++	++	-
TDF158	Histone deacetylase phd1	*F. oxysporum*	FOXG_00027	$4.0e^{-23}$	GO: 0017136	+	++	-
TDF161	Hypothetical protein similar to cohesin complex subunit Psm1	*F. oxysporum*	FOXG_04230	$4.0e^{-12}$	GO: 0008280	++	++	-

Transcript derived fragment	Most similar homologous protein	Species of homologous protein	Accession number	E-value	GO number	Transcript abundance patterns[a]		
						Foc STR4	*Foc* TR4	NP
TDF168	Hypothetical protein similar to laccase	*F. oxysporum*	FOXG_06344	$7.0e^{-23}$	GO: 0052716	+	+	-
TDF174	Small G-protein Gsp1p	*Magnaporthe grisea*	MGG_09952	$5.0e^{-13}$	GO: 0003924	-	+	-
TDF176	Hypothetical protein similar to chitin biosynthesis protein	*F. oxysporum*	FOXG_03146	$4.0e^{-19}$	GO: 0006031	++	-	-
TDF179	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase precursor	*F. oxysporum*	FOXG_04115	$3.0e^{-30}$	GO: 0004067	+	+	-
TDF186	Hypothetical protein similar to MutT/nudix family protein	*F. oxysporum*	FOXG_01294	$9.0e^{-15}$	GO: 0005515	+	-	-
TDF190	Hypothetical protein similar to glutamine-dependent NAD(+) synthetase	*F. verticillioides*	FVEG_07876	$1.0e^{-16}$	GO: 0003952	-	+	-
TDF193	Hypothetical protein similar to SAC3/GANP domain protein	*Neurospora crassa*	NCU06594	$1.0e^{-53}$	GO: 0005515	++	++	+
TDF204	Hypothetical protein similar to ATP-cone	*F. oxysporum*	FOXG_11977	$2.0e^{-53}$	GO: 0031250	+	-	+
TDF206	Hypothetical protein similar to vacuole-associated enzyme activator complex component Vac14	*F. graminearum*	FGSG_09846	$5.0e^{-24}$	GO: 0008047	-	++	-
TDF214	Hypothetical protein similar to serine/threonine protein kinase	*F. graminearum*	FGSG_05764	$3.0e^{-12}$	GO: 0004674	+	+	-

Transcript derived fragment	Most similar homologous protein	Species of homologous protein	Accession number	E-value	GO number	Transcript abundance patterns[a]		
						Foc STR4	*Foc* TR4	NP
TDF215	O-acetylhomoserine	*F. oxysporum*	FOXG_11296	$4.0e^{-16}$	GO: 0003961	+++	-	-
TDF217	Hypothetical protein similar to DUF895 domain membrane protein	*F. verticillioides*	FVEG_08610	$9.0e^{-18}$	GO: 0015572	+	+	-
TDF223	GTPase activating protein	*Verticillium albo-atrum*	XM_003005785.1	$1.0e^{-20}$	GO: 0003779	+	+	-

[a] Indicates the visual intensity of a specific cDNA-AFLP band in *Fusarium oxysporum* f. sp. *cubense* (*Foc*) 'subtropical' race 4 (STR4), *Foc* 'tropical' race 4 (TR4) and non-pathogenic *F. oxysporum* (NP). - = no transcripts detected, + = low level of transcript abundance, ++ = moderate level of transcript abundance and +++ = high level of transcript abundance.

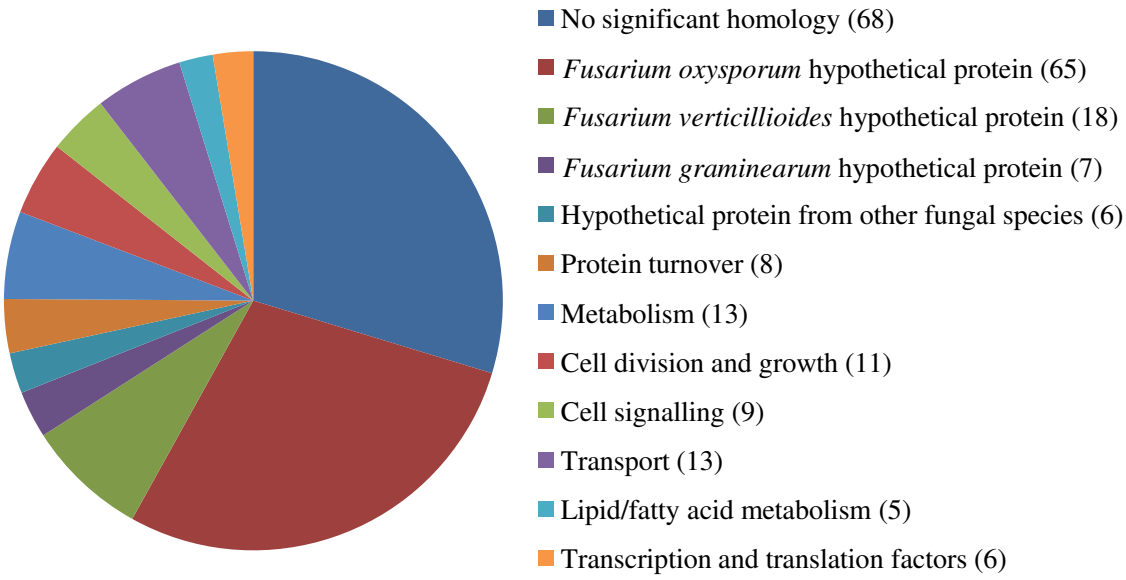

Figure 2.1. Classification of differentially accumulated transcript derived fragments (TDFs) after growth of *Fusarium oxysporum* f. sp. *cubense* and non-pathogenic *F. oxysporum* in minimal medium without a carbon source. A total of 229 TDFs were classified based on the BLASTx homology search on the Broad Institute Database. Numbers in parentheses indicate the amount of TDFs found in each category.

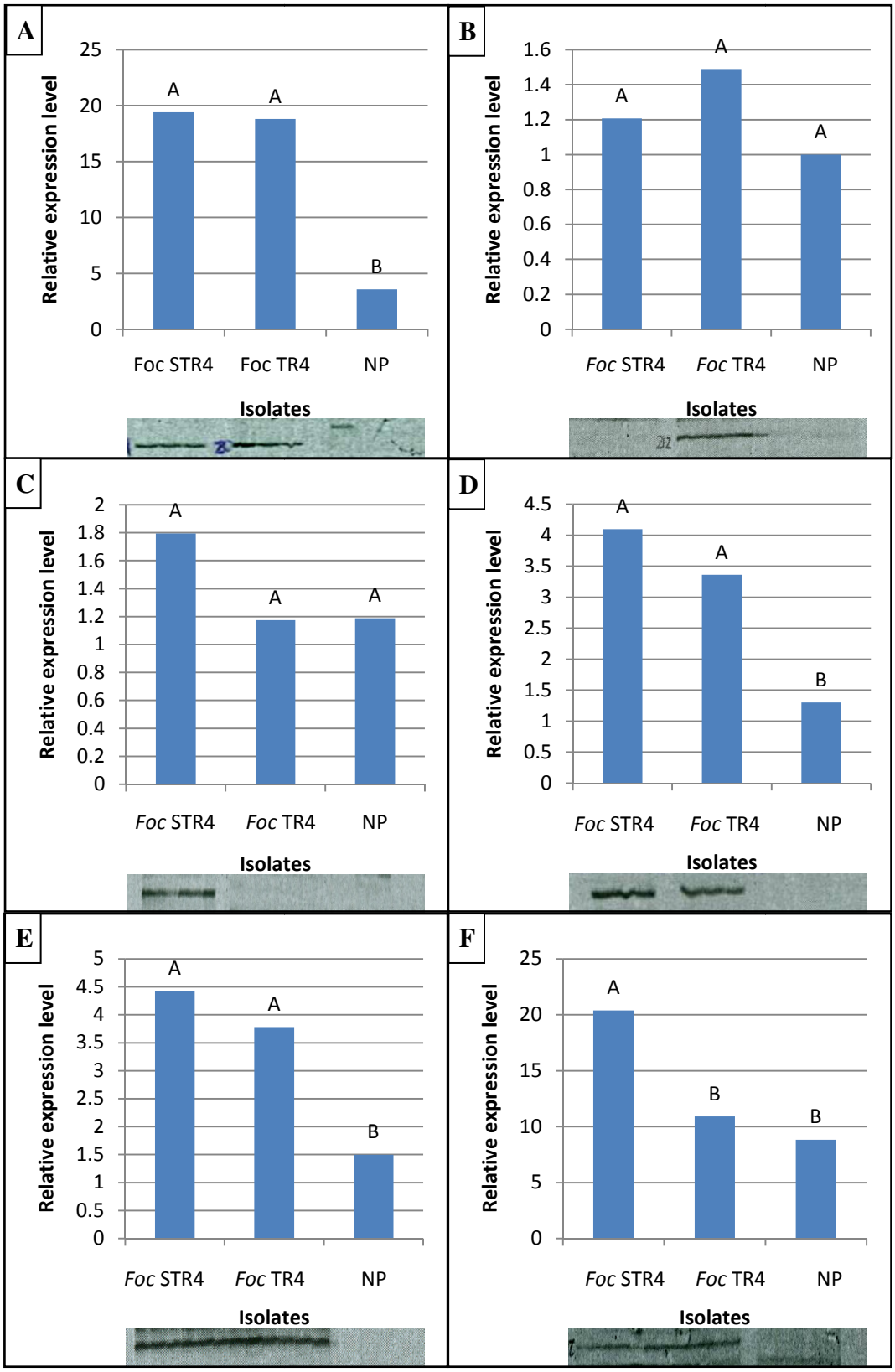

Figure 2.2. Verification of the relative expression of selected genes by quantitative reverse transcriptase PCR in *Fusarium oxysporum* f. sp. *cubense* (*Foc*) 'subtropical' race 4 (STR4), *Foc* 'tropical' race 4 (TR4) and non-pathogenic *F. oxysporum* (NP) namely A. MFS multidrug transporter (TDF9), B. L-aminoadipate-semialdehyde dehydrogenase (TDF64), C. Aspartyl-tRNA synthetase (TDF52), D. Chitinase class V (*chsV*) (TDF107), E. Serine/threonine protein kinases (*ste12*) (TDF214) and F. GTPase activating protein (*rhoI*) (TDF223). Segments of the original polyacrylamide cDNA-AFLP gels are shown below the horizontal axis. The difference in Ct values was determined statistically by One-way ANOVA, followed by Tukey's post-hoc analysis where a p value of < 0.05 was considered statistically significant. The letters above the bars indicate significant differences between the genotypes.

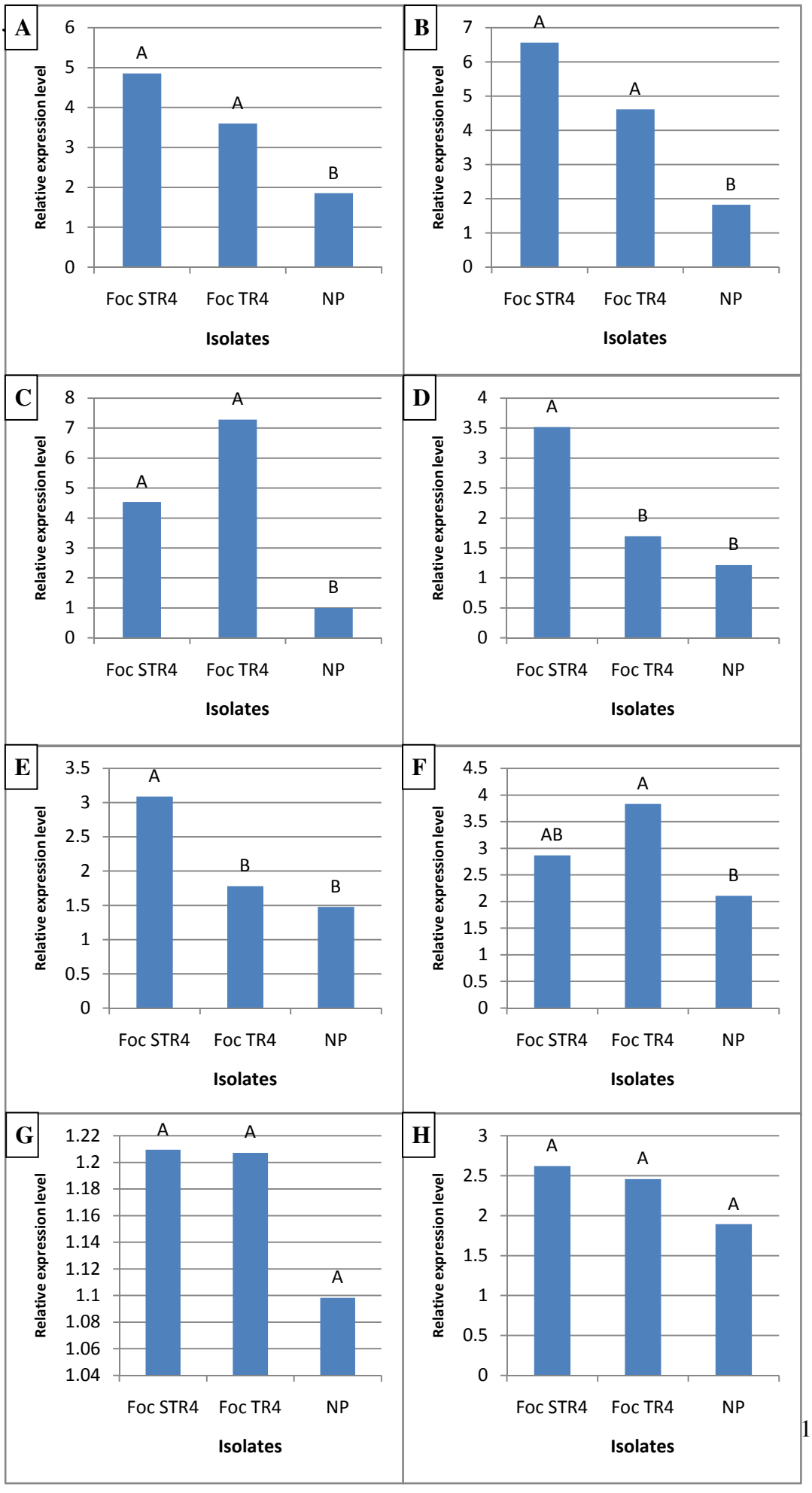

Figure 2.3. Relative transcript abundance of known virulence genes by quantitative reverse transcriptase PCR in *Fusarium oxysporum* f. sp. *cubense* (*Foc*) 'subtropical' race 4 (STR4), *Foc* 'tropical' race 4 (TR4) and non-pathogenic *F. oxysporum* (NP) namely A. Sucrose non-fermenting gene (*snf*), B. F-box protein required for pathogenicity (*frp1*), C. Cytochrome P450 (*cyp55*), D. *Fusarium* MAP kinase (*fmk1*), E. Chloride channel gene (*clc*), F. $Zn(II)_2Cys_6$-type transcription regulator (*fow2*), G. Arginine biosynthesis gene (*arg1*) and H. Mitochondrial protein (*fow1*). The letters above the bars indicate the significant differences between the samples. The difference in Ct values was determined statistically by One-way ANOVA, followed by Tukey's post-hoc analysis where a p value of < 0.05 was considered statistically significant.

CHAPTER 3

PREDISPOSING CAVENDISH BANANA PLANTS TO COLD STRESS DELAYS THE DEFENCE RESPONSE AGAINST *FUSARIUM OXYSPORUM* F. SP. *CUBENSE* 'SUBTROPICAL' RACE 4

ABSTRACT

Cold temperature is a major abiotic stress condition that reduces the yield of Cavendish bananas in the subtropics. It also predisposes plants to diseases such as Fusarium wilt. In this study, the hypothesis that the defence response of Cavendish bananas against *Fusarium oxysporum* f. sp. *cubense* (*Foc*) 'subtropical' race 4 (STR4), is negatively affected by low temperatures, was investigated. Greenhouse trials showed a significant increase in disease development in Cavendish bananas grown at 10°C compared to plants grown at 28°C. Numerous genes, involved in early plant response following fungal infection and cold temperature treatment, were identified using the 454 GS FLX sequencing platform. These included genes encoding pathogenesis related (PR) proteins, 1-aminocyclopropane-1-carboxylic acid oxidase, abscisic stress ripening protein, late embryogenesis abundant protein 5, metallothionein, cinnamate-4-monooxygenase, harpin-induced protein 1, lipid transfer protein, germins, peroxidase and defensins. Defence mechanisms in banana against *Foc* STR4 included the activation of transcripts involved in the salicylic acid, jasmonic acid and ethylene pathways. Similar transcripts were produced in Cavendish bananas exposed to 10°C and 28°C following *Foc* infection. However, qRT-PCR analysis showed that plant response was delayed and suppressed at the cooler temperature, thereby allowing *Foc* STR4 to invade the root xylem vessels and cause increased disease development. Thus, cold stress may enhance fungal infection, however disease development occurs only at 28°C, once water uptake increases. The transcriptome data obtained in this study can serve as a resource for gene expression and functional genomics studies.

INTRODUCTION

Plants are often exposed to cold stress under temperate and subtropical climatic conditions. When temperatures drop to freezing point, irreversible damage can occur on sensitive plants. Cold resistant plants, however, can withstand freezing temperatures through a process called cold acclimation (Ruelland *et al.*, 2009). Tropical plants like banana, cucumber, mango, tomato and maize are unlikely to acclimatize to freezing temperatures and are, therefore, more sensitive to low, non-freezing temperatures (Lyons, 1973). In these plants, cold stress is a serious threat to sustainable crop production.

Low temperatures have a significant impact on bananas grown in the subtropics. Frost damage destroys the functional leaves of the plant, which reduces their photosynthetic capacity and leads to a reduction in yield. The growth of a banana plant ceases at approximately 14°C with irreversible damage occurring below freeze point (Robinson and Galán Saúco, 2010). Symptoms associated with low winter temperatures include 'choking', 'choke throat', 'November dump' (May flowering) as well as under-peel discolouration (Robinson and Galán Saúco, 2010). A good example of the magnitude of damage that cold stress can cause occurred in 1999, when 150 000 ha of banana plantations were destroyed in China by frost damage (Linbing *et al.*, 2003).

Cold stress during winter does not only decrease the yield in banana, but can also predispose plants to Fusarium wilt, a disease caused by a soil-borne fungus called *Fusarium oxysporum* f. sp. *cubense* (*Foc*) (Viljoen, 2002). With the onset of spring, the daily temperature and transpiration rate in plants begin to rise, and disease incidence is significantly increased. Fusarium wilt (Panama disease) is considered one of the most devastating diseases of banana and has destroyed many plantations worldwide (Ploetz, 2006). Damage caused by the disease during the first half of the 20th century established it as one of the greatest epidemics in agricultural history (Ploetz and Pegg, 2000), with over $400 million (US$ 2.3 billion in 2000-value) in losses recorded in the 1950's (Ploetz, 2005). There is no effective means to control Fusarium wilt, except for replacing susceptible banana varieties with resistant cultivars. However there is currently no resistant dessert banana variety available to replace the popular Cavendish banana, which today dominates the export and fresh fruit markets.

Cavendish bananas succumb to Fusarium wilt both in the tropics and subtropics. The variant of the fungus causing disease in the two climate zones, however, differ. In the subtropics, the disease is caused by *Foc* 'subtropical' race 4 (STR4), which belongs to vegetative compatibility group (VCG) 0120. *Foc* STR4 usually infects Cavendish bananas after cold predisposition, and seldom causes Fusarium wilt in Cavendish plants in tropical climates. *Foc* 'tropical' race 4 (TR4), however, does not require any predisposition by abiotic stresses for causing disease in Cavendish bananas. *Foc* races 1 and 2 do not cause Fusarium wilt of Cavendish bananas, neither in the subtropics nor in the tropics.

The development of Fusarium wilt can also be influenced by other abiotic stress factors also, such as hypoxia, drought, pH and salinity (Rishbeth, 1955; Simmonds, 1959; Stover, 1962). In Western Australia, the Cavendish cv. Williams showed increased disease severity under waterlogged and drought conditions after infection with *Foc* race 4 (Shivas *et al.*, 1995). Low pH and high salinity favours disease development and severity in banana plants (Stover, 1962).

Despite numerous reports on the increased disease susceptibility of plants following cold stress (Line and Chen, 1995; Kim and Bockus, 2003; Bhuiyan *et al.*, 2009), the molecular mechanisms underlying plant response is still poorly understood. This study, therefore, investigated the hypothesis that the defence response against *Foc* STR4 in Cavendish bananas is delayed and suppressed during cold stress. Greenhouse trials were performed to confirm the phenotypic effect of cold stress, and 454 GS FLX sequencing to identified transcripts expressed during cold stress and/or *Foc* infection. A subset of defence/cold stress-related genes was further studied by quantitative expression analysis.

MATERIALS AND METHODS

Fungal isolates

Foc STR4 isolates CAV 045, CAV 092 and CAV 105 (maintained at the Department of Plant Pathology, Stellenbosch University, South Africa) were cultured on half strength potato dextrose agar (PDA) and incubated for five to seven days at ± 25°C. To ensure that they were pathogenic, the isolates were first inoculated in susceptible banana plants and re-isolated

from diseased rhizome material. The re-isolated cultures were then grown on half-strength PDA, and their mycelium transferred to Armstrong's sporulation media (Booth, 1971). After five days' growth on a shaking incubator, rotating at 120 rpm at 25°C, the spore concentration was adjusted to $1x10^5$ spores/ml by using a hemacytometer (Laboratory & Scientific Equipment Company (Pty) Ltd. (LASEC), Randburg, South Africa).

Inoculation of banana plants

Three hundred tissue-cultured Cavendish (cv Grand Naine) banana plants were obtained from Du Roi Laboratories in Letsitele, South Africa. Grand Naine is susceptible to *Foc* STR4 in the subtropics, especially under stressful conditions. Plants were transplanted into a hydroponic system using 250-ml black plastic cups containing tap water, and fertilized (0.6 g/L $Ca(NO_3)_2H_2O$, 0.9 g/L Agrasol, and 3 g/L Micromax) every fortnight (Nel *et al.*, 2006). After approximately four weeks' growth at 28°C, sufficient root growth was observed for infection.

Before inoculation, the plants were removed from the black plastic cups and their roots gently squeezed by hand to induce wounds. The plants were then replanted in polystyrene cups containing 200 ml of either a *Foc* STR4 spore suspension or sterile distilled water. The plants were divided into five groups and treated as follows: A. Inoculated and incubated at 28°C (infected), B. Inoculated and incubated at 10°C (coldinf), C. Incubated at 10°C for two weeks, then inoculated and transferred to 28°C (precold), D. Incubated at 10°C without inoculation (cold control) and E. Incubated at 28°C without inoculation (control). Four weeks after infection (wai), the plants incubated at 10°C (coldinf and cold) were transferred to 28°C.

Six weeks after inoculation, the rhizomes of the banana plants were dissected horizontally and the degree of discolouration determined according to the INIBAP rating scale (Carlier *et al.*, 2002). Disease severity was calculated from ten plants per treatment as: DSI% = Σ (number of scale x number of plants in that scale)/Σ (number of treated plants) (Sherwood and Hagedorn, 1958). All the data were analyzed by JMP® (SAS Institute, Cary, North Carolina) using analysis of variance (ANOVA) test with significant difference values at $p<0.05$ using the Student t-test. The plants used for the quantitative reverse transcriptase PCR (qRT-PCR) were stripped of most of their roots and placed back into the cups. Eight to ten

weeks later the rhizome was sliced open and used to determine disease severity as described above.

RNA extraction and cDNA generation

For transcriptome analysis, roots were harvested at 3 and 12 hours post infection (hpi), while for qRT-PCR, roots were harvested at 0, 3, 12, 24, 48 hpi and five days post infection (dpi). The roots were then rapidly frozen in liquid nitrogen and stored at -80^{o}C. RNA was extracted from the roots of six plants each that were collected 3 and 12 hpi. The RNA of the two collection points was then combined for each of the treatments (infected, coldinf and precold) to obtain a representative sample of early plant response after infection. For quantitative gene expression studies, RNA (60 µg) from two plants was combined of each time point and regarded as a biological repeat.

RNA was extracted from banana roots with a CTAB extraction buffer and LiCl precipitation (Chang *et al.*, 1993). For transcriptome analysis, 360 µg RNA was treated with *DNase*I (Fermentas, Life Sciences, Hanover, USA) and purified with a RNeasy mini kit (Qiagen, Valencia, California, USA). The quantity of RNA was determined with a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Inc., Montchanin, USA) and quality was assessed by gel electrophoresis under non-denaturing conditions on 2% agarose gel. mRNA was isolated from total RNA using the oligotex mRNA mini kit (Qiagen) according to the manufacturer's instructions. From the mRNA, double stranded cDNA was synthesized with cDNA Synthesis System (Roche Diagnostics, Mannheim, Germany). For quantitative gene expression studies, 60 µg RNA was treated with *DNase*I and purified with an RNeasy mini kit. Single-stranded cDNA was synthesized using the Transcriptor first strand cDNA synthesis kit (Roche Diagnostics). In both cases, DNA contamination was verified by PCR using intron flanking actin primers for plant cDNA (Van den Berg *et al.*, 2007).

Sequencing of Cavendish banana root transcripts

cDNA from infected, coldinf and precold libraries were separately tagged and sequenced in a single lane using the 454 Titatium GS FLX platform at Inqaba Biotechnologies (Pretoria, South Africa). The tags were manually removed and the sequences were assembled with

CAP3 (Huang and Madan, 1999) and Newbler version 2.5.3 (454 Life Sciences, Branford CT) and annotated with dCAS (Desktop cDNA Annotation System) (Guo *et al.*, 2009) and CIRAD's Genome Browser (D'Hont *et al.*, 2012). Functional groups were defined according to MIPS (Ruepp *et al.*, 2004) and GO (Ashburner *et al.*, 2000) databases. Genes that were highly abundant and known to play a significant role in either defence or cold stress were studied further. The conserved domains were identified using a conserved domain database (CDD) search (Marchler-Bauer *et al.*, 2011) and multiple alignments of nucleotide or protein sequences were constructed by using MUSCLE (Edgar, 2004). In the case of abscisic acid stress ripening (*ASR*) transcripts, the transcripts were further characterized into their respective groups. A phylogenetic tree was constructed using the neighbour-joining algorithm in MEGA 5.05 (Tamura *et al.*, 2011) with bootstrap values (>50%) after 1000 replicates shown.

Quantitative gene expression profiling

The regulation of ten putative defence and stress-associated genes identified by transcriptome sequencing was assessed by qRT-PCR on a Light Cycler version 480 instrument (Roche Diagnostics). Primers were designed using Primer3 (Whitehead Institute, MIT, Cambridge, MA, USA) and Netprimer (Premier Biosoft, Palo Alto, CA, USA) and synthesized by Integrated DNA Technologies (Coralville, United States) (Table 3.1). qRT-PCR reactions were executed in 10-µl volumes containing 1 µl cDNA template (1:5 dilution), 1 µl of each of the forward and reverse primers (10 µM) (Table 3.1), 2 µl sterile water and 5 µl SensiMix™ SYBR (Bioline, London, UK). All reactions were run in triplicate with three independent biological replicates of which two plants were combined, as well as a negative control (no template), for all the genes. A standard curve was generated by preparing a dilution series (1:5, 1:10, 1:50, 1:100, 1:500 and 1:1000) for each gene. The gene expression stability (M-value) and pairwise variation (V-values) was determined using Genorm (Vandesompele *et al.*, 2002). Ct values were imported into qbase^{PLUS} (Biogazelle, Ghent, Belgium) for further analysis. Four reference genes, namely *ubi*, *actin*, *Musacont1* and *25S*, were used to normalize the data (Van den Berg *et al.*, 2007; Munro, 2008) (Table 3.1). The expression data were analyzed by JMP® (SAS Institute, Cary, North Carolina) using analysis of variance (ANOVA) test and significant difference values at $p<0.05$ using the Student t-test.

RESULTS

Greenhouse assessment of Fusarium wilt severity in Cavendish bananas grown under cold stress conditions

Chilling injury was visually observed in banana plantlets within five to seven days after they were exposed to cold temperatures (10°C). The first visual symptom of cold stress involved the yellowing of the leaves (Fig. 3.1A, B), followed by the browning of leaf margins and a reduced growth rate two weeks later. When the same plants were transferred to warmer temperatures (28°C) four weeks later, they recovered within three days and new leaves emerged. In plantlets inoculated with *Foc* at 28°C, the older leaves turned yellow 5 wpi (Fig. 3.1C). Similar disease symptoms were observed in plantlets that were predisposed to cold stress before being inoculated at 28°C (Fig. 3.1D). The control plants remained healthy and no external symptoms were observed throughout the study (Fig. 3.1E).

Grand Naine banana plants, with and without predisposition to cold temperatures, were highly susceptible to *Foc* STR4 after infection at 28°C. Severe brown discolouration was observed in the vascular tissue of the infected plants 6 wpi. A disease severity rating of 82% was calculated for plants predisposed to cold temperatures and a rate of 84% in plants infected at 28°C (Fig. 3.2). Plants inoculated at 10°C exhibited a 23% disease severity after six weeks. As expected, no internal symptoms were observed in the control plants and plants subjected to cold temperatures only.

As time progressed, disease severity in plants inoculated with *Foc* increased (Fig. 3.3). After eight weeks, disease severity was significantly lower in the infected plants predisposed to cold temperature compared to the plants infected at 28°C. However, at 9 wpi, the plants infected at 10°C showed a significant increase in symptom development (78%) compared to plants infected at 28°C with/without predisposition to cold temperature (38% and 60%, respectively). The plants infected at 10°C showed a 90% disease severity after ten weeks, significantly higher than the 64% disease severity of plants predisposed to 10°C before infection. We further observed a delay in disease development in plants from which roots were harvested for qRT-PCR, as they had limited tissue for the pathogen to enter.

Sequencing of Cavendish banana root transcripts

cDNA libraries from the infected, cold predisposed and cold infected banana samples produced a total of 15 464 reads (3.9 Mb) (Table 3.2). CAP3 assembly resulted in 1251 contigs of between 111 and 1 726 bp, with an average contig length of 615 bp. Assembly through Newbler gave rise to 74 contigs varying from 69 to 1 763 bp in size, with an average contig length of 669 bp. Newbler N50 contig size was 631 bp, compared to 616 bp with CAP3.

To speculate the putative functions of contigs, homology to sequences from other plants was determined. More than 50% of the contigs showed homology to *Musa* spp. (Fig. 3.4). Homology was also shown to *Oryza* spp. (5.2%), *Vitis* spp. (3.6%), *Zea* spp. (3.6%) and *Populus* spp. (3.3%). To determine the extent of homology, E-value distribution maps were obtained in the infected, precold and coldinf libraries. The E-value distribution of the best hits in the NR database showed that 19.7% of the contigs had very high homology (E-value smaller than $1.0e^{-50}$), while 54.7% showed high homology (E-value smaller than $1.0e^{-25}$) (Fig. 3.5). In total, 13.0% of the contigs from banana roots showed no homology to any sequences in the NR database or *Musa acuminata* 'Pahang' database. The GO database grouped contigs from banana roots into 12 functional categories. Of these, 14.8% of the contigs were linked to defence and stress, 16.2% were involved in transcription and translation, and 8.6% in cell division and growth (Fig. 3.6). Seventeen precent of the contigs had no known functions.

Certain contigs were present in high abundance in all three libraries (infected, coldinf and precold), like the metallothionein type 2 (MT2) and hypothetical protein BOS_23236 (Table 3.3-3.5). High abundance transcripts that showed homology to defence/stress proteins included the putative senescence-associated protein, pathogenesis-related protein, cold induced plasma membrane protein and abscisic stress ripening protein. Other putative defence/stress transcripts identified in the three different libraries included those involved in pathogen-associated molecular pattern (PAMP) and resistance (*R*)-gene triggered immunity, transcription factors, the hypersensitive response (HR) and signalling (Table 3.6). Transcripts associated with PAMP-triggered immunity included receptors and elicitor like receptor kinase, avr9/cf-9 elicitor response protein, brassinosteroid insensitive 1-associated receptor kinase and proline extensin-like receptor kinase. Five putative disease resistance transcripts

were expressed in the transcriptome, as well as several contigs involved in regulating ion fluxes, such as calmodulin, calcineurin, vacuolar H+ ATPase and voltage-gated potassium channel. Transcription factors possibly involved in defence include WRKY, BHLH, AP2/ERF domain-containing transcription factor and bZIP transcription factor. Transcripts involved in signalling pathways include PAL and NPR1, which are involved in salicylic acid (SA) signalling; lipoxygenase and pnFL-2, which are involved in jasmonic acid (JA) signalling; ACS and ACO, which are involved in ethylene (ET) signalling; and ASR, which is involved in ABA signalling. Catalase and peroxidase, involved in the oxidative burst; Hin1, involved in programmed cell death; and the pathogenesis-related (PR) proteins PR-1, PR-4, PR-5, PR-6, PR-10 and PR-14 were also induced upon pathogen attack. Cell wall strengthening proteins that restrict the pathogen's movement included cold induced plasma membrane protein and dirigent protein.

Gene-specific domains and motifs

To characterize transcripts in more detail, we selected ten transcripts and subjected them to multiple alignments for the presence of gene-specific domains and motifs. The transcripts were selected based on a low E-value, high abundance and a known role of defence or cold stress in other crops (Tables 3.3-3.6). These included *PR-1*, *PR-4*, *PR-6*, *PR-10*, *LEA5*, *ASR*, *MT2*, *C4H*, *ACO* and *Hin1*.

PR-1 was highly expressed in all three libraries. Three motifs were found in the *PR-1* contigs namely cysteine-rich secretory protein-1 precursor (CRISP) family signature 1 and 2 and the SCP-like extracellular protein (Fig. 3.7). *PR-4* was only detected in the infected library after transcriptome analysis, and the contig contained Barwin domain signature 1 and 2 and Barwin family domain (PF00967) (Fig. 3.8). As only the 5' end of the *PR-4* was sequenced, it could not be determined if the transcript belonged to class I or II. The potato inhibitor I family domain (PF00280) was identified in contig infect_49 and showed 60% similarity to PR-6 protein from *Sambucus nigra* (Fig. 3.9). Amino acid sequences obtained by translated cDNA sequences; namely contigs infect_14, infect_15 and precold_5; showed homology to the PR-10 protein and contained pathogenesis-related protein Bet v I family (PF00407) domain (Fig. 3.10).

LEA5 proteins are well known to be induced by either cold or drought stress and play a role in protection by helping the folding of proteins (Tunnacliffe and Wise, 2007). Three contigs, infect_385, coldinf_387 and precold_1007, showed similarity to the LEA5 protein after BLASTx searches on the NCBI database, with a 63, 63 and 45% identity, respectively, to a LEA protein from *Sesuvium portulacastrum*. After translation, all three contigs contained the conserved domain W(A/V)PDP(V/I)TGYYRPE found in all LEA5 plant proteins, except for the second amino acid (Tao *et al.*, 2006). A motif search confirmed the fragments contained a LEA group 3 motif (PF03242) (Fig. 3.11).

The function of *ASR* is proposed to help in protection against abiotic stresses as well as involvement in the regulation of gene expression. Eleven of the contigs showed homology to *ASR* in the NR database of NCBI. The conserved abscisic acid/water deficit stress amino acid domain (ABA/WDS) (PF02496) was found in all the contigs except precold_2489 (Fig. 3.12). Three different groups of *ASR* were identified in this study (Fig. 3.13). *ASR1* and *ASR4* were identified in all three libraries. *ASR1* transcripts clustered with other *ASR1* sequences from banana plants. *ASR4* transcripts grouped with *ASR4* sequences from banana plants with an ABB and AAA genomic composition as well as *M. banksii*. *ASR2* was only detected in the cold-infected library, whereas *ASR3* transcripts were absent from all the libraries.

Metallothionein (MT) is a family of cysteine-rich, low molecular weight proteins and has different functions including scavenging ROS, detoxification of metal ions and control of redox potential (Hassinen *et al.*, 2010). MTs are divided into four families (Grennan, 2011) and MT type 2 and 3 were highly expressed in all three libraries. All the MT type 2 transcripts contained the MT type 2 domain (PF01439) (Fig. 3.14).

Cinnamate 4-mono-oxygenase (C4H) catalyzes the hydroxylation of trans-cinnamate 3 to trans-4-coumarate 4 in the second step in the phenylpropanoid biosynthetic pathway leading to the production of phytoalexins and lignin (Vogt, 2010). Cinnamate 4-mono-oxygenase is also referred to as cinnamate 4-hydroxylase. *C4H* was identified in all three libraries according to the NR database. The contigs contained a cytochrome P450 (secondary metabolites biosynthesis, transport, and catabolism) motif (PLN02394) which is part of the cl12078 superfamily (Fig. 3.15). Contigs precold_658, infect_3422 and coldinf_704 showed 83, 83 and 93% homology, respectively, to a cinnamate 4-hydroxylase from *Populus*

tremuloides, whereas precold_23, precold_438 and coldinf_551 showed 83, 72 and 83% homology to a *Zea mays* cinnamate 4-mono-oxygenase, respectively.

Aminocyclopropane-1-carboxylic acid (ACC) is used in the production of ET by catalysing the reaction with ACC synthase followed by oxidation by ACC oxidase (ACO). ACC synthase was only discovered in the precold library whereas ACO was more common and was identified in all three libraries, infected, precold and coldinf. Contigs that showed homology to ACO contained 2 oxoglutarate -FeII_Oxy domains (PF03171) (Fig. 3.16).

Harpin inducing protein (*Hin1*) triggers the HR as a defence mechanism. *Hin1* was detected in all the libraries but higher abundance levels were observed in the infected library. Contig infect_3095 showed 62% homology to harpin-induced protein from *Zea mays* (E-value $3e^{-24}$) while precold_2399 showed 63% similarity to the same protein sequence with an E-value of $8e^{-31}$. Furthermore, these contigs contained a harpin-induced protein 1 domain (PF07320) (Fig. 3.17).

Quantitative gene expression profiling

Cold stress (precold and coldinf) delayed the induction of *PR-1* transcripts in banana infected with *Foc* and suppressed the levels of expression (Fig. 3.18A). The same relative expression of *PR-1* found 3 hpi at 28°C was obtained 12 hpi in bananas predisposed to 10°C before infection at 28°C, and 24 hpi in plants infected at 10°C. *PR-1* expression in both cold treatments was significantly lower at 3 and 12 hpi compared to the infected plants at 28°C. When bananas were inoculated with *Foc* at 28°C, *PR-1* was significantly induced 3 hpi, and reached 16.6-fold higher levels compared to plants subjected to infection under cold temperatures. In comparison, a non-significant increase was obtained in the 'control' plants from 0-3 h, and from 3-12 h. Coldinf plants responded similar to plants exposed to temperatures of 10°C without infection, except after 12 h when *PR-1* was significantly up-regulated in the non-inoculated plants.

The induction of *PR-4* in response to *Foc* was significantly inhibited during cold treatments (Fig. 3.18B). Banana plants infected with *Foc* at 10°C, as well as plants predisposed to 10°C before infection at 28°C, showed no significant up-regulation of *PR-4* over the whole time

course. In stark contrast, a highly significant up-regulation in transcript expression was obtained in plants inoculated with *Foc* 3 hpi (6.2-fold more than the control plants). *PR-4* levels in these plants were significantly reduced at 12 hpi but still remained significantly higher than all other treatments. Plants subjected to cold stress only, without infection with *Foc,* showed no significant induction of *PR-4* at early time points (3, 12, 24 hpi), but showed a significant induction at 48 hpi compared to 0 h that remained at this level until 5 dpi. Uninfected control plants maintained at 28°C had a significant increase in *PR-4* expression at 3 hpi, but this was significantly lower compared to plants infected with *Foc* at the same temperature.

A significant increase in *PR-6* levels occurred in banana plants infected with *Foc* at 28°C after 12 h when compared to the uninfected control plants (Fig. 3.18C). At 12 hpi, *PR-6* levels were 15.9-fold more compared to roots infected at 10°C, and 14.8-fold more compared to the control plants. Interestingly, non-inoculated plants subjected to cold temperatures (10°C) showed no significant difference in *PR-6* expression up to 24 h, but expression levels increased significantly thereafter. This increase was not found in the non-inoculated control plants. The expression pattern of *PR-6* transcripts in plants infected with *Foc* at 10°C, as well as in those predisposed to 10°C before infection at 28°C, were similar to each other. The uninfected control plants maintained *PR-6* levels at 28°C at all time points.

The expression level of *PR-10* in bananas infected and uninfected with *Foc* under cold stress was significantly delayed (Fig. 3.18D). Plants infected at 28°C resulted in a significantly higher expression of *PR-10* compared to those infected with *Foc* at 10°C and ones predisposed to 10°C before infection at 28°C. At 28°C with infection, *PR-10* expression increased significantly at each time interval up to 12 hpi, after which it was slowly reduced until 5 dpi. Similarly, expression levels in the non-infected control plants maintained at 28°C increased significantly at the early time intervals (3 and 12 hpi), but this was significantly lower compared to plants infected with *Foc* at the same temperature. Plants infected with *Foc* at 10°C showed a significant increase in *PR-10* levels from 0 to 48 hpi, whereas plants predisposed to 10°C before infection at 28°C showed significant up-regulation at 24 hpi compared to 0 h.

Cold stress in combination with *Foc* infection hindered the early induction of *ACO* levels in bananas (Fig. 3.18E). Plants infected at 28°C and uninfected control plants at 10°C and 28°C showed a significant increase in *ACO* expression at 3 hpi. Cold stress (10°C) applied before or at infection, significantly suppressed the up-regulation of *ACO* levels compared to the control plants when measured at 3 hpi, with 4.7- (precold) and 38.9- (coldinf) fold less *ACO* transcripts, respectively. Plants infected at 10°C with *Foc* showed a later induction of *ACO* transcripts (12 hpi), compared to the control plants (uninfected), plants with cold stress (uninfected) as well as plants infected at 28°C. In spite of this, at 5 dpi difference in *ACO* levels were not significant between the different treatments.

ASR4 transcripts, however, were not significantly up or down-regulated over the time course in bananas subjected to all treatments, except for two instances (Fig. 3.18F). Plants predisposed to cold temperature before infection at 28°C showed significant up-regulation of *ASR4* from 12 to 24 hpi, while plants subjected to cold temperatures without infection showed significant up-regulation from 24 to 48 h.

Plants predisposed to 10°C before being infected with *Foc* at 28°C showed a significant up-regulation of *LEA5* 12 and 24 hpi compared to all the other treatments (Fig. 3.18G). At 24 hpi, this level was 12.6-fold more than in the control plants. *LEA5* transcript expression was also significantly increased 12 and 24 h after infection in plants kept at 10°C, with and without infection by *Foc*, when compared to earlier expression levels. Control plants had an early induction of *LEA5* at 3 h compared to plants kept at 10°C with and without *Foc* infection, and then showed an increase again at 48 h. *LEA5* was not significantly up-regulated during the early time intervals (3 and 12 h) after *Foc* infection at 28°C, but after 24 and 48 h, *LEA5* was significantly higher compared to 0 h. *LEA5* expression did not differ significantly between the different treatments at 5 dpi.

Banana plants predisposed to 10°C for two weeks and then infected with *Foc* at 28°C responded with a significant induction in *MT2* expression levels at 3 hpi when compared to other treatments, followed by a significant down-regulation at 12 hpi (Fig. 3.18H). By 12 and 24 h, there were no significant differences in *MT2* expression among treatments, except for plants kept at 10°C without infection, which showed significantly lower levels of *MT2* transcribed after 12 h. Plants exposed to *Foc* at 28°C had a significant increase in *MT2* at 48

hpi that remained high until 5 dpi. Banana exposed to 10°C for two weeks prior to *Foc* infection at 28°C showed a significant decline in *MT2* at 5 dpi. Control plants at 28°C showed no significant change in *MT2* levels during the entire experiment.

Constant cold stress at 10°C without infection suppressed the induction of *C4H* compared to plants predisposed to cold and infected with *Foc* at 28°C (Fig. 3.18I). The later treatment showed a highly significant up-regulation of *C4H* compared to all the other treatments during the first 24 h after inoculation with *Foc*. Plants treated and non-treated with *Foc* at 28°C also resulted in a significant induction of *C4H* at 3 hpi. Banana plants infected with *Foc* at 10°C, however, exhibited a delayed response by only inducing *C4H* expression after 12 h. Plants exposed to 10°C for five days without pathogen infection showed very low levels of *C4H* throughout the experiment.

The early up-regulation of *Hin1* transcripts in response to *Foc* were suppressed under constant cold stress (Fig. 3.18J). Plants infected with *Foc* at 28°C, as well as plants predisposed to 10°C but infected at 28°C, transcribed significantly more *Hin1* compared to any other treatment as early as 3 hpi. This pronounced induction of *Hin1* quickly declined, less rapidly following infection with *Foc* at 28°C, until 5 dpi. The control plants also responded with a significant up-regulation of *Hin1* at 3 h, but this was significantly lower compared to that in bananas exposed to *Foc* at 28°C. Plants infected with *Foc* at 10°C only showed a significant up-regulation of *Hin1* transcripts at 12 hpi, which did not differ significantly from the precold-treated plants, but which was significantly lower than expression levels in plants infected at 28°C. Plants exposed to cold stress (10°C) without infection showed a significant induction of *Hin1* transcripts at 3 h, similar to the control plants, which remained unchanged until 48 h. Transcript levels in the control plants, however, declined quickly thereafter.

DISCUSSION

The complex relationship between *Foc* STR4 and Cavendish bananas following cold stress treatment was elucidated in this study by using greenhouse inoculation and gene expression analysis. Bananas treated with cold temperatures were more severely affected with *Foc* than those grown at 28°C, probably as a result of the slowdown of metabolic processes which

manifested as chlorotic leaves and stunted growth. The expression of genes such as *PR1*, *PR4*, *PR-6*, *PR-10*, *MT2* and *Hin1*, all known to be associated with plant defence responses, were also delayed following exposure to cold temperatures.

In order for plants to protect themselves against pathogen attacked, pattern recognition receptors (PRRs) in the plant recognize PAMPs to initiate PAMP-triggered immunity (PTI) (Mengiste, 2012). In this study brassinosteroid insensitive 1-associated receptor kinase and proline-rich extensin receptor protein kinase, both shown to be involved in activation of PTI (Sanabria *et al.*, 2010; Kemmerling *et al.*, 2011), were identified during the early response of banana roots against *Foc* STR4. A second defence response is activated upon effector-triggered immunity (ETI), in which resistance (*R*)-genes are induced (Mengiste, 2012). After Cavendish banana plants were infected at 28^{o}C or 10^{o}C, five different disease resistance genes were expressed namely two disease resistance response genes, coiled-coil nucleotide-binding site leucine-rich repeat (CC-NBS-LRR) gene, *Hs1pro-1* (nematode resistance) and calcineurin B-like (CBL)-interacting ptotein kinase 4 gene. Transcription factors play a major role in PTI and ETI immunity. Transcription factors possibly involved in defence such as WRKY and BHLH transcription factors (Niu *et al.*, 2011; Van Verk *et al.*, 2011) were also identified in this study. Additionally, ion fluxes are further regulated during defence, and transcripts identified in this study include those coding for calmodulin and voltage-gated potassium channel. The host defence response is mediated by signalling pathways. During the early response of banana roots to *Foc* infection, we identified PAL and NPR1 involved in SA signalling (Vlot *et al.*, 2009); lipoxygenase and pnFL-2 involved in JA signalling (Schaller and Stintzi, 2009); ACS and ACO involved in ET signalling (Broekaert *et al.*, 2006) and ASR regulated in ABA signalling (Henry *et al.*, 2011). Importantly, several transcripts linked to various defence pathways in other plants were also identified from the banana transcriptome following infection with *Foc* STR4. Oxidative burst is mediated by catalase and peroxidase (Nanda *et al.*, 2010); Hin1 is required in programmed cell death (Lam and Zhang, 2012), while PR-1, PR-4, PR-5, PR-6, PR-10 and PR-14 are induced upon pathogen attacked (Sels *et al.*, 2008).

During early infection, high levels of PR proteins (PR-1, PR-4, PR-6 and PR-10) were expressed in Cavendish bananas grown at 28^{o}C, which presumably prevented Fusarium wilt development. Cold stress, however, delayed *PR* transcript expression which most likely

provided *Foc* STR4 with an opportunity to invade the xylem tissue. The expression of PR proteins in banana roots is important for protection against root pathogens such as *Foc*. For instance, PR-1 has antifungal activity and accumulates in xylem vessels (Houterman *et al.*, 2007), and PR-4 proteins are chitin-binding and hamper the growth of fungal pathogens (Van Loon *et al.*, 2006). Thus, low levels may have led to unrestricted infection of *Foc* STR4 and upon increased temperature resulted in increased disease severity. This was correlated with the increased disease development in plants subjected to cold stress. PR-6 forms part of the serine proteinase inhibitors and inhibits fungal growth by degrading lytic enzymes of the pathogen (Sels *et al.*, 2008) or by stimulating the JA pathway (Glazebrook, 2001), while PR-10 proteins have nuclease activity (both DNase and RNase) which is hypothesised to play an important role in the protection of the plant cell (Liu and Ekramoddoullah, 2006). Thus, during cold stress, *Foc* was not constrained by the protection of the PR transcripts and this accelerated disease symptom development. Infection was not hampered by the inhibitory effect of *PR* genes in the plant tissue which resulted in an increase in disease severity.

Cavendish banana roots exposed to temperature below 10^{o}C may compromise plant defence responses, thereby allowing *Foc* to infect the roots and colonise the xylem vessels. In this study, *C4H* was suppressed in plants subjected to cold stress, and up-regulated in control plants at 28^{o}C. C4H is a key enzyme in the phenylpropanoid pathway, and is important for phytoalexin and lignin production against pathogen infection (Dixon *et al.*, 2002). It is induced during different conditions, like pathogen invasion (Bi *et al.*, 2011), wounding (Batard *et al.*, 1997; Mizutani *et al.*, 1997), light (Mizutani *et al.*, 1997), ET treatment (Kim *et al.*, 2005), methyl jasmonate (MeJA) (Kim *et al.*, 2005) and biocontrol (yeast antagonist (*Metschnikowia fructicola*) (Hershkovitz *et al.*, 2012). We reported a significant up-regulation in roots predisposed to cold stress (10^{o}C) before infection at 28^{o}C. Thus, it can be concluded that the cold temperature inhibited the production of *C4H*.

LEA5 was induced upon cold stress in banana plants and is known to increase in the presence of external stresses like cold, dehydration and salt (Tunnacliffe and Wise, 2007). The LEA proteins act as molecular chaperons that aid in folding of denatured proteins as well as scavenging ions (Shih *et al.*, 2008). Our study showed a significant increase in *LEA5* levels after 12 and 24 h of cold stress. This is in accordance with the work by Zhang *et al*. (2009), that showed a rapid increase in *LEA* transcripts in the early stages of cold acclimation in

perennial ryegrass. Furthermore, *LEA5* levels also increased after wounding of the banana root tissue. Similar results were obtained in tomato roots where *ER5*, a *LEA* homolog showed increased levels 3 h after wounding (Zegzouti *et al.*, 1997). We hypothesize that the increased levels of *LEA5* shortly after wounding enabled the plants to protect the membrane structure, aided in refolding proteins after damage and also bound to proteins involved in ROS signalling (Salleh *et al.*, 2012). *LEA5* was further induced when the plants were transferred from 10°C to 28°C. This up-regulation might be due to the fact that plants acclimatise to the new environment. As expected, *LEA5* was involved in the cold stress response in Cavendish banana; but was not important in the defence response against *Foc*.

Hin1 is not only significantly suppressed at an early stage with cold stress (10°C) but is also significantly increased upon pathogen attack. Hin1 is a hairpin inducing protein that elicits the HR (Gopalan *et al.*, 1996; Takahashi *et al.*, 2004). Since non-race-specific disease resistance genes (NDR1) and Hin1-like genes share structural motifs, they are grouped into the NHL superfamily (Dormann *et al.*, 2000) and predicted to have a similar role in defence. *Arabidopsis* plants infected with an avirulent cucumber mosaic virus strain showed *NHL10* accumulation as well as local necrotic lesions, which is an HR response (Zheng *et al.*, 2004). Our results showed early mRNA accumulation of *Hin1* at 3 h, except with infection under cold temperatures, in which *Hin1* expression is suppressed at an early stage. As *Hin1* is a marker gene for the HR response (Gopalan *et al.*, 1996), we suggest that banana plants initiate the HR response as a defence mechanism to control *Foc* but the defence is hampered by cold temperatures. *Hin1* was significantly induced upon wounding in the uninfected control plants in our study, as well as in *Casuarina glauca* nodules for protection against pathogen attack (Santos *et al.*, 2010). In the study by Taylor (2005), the author identified a *NHL* gene in Calcutta 4 as well as in a Cavendish cultivar. The role of these *NHL* genes has not been studied during plant-pathogen interactions in banana.

ACO was suppressed in Cavendish bananas during the early stages of cold stress. ACO is a key enzyme in the production of ET (Bleecker and Kende, 2000), which is a hormone involved in several signalling processes such as plant defence and programmed cell death (Ecker and Davis, 1987; Greenberg, 1997; Broekaert *et al.*, 2006). High levels of ACO contribute to MeJA induced resistance (Yu *et al.*, 2011). Although there was a significant induction of *ACO* transcripts at 3 hpi in plants infected with *Foc* at 28°C, this is most

probably not related to pathogen defence, since the uninfected control plants showed the same type of reaction. Suppression of *ACO* levels by cold stress lead to limited signalling responses through ET. These limited signalling responses in the roots may weaken the defence responses and enable the pathogen, *Foc,* to overcome the plant's defence, which results in elevated disease development. This hypothesis is substantiated by the fact that heat treatment of banana fruit not only induced the expression of *MaACO1,* but increased the tolerance to *Colletotrichum musae* (Zhu *et al.*, 2011). Additionally, *Arabidopsis* plants overexpressing ethylene response factor 1 (*ERF1*), which regulate ethylene responsive defence-related genes, decrease the susceptibility towards *Fusarium oxysporum* sp. *conglutinans* and *F. oxysporum* f. sp. *lycopersici* (Berrocal-Lobo and Molina, 2004).

Cold stress in combination with *Foc* infection led to the significant reduction of *MT2* transcripts at a later stage (48 h and 5 dpi) in our study. Plant MTs are grouped into four subfamilies and are involved in scavenging ROS, detoxifying metal ions and control of redox potential (Hassinen *et al.*, 2010). We hypothesize that ROS is formed during the early response to infection with *Foc* and that the increase in *MT2* transcripts at a later stage will scavenge the harmful ROS. Our results support that, since *MT2* was significantly up-regulated in roots at the later infection stages (48 h and 5 dpi). As *MT2* is known to scavenge ROS (Wong *et al.*, 2004), the up-regulation of *MT2* in the later stages of infection may help to scavenge the ROS which was formed in signalling during the early response of the plant's defence. Similar results were reported by Van den Berg *et al*. (2007), who showed significant up-regulation of *MT2* in roots after infection with *Foc*. In all the other treatments, with and without infection, *MT2* was down-regulated at five days compared to the basal level. As *MT2* transcripts are suppressed by cold stress, ROS may cause damage to cellular components and DNA by oxidation. Furthermore, *MT* was highly abundant in all the libraries sequenced. Similar results were obtained in banana leaf tissue before and after temperature stress (Santos *et al.*, 2005; Carpentier *et al.*, 2008), and also in banana fruit (Liu *et al.*, 2002).

Absiscic stress ripening proteins are known to be induced upon different abiotic and biotic stresses as well as fruit ripening (Liu *et al.*, 2010). Four different classes of ASR have been identified in banana (Henry *et al.*, 2011), of which three classes, ASR1, ASR2 and ASR4 were found in this study. *ASR4* was present in high numbers in root tissue in all the libraries. Henry *et al*. (2011) found similar results where *ASR4* was significantly more expressed in

roots of cv. Cachaco (ABB) compared to leaf tissue. We further characterized the expression profile of *ASR4* in which the expression level did not significantly differ during cold stress. Although the control plant's roots were crushed to induce wounding, *ASR4* was not significantly up-regulated. This is in accordance with the work by Henry *et al.* (2011), who found that *ASR4* is not induced upon wounding. Therefore, we suggest that *ASR4* does not play a significant role in cold stress nor in plant defence against *Foc*. However, in the study by Liu *et al.* (2010), *ASR1* was found to be induced upon infection with *Foc*, and also to play a role in drought stress (Carpentier *et al.*, 2007). The expression profiling of other *ASRs* after *Foc* infection with *Foc* under cold stress could reveal more information about its functionality during defence at cold temperatures.

Plants defend themselves against pathogens by activating complex signalling pathways involving SA, JA and ET. These pathways can interact with each other synergistically or antagonistically. *PR-1* is an indicator of the SA response (Vlot *et al.*, 2009), and the early up-regulation of this transcript indicates that banana exploits the SA pathway as defence signalling pathway against *Foc* infection. Additionally, *PR6* showed up-regulation upon pathogen attack 12 hpi and is essential in the activation of the JA pathway (Hase *et al.*, 2008). The expression of *PR4* is known to be induced by ET (Broekaert *et al.*, 2006) and was highly expressed in infected root tissue at 3 hpi. These results show that SA, JA and ET pathways synergistically interact with each other to defend against *Foc*. Controversially, the interaction of banana and *Foc* 'tropical' race 4 has recently been studied by Li *et al.* (2012) and they have found that SA was not significantly involved in defence. Although, several studies indicated an antagonistic relationship between SA and JA (Koornneef and Pieterse, 2008; El Oirdi *et al.*, 2011), the ability of banana plants to protect themselves from invasion by *Foc* STR4 utilizing several defence pathways has been reported in other studies (Van Wees *et al.*, 2000; Yarullina *et al.*, 2011). For example, in *Arabidopsis* plants, resistance against *Pseudomonas syringae* pv. *tomato* is achieved through simultaneous activation of SA and JA pathways (Van Wees *et al.*, 2000).

CONCLUSIONS

This study provides the first investigation of the response of Cavendish banana roots following *Foc* infection after cold predisposition. Greenhouse trials showed that disease

severity was significantly increased following cold stress, and that a number of defence genes were activated in banana roots exposed to and without cold stress. These genes included those involved in the activation of PTI, the SA, JA and ET signalling pathways, oxidative burst and HR. Cold temperature delayed the response time and suppressed defence related genes which presumably enabled *Foc* STR4 to invade the xylem vessels and contribute to disease development. We hypothesize that cold stress (10^{o}C) increases fungal infection, but that disease development occurs only at 28^{o}C. Higher temperatures increase evaporation, which enhance transport of *Foc* in the xylem vessels and increase the growth rate of the pathogen (Beckman *et al.*, 1962). The EST sequence data obtained in this study will be useful in subsequent studies escepically for gene expression and functional genomics studies.

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TABLES AND FIGURES

Table 3.1. Primer sequences used in quantitative reverse transcription PCR analysis to quantify reference and putative defence/stress related genes after infection of Cavendish banana roots with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 with and without cold temperatures.

PrimerID	Gene identity	Accession number	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Product size (bp)	Annealing tempera-ture (°C)
ubi[a]	Ubiquitin	AY651067	AGGCCTGCTGCTAGAGTTCA	TAGCAACCACCAACCAGATG	77	60
actin[a]	Actin	-	GCTATTCAGGGCGTCCTTTC	GCTGACACCATCACCAGAATC	78	61
musacont1[b]	Ribososomal protein S23 *Medicago truncatula*	ABE78299	TGACGAAGTCTTGATCGCTGG	AATAGCGCCAACAGCGACA	145	60
25S[a]	Ribosomal protein	AF 399949	GTAAACGGCGGGAGTCACTA	TCCCTTTGGTCTGTGGTTTC	106	61
PR-1[a]	Pathogenesis related protein class 1	DQ531622	TCCGGCCTTATTTCACATTC	GCCATCTTCATCATCTGCAA	126	61
PR-4	Pathogenesis related protein class 4	-	GCAGAAATGTCCGGTCTCAT	CTTGGCTCCTACACCAGCTT	105	61
PR-6	Pathogenesis related protein class 6	-	GTTGACAAGGCTGGAATCGT	TATTCATGTCGGTGGCACAA	123	61

PrimerID	Gene identity	Accession number	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Product size (bp)	Annealing tempera-ture (^{o}C)
PR-10	Pathogenesis related protein class 10	-	TCCGTAAGGCAGCTCAACTT	TGCTTGCACTCGAACTTGTC	95	61
ACO	1-aminocyclopropane-1-carboxylic acid	-	CCATCGCCTCCTTCTACAAC	TAGTCGCCGAAGACGAACTT	118	61
ASR4	Abscisic stress ripening	-	CTTATGCCCTGCACGAGAA	CTCATGGTGCTCATGGAATG	131	61
LEA5	Late embryogenesis abundant protein 5	-	CTCAAAACATCGCCCTCTTG	CGAGGAGTCTACCACCTTGC	119	61
MT2	Metallothionenin type 2	-	GGCACTTTGAGGAGCTTGAG	CTCCTTCCTTCCTCCAAACC	124	61
C4H	Cinnamate-4-monoxygenase	-	CGGGATCATACTGGCATTG	CCCTTCTCTGTCACGTCGAT	111	61
Hin1	Harpin-induced protein 1	-	GGAGCGGAGGGACTTTAACT	AGTGGCCGATCTTGATGAAC	80	61

[a] Primer sequences previously identified by Van den Berg *et al*. (2007)

[b] Primer sequences previously identified by Munro (2008)

Table 3.2. Results of *de novo* assembly of sequences with CAP3 and Newbler from root tissue of Cavendish bananas after infection with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 (*Foc* STR4) obtained from the 454 GS FLX with three treatments namely A. Infected with *Foc* STR4 at 28°C (infected), B. Plants predisposed to 10°C for two weeks and transferred to 28°C and infected with *Foc* STR4 (precold) and C. Infected with *Foc* STR4 at 10°C (coldinf).

Library	Reads	Contigs		Singletons		Average contig length (bp)		Largest contig		N50 contig size	
		CAP3	Newbler	CAP3	Newbler	CAP3	Newbler	CAP3	Newbler	CAP3	Newbler
Infected	5935	489	35	3699	4602	623	670	1385	1629	630	645
Precold	4840	388	23	3303	3985	611	616	1234	942	614	584
Coldinf	4689	373	16	3208	3697	611	720	1726	1763	604	663

Table 3.3. The ten most abundant transcripts identified in Cavendish banana root tissue after infection with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 at 28°C.

Contig name	Length (bp)	Abun-dance[a]	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
infect_485	646	98	ABQ14530.1	Metallothionein type 2	*Typha angustifolia*	$3e^{-26}$
infect_570	657	48	XP_002698352.1	Hypothetical protein BOS_23236	*Bos taurus*	$3e^{-33}$
infect_670	621	37	ACA30301.1	Putative senescence-associated protein	*Cupressus sempervirens*	$4e^{-23}$
infect_5	214	36	XP_001892072.1	Putative T-cell receptor beta chain ANA 11	*Brugia malayi*	$3e^{-16}$
infect_546	794	30	CBK22332.2	Unnamed protein product	*Blastocystis hominis*	$5e^{-50}$
infect_593	608	28	XP_002698352.1	Hypothetical protein BOS_23236	*Bos taurus*	$2e^{-33}$
infect_461	531	23	ACV50425.1	Cold induced plasma membrane protein	*Jatropha curcas*	$3e^{-23}$
infect_496	892	22	ACF06544.1	Pathogenesis-related protein	*Elaeis guineensis*	$2e^{-73}$
infect_440	837	17	pdb|1X1V	Lectin	*Musa acuminata* AAA Group	$4e^{-51}$
infect_642	649	16	ABS86033.1	Mannose-specific recombinant lectin [synthetic construct]	-	$8e^{-61}$

[a] The number of transcripts used to assemble the contig

Table 3.4. The ten most abundant transcripts identified in Cavendish banana root tissue predisposed to cold temperatures (10°C) for two weeks followed by infection with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 at 28°C.

Contig name	Length (bp)	Abun-dance[a]	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
precold_412	681	41	ABQ14530.1	Metallothionein type 2	*Typha angustifolia*	$4e^{-26}$
precold_543	792	19	XP_002698352.1	Hypothetical protein BOS_23236	*Bos taurus*	$4e^{-33}$
precold_403	648	18	ZP_06798900.1	3-Hydroxyisobutyrate dehydrogenase	*Mycobacterium tuberculosis*	$5e^{-44}$
precold_374	935	15	ABK41053.2	Pathogenesis-related protein 1	*Musa acuminata*	$8e^{-56}$
precold_390	743	15	ACZ50734.1	Abscisic stress ripening	*Musa* ABB Group	$1e^{-56}$
precold_836	604	15	XP_002698352.1	Hypothetical protein BOS_23236	*B. taurus*	$2e^{-33}$
precold_507	571	14	EAY73411.1	Hypothetical protein OsI_01294	*Oryza sativa* Indica Group	$3e^{-50}$
precold_400	587	14	ABQ14530.1	Metallothionein type 2	*T. angustifolia*	$3e^{-26}$
precold_593	509	14	ADG57923.1	Transcription factor	*Lycoris longituba*	$1e^{-11}$
precold_1272	706	13	EEE66724.1	Hypothetical protein OsJ_23408	*O. sativa* Japonica Group	$6e^{-05}$

[a] The number of transcripts used to assemble the contig

Table 3.5. The ten most abundant transcripts identified in Cavendish banana root tissue after infection with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 at 10°C.

Contig name	Length (bp)	Abun-dance[a]	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
coldinf_383	607	78	XP_002698352.1	Hypothetical protein BOS_23236	*Bos taurus*	$2e^{-33}$
coldinf_443	667	70	XP_002698352.1	Hypothetical protein BOS_23236	*B. taurus*	$3e^{-33}$
coldinf_429	538	31	ABQ14530.1	Metallothionein type 2	*Typha angustifolia*	$2e^{-26}$
coldinf_369	1726	26	BAB33421.1	Putative senescence-associated protein	*Pisum sativum*	$2e^{-85}$
coldinf_417	597	21	ZP_06798900.1	3-hydroxyisobutyrate dehydrogenase	*Mycobacterium tuberculosis*	$3e^{-29}$
coldinf_13	1443	19	YP_001949468.1	Cell wall-associated hydrolase	*Burkholderia multivorans*	$1e^{-108}$
coldinf_366	776	18	ZP_06800983.1	Hypothetical protein Mtub2_12463	*M. tuberculosis*	$3e^{-43}$
coldinf_638	497	17	EEC74585.1	Hypothetical protein OsI_10164	*Oryza sativa* Indica Group	$8e^{-28}$
coldinf_505	642	17	AAB82774.1	Ripening-associated protein	*Musa acuminata* AAA Group	$3e^{-46}$
coldinf_375	782	14	1X1V	Lectin	*M. acuminata* AAA Group	$1e^{-50}$

[a] The number of transcripts used to assemble the contig

Table 3.6. Putative identities of transcripts involved in defence/stress in other crops identified in banana root tissue after infection with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 generated by a 454 GS FLX sequencer. Annotation is based on BLAST results obtained from the NR database.

Contig	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
Receptors and elicitors involved in pathogen-associated molecular patterns triggered immunity				
infect_1306	XP_002510650.1	Receptor protein kinase, putative	*Ricinus communis*	$1e^{-44}$
infect_2313	ACG28793.1	Receptor protein kinase PERK1	*Zea mays*	$1e^{-23}$
infect_711	ABF96847.1	Brassinosteroid insensitive 1-associated receptor kinase 1	*Oryza sativa* Japonica Group	$7e^{-39}$
coldinf_1372	ADN34056.1	GABA(A) receptor-associated protein	*Cucumis melo* subsp. *melo*	$4e^{-43}$
coldinf_2336	ABS83497.1	Receptor-like serine threonine kinase	*O. sativa* Japonica Group	$1e^{-14}$
infect_3220	AAV92899.1	Avr9/Cf-9 rapidly elicited protein 140	*Nicotiana tabacum*	$7e^{-11}$
precold_880	BAD16491.1	Putative Avr9/Cf-9 rapidly elicited protein	*O. sativa* Japonica Group	$6e^{-26}$
coldinf_2146	NP_189098.1	Proline extensin-like receptor kinase (Atperk1)	*Arabidopsis thaliana*	$9e^{-7}$
infect_790	Q9SX31	Proline-rich receptor-like protein kinase PERK9	*A. thaliana*	$3e^{-9}$
Resistance genes involved in effector-triggered immunity				
coldinf_2124	NP_173703.2	Putative disease resistance response protein	*A. thaliana*	$1e^{-7}$
infect_2269	CBW30238.1	Disease resistance protein (CC-NBS-LRR)	*Musa balbisiana*	$7e^{-21}$
infect_472	XP_002513105.1	Disease resistance response protein, putative	*R. communis*	$4e^{-28}$
precold_601	BAD82302.1	Putative nematode resistance protein Hs1pro-1	*O. sativa* Japonica Group	$1e^{-36}$
infect_3190	CBW30552.1	CBL-interacting protein kinase 04	*Vitis vinifera*	$1e^{-20}$

Contig	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
Ion fluxes				
infect_968	NP_001167666.1	Calmodulin	*Z. mays*	$2e^{-56}$
infect_967	NP_001167666.1	Calmodulin	*Z. mays*	$6e^{-57}$
infect_1845	XP_002512195.1	Calcium ion binding protein, putative	*R. communis*	$3e^{-10}$
coldinf_1306	AAA33705.1	Calmodulin-related protein	*Petunia x hybrid*	$6e^{-45}$
coldinf_1808	NP_001105547.1	Calmodulin2	*Z. mays*	$2e^{-50}$
precold_1352	NP_197748.1	CDPK9 (calmodulin-like domain protein kinase 9)	*A. thaliana*	$3e^{-45}$
precold_1598	ACQ83560.1	Calcineurin B-like protein	*V. vinifera*	$4e^{-24}$
precold_2621	BAD08916.1	Calcium-binding EF-hand family protein-like	*O. sativa* Japonica Group	$6e^{-13}$
infect_ 2453	XP_002514898.1	Calmodulin-binding transcription activator	*R. communis*	$4e^{-30}$
precold_1128	ACG31559.1	Calmodulin	*Z. mays*	$1e^{-18}$
precold_1219	AAR99409.1	Calmodulin	*Arachis hypogaea*	$7e^{-41}$
coldinf_ 2242	NP_001150755.1	Calmodulin binding protein	*Z. mays*	$1e^{-17}$
infect_ 790	XP_002368679.1	Voltage gated chloride channel domain-containing protein	*Toxoplasma gondii* ME49	$1e^{-10}$
infect_ 1614	ACF06516.1	Vacuolar H+-ATP synthase 16kDa proteolipid subunit	*Elaeis guineensis*	$1e^{-54}$
infect_476	ABS72193.1	Vacuolar proton pump subunit F	*Corchorus olitorius*	$8e^{-43}$
precold_1085	XP_002532256.1	Vacuolar proton ATPase, putative	*R. communis*	$1e^{-14}$

Contig	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
coldinf_1148	ACG48688.1	Vacuolar ATP synthase catalytic subunit A	*Z. mays*	$1e^{-33}$
coldinf_630	XP_002515334.1	H+ transporting ATPase plant/fungi plasma membrane type, putative	*R. communis*	$1e^{-75}$
coldinf_688	XP_645434.1	Vacuolar H+ ATPase F subunit	*Dictyostelium discoideum* AX4	$5e^{-35}$
Transcription factors				
infect_ 1343	ABS18436.1	WRKY39	*Glycine max*	$1e^{-46}$
precold_397	ACY25182.1	WRKY	*V. vinifera*	$1e^{-24}$
coldinf_1093	NP_001148212.1	WRKY23 - superfamily of TFs having WRKY and zinc finger domains	*Z. mays*	$1e^{-31}$
coldinf_2349	ABF69964.1	DNA-binding WRKY domain-containing protein	*M. acuminata*	$1e^{-6}$
infect_2623	NP_194827.2	Basic helix-loop-helix (bHLH) family protein	*A. thaliana*	$1e^{-13}$
infect_ 1179	XP_002307142.1	AP2/ERF domain-containing transcription factor	*Populus trichocarpa*	$4e^{-18}$
infect _1503	XP_002310127.1	AP2/ERF domain-containing transcription factor	*P. trichocarpa*	$1e^{-7}$
infect_ 1661	ADB85099.1	Putative transcription regulator	*Jatropha curcas*	$3e^{-30}$
infect_ 1719	ADL36657.1	C3HL domain class transcription factor	*Malus x domestica*	$8e^{-23}$
infect_ 2638	ADG58085.1	Transcription factor	*Lycoris longituba*	$1e^{-6}$
infect_ 3646	XP_002284400.1	MYB transcription factor MYB139	*V. vinifera*	$2e^{-31}$
infect_ 432	AAS19479.1	MYB5	*Tradescantia fluminensis*	$2e^{-26}$
infect_ 462	ADG57949.1	Transcription factor	*L. longituba*	$3e^{-30}$

Contig	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
precold_1045	ADG57979.1	Transcription factor	*L. longituba*	$3e^{-52}$
precold_1057	ADG58020.1	Transcription factor	*L. longituba*	$2e^{-13}$
precold_593	ADG57923.1	Transcription factor	*L. longituba*	$1e^{-11}$
precold_938	ADE41103.1	AP2 domain class transcription factor	*Malus x domestica*	$9e^{-8}$
coldinf_1144	ACN71235.1	bZIP transcription factor	*Tamarix hispida*	$3e^{-37}$
coldinf_1955	XP_002518952.1	Transcription factor, putative	*R. communis*	$2e^{-13}$
coldinf_2764	ADK25058.1	AN1-like transcription factor	*G. max*	$2e^{-21}$
coldinf_447	ADG57979.1	Transcription factor	*L. longituba*	$8e^{-54}$
Transcription factors				
precold_723	A2YH64	Catalase isozyme B	*O. sativa* Indica Group	$9e^{-18}$
precold_869	XP_002306976.1	Catalase	*P. trichocarpa*	$3e^{-31}$
infect_ 1512	ADN96694.1	Peroxidase 7	*Rubia cordifolia*	$3e^{-38}$
infect_ 1656	AAD43561.1	Bacterial-induced peroxidase precursor	*Gossypium hirsutum*	$9e^{-42}$
infect_ 1698	BAA03373.1	Putative peroxidase	*O. sativa* Japonica Group	$5e^{-36}$
infect_ 2293	ACN25040.1	Peroxidase	*Doritis pulcherrima* x *Phalaenopsis* hybrid cultivar	$6e^{-35}$
precold_452	NP_001147254.1	Peroxidase 52	*Z. mays*	$7e^{-27}$
coldinf_1444	NP_001147254.1	Peroxidase 52	*Z. mays*	$1e^{-32}$
coldinf_2097	ACO90366.1	Peroxidase precursor	*Triticum aestivum*	$1e^{-11}$
coldinf_428	BAA01950.1	Peroxidase	*Vigna angularis*	$1e^{-29}$

Contig	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
coldinf_437		Peroxidase 7	*R. cordifolia*	$1e^{-21}$
Hypersensitive response				
infect_652	CAA68848.1	Hin1	*N. tabacum*	$3e^{-12}$
infect_ 2524	XP_002519736.1	Programmed cell death, putative	*Ricinus communis*	$1e^{-10}$
Pathogenesis related				
infect_1123	ACF06544.1	Pathogenesis-related protein	*E. guineensis*	$5e^{-27}$
infect_483	XP_002945771.1	Pathogenesis-related protein 1-like protein	*Volvox carteri* f. *nagariensis*	$9e^{-18}$
infect_486	ACF06544.1	Pathogenesis-related protein	*E. guineensis*	$3e^{-17}$
infect_496	ACF06544.1	Pathogenesis-related protein	*E. guineensis*	$2e^{-73}$
precold_1574	ACF06544.1	Pathogenesis-related protein	*E. guineensis*	$2e^{-12}$
precold_3062	XP_002945770.1	Pathogenesis-related protein 1-like protein	*Volvox carteri* f. *nagariensis*	$1e^{-8}$
precold_374	ABK41053.2	Pathogenesis-related protein 1	*Musa acuminata*	$8e^{-56}$
coldinf_1399	ACF06544.1	Pathogenesis-related protein	*E. guineensis*	$2e^{-65}$
infect_2033	XP_002884462.1	PR4-type protein	*A. lyrata* subsp. *lyrata*	$1e^{-57}$
infect_3540	ACM45716.1	Chitinase class IV	*Pyrus pyrifolia*	$7e^{-63}$
precold_883	BAD34224.1	Putative thaumatin-like protein	*Oryza sativa* Japonica Group	$8e^{-27}$
infect_467	CAA87073.1	Pathogenesis-related protein PR-6 type	*Sambucus nigra*	$2e^{-7}$
coldinf_399	CAA87073.1	Pathogenesis-related protein PR-6 type	*S. nigra*	$9e^{-17}$
infect_438	AAF60972.2	Pathogenesis-related protein psem	*Pseudotsuga menziesii*	$2e^{-18}$

Contig	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
infect_444	AAL50005.1	PR10 protein	*Pinus monticola*	$1e^{-24}$
infect_499	ACF06599.1	Pathogenesis-related protein 10c	*E. guineensis*	$8e^{-25}$
infect_501	ACF06599.1	Pathogenesis-related protein 10c	*E. guineensis*	$3e^{-57}$
infect_503	ACF06599.1	Pathogenesis-related protein 10c	*E. guineensis*	$1e^{-22}$
infect_949	ACF06599.1	Pathogenesis-related protein 10c	*E. guineensis*	$4e^{-43}$
precold_510	ACF06599.1	Pathogenesis-related protein 10c	*E. guineensis*	$1e^{-50}$
precold_511	ACF06599.1	Pathogenesis-related protein 10c	*E. guineensis*	$4e^{-57}$
precold_653	AAL50005.1	PR10 protein	*P. monticola*	$1e^{-20}$
coldinf_432	AAL50005.1	PR10 protein	*P. monticola*	$1e^{-24}$
coldinf_754	ACF06599.1	Pathogenesis-related protein 10c	*E. guineensis*	$6e^{-52}$
infect_951	ACG69488.1	Germin-like protein 12	*Glycine max*	$9e^{-36}$
infect_952	Q2QXJ0	Putative germin-like protein	*O. sativa* Japonica Group	$2e^{-7}$
infect_953	AAL05886.1	Germin-like protein	*M. acuminata* AAA group	e^{-109}
coldinf_568	Q2QXJ1	Putative germin-like protein	*O.sativa* Japonica Group	$2e^{-5}$
coldinf_736	ACJ64505.1	Germin-like protein	*Z. mays*	$2e^{-78}$
infect_1245	NP_190966.1	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	*A. thaliana*	$2e^{-28}$
infect_2210	AAF35184.1	Lipid transfer protein precursor	*Gossypium hirsutum*	$1e^{-16}$
precold_1320	ABQ88334.1	Lipid transfer protein	*Capsicum annuum*	$1e^{-32}$
precold_2267	AAF35184.1	Lipid transfer protein precursor	*G. hirsutum*	$3e^{-23}$
coldinf_431	P10976	Non-specific lipid-transfer protein	*Spinacia oleracea*	$1e^{-11}$

Contig	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
coldinf_711	NP_568160.1	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	*A. thaliana*	$2e^{-32}$
coldinf_892	XP_002524256.1	Lipid binding protein, putative	*R. communis*	$1e^{-34}$
Signalling				
Salicylic acid				
infect _1238	ABL63913.1	NPR1-like protein	*M. acuminata* AAA Group	$4e^{-5}$
infect _1026	ACG80828.1	Phenylalanine ammonia lyase	*M. acuminata* AAA group	$2e^{-14}$
precold_440	ADM74255.1	Phenylalanine ammonia lyase-like protein	*Picea sitchensis*	$3e^{-42}$
Jasmonic acid				
coldinf_1453	AAD09861.1	Lipoxygenase	*Persea americana*	$2e^{-67}$
infect_2409	NP_001148852.1	pnFL-2	*Z. mays*	$3e^{-15}$
Ethylene				
infect_407	AAN87846.1	1-aminocyclopropane-1-carboxylic acid oxidase	*Populus trichocarpa*	$7e^{-70}$
precold_29	AAQ13435.1	1-aminocyclopropane-1-carboxylate synthase	*M. acuminata*	$3e^{-54}$
coldinf_1591	XP_002300962.1	1-aminocyclopropane-1-carboxylate	*P. trichocarpa*	$3e^{-34}$
ABA				
infect_460	ACZ50734.1	Abscisic stress ripening	*Musa* ABB Group	$1e^{-56}$
precold_2489	ACZ60123.1	Abscisic stress ripening	*Musa* ABB Group	$5e^{-18}$
precold_390	ACZ50734.1	Abscisic stress ripening	*Musa* ABB Group	$1e^{-56}$
coldinf_1866	ACZ50734.1	Abscisic stress ripening	*Musa* ABB Group	$9e^{-18}$
coldinf_377	ACZ50734.1	Abscisic stress ripening	*Musa* ABB Group	$1e^{-55}$

Contig	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
Phytoalexin production				
infect_ 477	ABF20067.1	Chalcone synthase	*Zingiber officinale*	$3e^{-36}$
coldinf_1600	ABG26444.1	Chalcone synthase	*Z. officinale*	$1e^{-44}$
infect_ 1678	ACH63235.1	Alcohol dehydrogenase	*Rheum australe*	$4e^{-59}$
precold_1452	P48977	Alcohol dehydrogenase	*Malus x domestica*	$9e^{-20}$
precold_2274	ACH63235.1	Alcohol dehydrogenase	*R. australe*	$1e^{-24}$
precold_580	ACZ48689.1	Alcohol dehydrogenase	*Salvia miltiorrhiza*	$4e^{-32}$
precold_581	ACF06607.1	Alcohol dehydrogenase	*Elaeis guineensis*	$1e^{-117}$
coldinf_1477	ACF06607.1	Alcohol dehydrogenase	*E. guineensis*	$9e^{-29}$
coldinf_424	AAB39597.1	Alcohol dehydrogenase B	*Washingtonia robusta*	$2e^{-37}$
precold_658	ACH56520.1	Cinnamate-4-hydroxylase	*G. hirsutum*	$9e^{-77}$
precold_438	XP_002331408.1	Trans-cinnamate 4-monooxygenase	*P. trichocarpa*	$2e^{-42}$
infect_454	AAT67247.1	Isoflavone reductase	*Musa acuminata*	$4e^{-51}$
precold_384	AAT67247.1	Isoflavone reductase	*M. acuminata*	$9e^{-48}$
Cell wall modification				
infect_461	ACV50425.1	Cold induced plasma membrane protein	*Jatropha curcas*	$3e^{-23}$
infect_1191	ACV50425.1	Cold induced plasma membrane protein	*J. curcas*	$6e^{-18}$
coldinf_398	ACV50425.1	Cold induced plasma membrane protein	*J. curcas*	$5e^{-23}$
infect_494	ACV50425.1	Cold induced plasma membrane protein	*J. curcas*	$1e^{-21}$
precold_524	ACV50425.1	Cold induced plasma membrane protein	*J. curcas*	$2e^{-23}$
precold_2523	ABR27717.1	Dirigent-like protein	*Picea sitchensis*	$1e^{-11}$
precold_2128	ACA04851.1	Dirigent-like protein pDIR14	*P. abies*	$2e^{-8}$

Contig	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
coldinf_2124	ABD52118.1	Dirigent-like protein pDIR	*P. glauca*	$4e^{-6}$
coldinf_2914	ACU55136.1	Dirigent-like protein 2	*G. hirsutum*	$7e^{-15}$
infect_3008	ABD52118.1	Dirigent-like protein pDIR7	*G. hirsutum*	$6e^{-12}$
Oxidative stress				
infect_510	CAA09193.1	Glutathione transferase	*Alopecurus myosuroides*	$2e^{-46}$
infect_1081	ACF06490.1	Glutathione-S-transferase	*E. guineensis*	$2e^{-34}$
infect_1832	XP_002509785.1	Glutathione-S-transferase	*Ricinus communis*	$4e^{-40}$
infect_797	ACF06541.1	Glutathione S-transferase	*E. guineensis*	$7e^{-15}$
precold_428	AAF22517.1	Glutathione S-transferase	*Papaver somniferum*	$1e^{-67}$
precold_752	ACF06541.1	Glutathione S-transferase	*E. guineensis*	$1e^{-35}$
infect_485	ABQ14530.1	Metallothionein type 2	*Typha angustifolia*	$3e^{-26}$
infect_1136	ABC60342.1	Putative type 2 metallothionein	*M. acuminata* AAA Group	$2e^{-15}$
infect_415	CAB52585.1	Metallothionein-like protein	*E. guineensis*	$3e^{-25}$
precold_412	ABQ14530.1	Metallothionein type 2	*T. angustifolia*	$4e^{-26}$
precold_400	ABQ14530.1	Metallothionein type 2	*T. angustifolia*	$3e^{-26}$
precold_556	ABC60342.1	Putative type 2 metallothionein	*M. acuminata* AAA Group	$6e^{-16}$
precold_399	ABQ14530.1	Metallothionein type 2	*T. angustifolia*	$3e^{-26}$
precold_411	ABC60342.1	Putative type 2 metallothionein	*M. acuminata* AAA Group	$1e^{-21}$
coldinf_429	ABQ14530.1	Metallothionein type 2	*T. angustifolia*	$2e^{-26}$
coldinf_1047	ABC60342.1	Putative metallothionein type 2	*M. acuminata* AAA Group	$2e^{-16}$
coldinf_425	ABQ14530.1	Metallothionein type 2	*T. angustifolia*	$5e^{-26}$
coldinf_427	ABQ14530.1	Metallothionein type 2	*T. angustifolia*	$2e^{-26}$

Contig	Genbank nr	Most similar protein homology	Species of homologous protein	E-value
Other				
infect_1070	BAA34919.1	Heat shock protein 70 cognate	*Salix gilgiana*	$5e^{-30}$
precold_376	AAL85887.1	70 kDa heat shock protein	*Sandersonia aurantiaca*	$3e^{-23}$
precold_375	ACJ11740.1	Heat shock protein 70	*G. hirsutum*	$4e^{-52}$
coldinf_1143	BAA34919.1	Heat shock protein 70 cognate	*Salix gilgiana*	$5e^{-31}$
infect_1085	ACG38807.1	Multiple stress-responsive zinc-finger protein ISAP1	*Zea mays*	$5e^{-22}$
infect_2874	XP_002864044.1	Wound-responsive 3	*Arabidopsis lyrata* subsp. *lyrata*	$2e^{-14}$

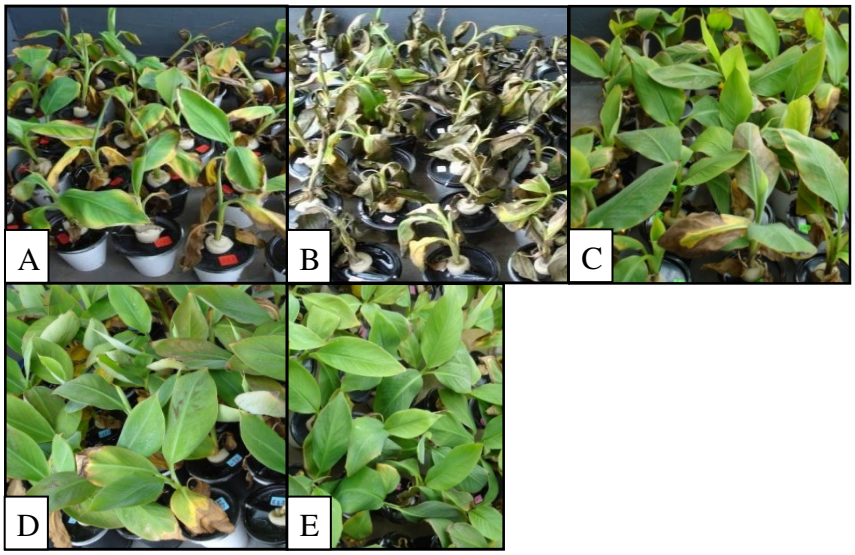


Figure 3.1. External symptom development of Grand Naine banana plants six weeks after infection with *Fusarium oxysporum* f. sp. *cubense* (*Foc*) 'subtropical' race 4 (STR4). Treatments included A. Plants subjected to 10°C and infected with *Foc* STR4 (coldinf), B. plants incubated at 10°C without infection (cold). Plants subjected to cold temperatures showed extensive leaf necrosis and stunted growth, C. Infection at 28°C with *Foc* STR4 (infected). The external symptoms included yellowing of the older leaves and necrosis of the leaf edges, D. Plants predisposed to 10°C for two weeks and transferred to 28°C and infected with *Foc* STR4 (precold) and E. Control plants at 28°C without infection (control).

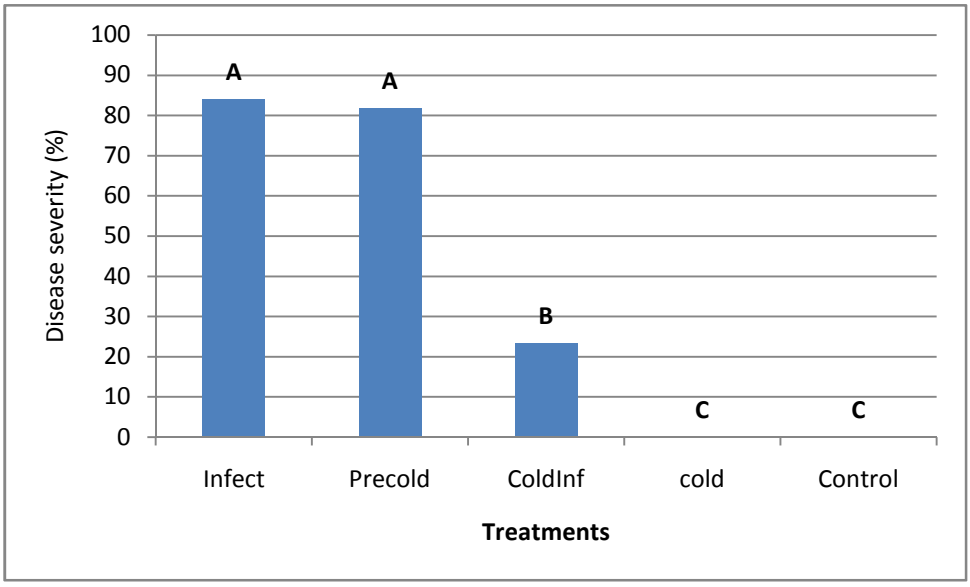


Figure 3.2. Fusarium wilt disease severity on Grand Naine banana plants (Cavendish subgroup) after infection with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 (*Foc* STR4) following five different treatments. Disease severity was calculated according to the degree of discolouration after six weeks according to the INIBAP rating scale (Carlier *et al.*, 2002) with ten plants per treatment. Treatments included: A. Infection at 28°C with *Foc* 'subtropical' race 4 (STR4) (infect), B. Plants predisposed to 10°C for two weeks then transferred to 28°C and infected with *Foc* STR4 (precold), C. Plants subjected to 10°C and infected with *Foc* STR4 (coldinf), D. Plants incubated at 10°C without infection (cold) and E. Control plants at 28°C without infection (control). All the plants were transferred to 28°C after four weeks. Statistical analysis was performed by JMP® (SAS Institute, Cary, North Carolina) using a multifactor analysis of variance (ANOVA) test and significant difference values were determined at $p<0.05$ using the Student t-test where different letters above the bar show significant differences.

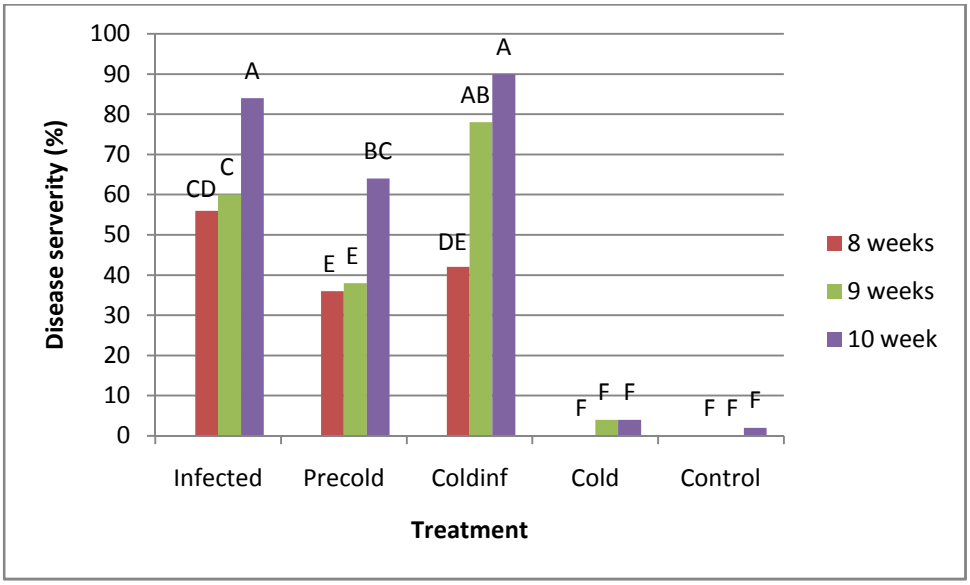


Figure 3.3. Fusarium wilt disease severity on Grand Naine banana plants (Cavendish subgroup) after infection with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 (*Foc* STR4) following five different treatments, namely A. Infection at 28°C with *Foc* 'subtropical' race 4 (STR4) (infect), B. Plants predisposed to 10°C for two weeks and transferred to 28°C and infected with *Foc* STR4 (precold), C. Plants subjected to 10°C and infected with *Foc* STR4 (coldinf), D. plants incubated at 10°C without infection (cold) and E. Control plants at 28°C without infection (control). After roots were harvested (between 0 hours to five days after infection) the plants were placed back into the cups and disease ratings were preformed after eight, nine and ten weeks according to the INIBAP rating scale (Carlier *et al.*, 2002). All the plants were moved to 28°C after four weeks after infection. There were ten plants per treatment per time point. The data were analyzed by using the JMP program (SAS Institute, Cary, North Carolina) using multifactor analysis of variance (ANOVA) test and significant difference values were determined at $p<0.05$ using the Student t-test. Different letters above the bars show significant differences.

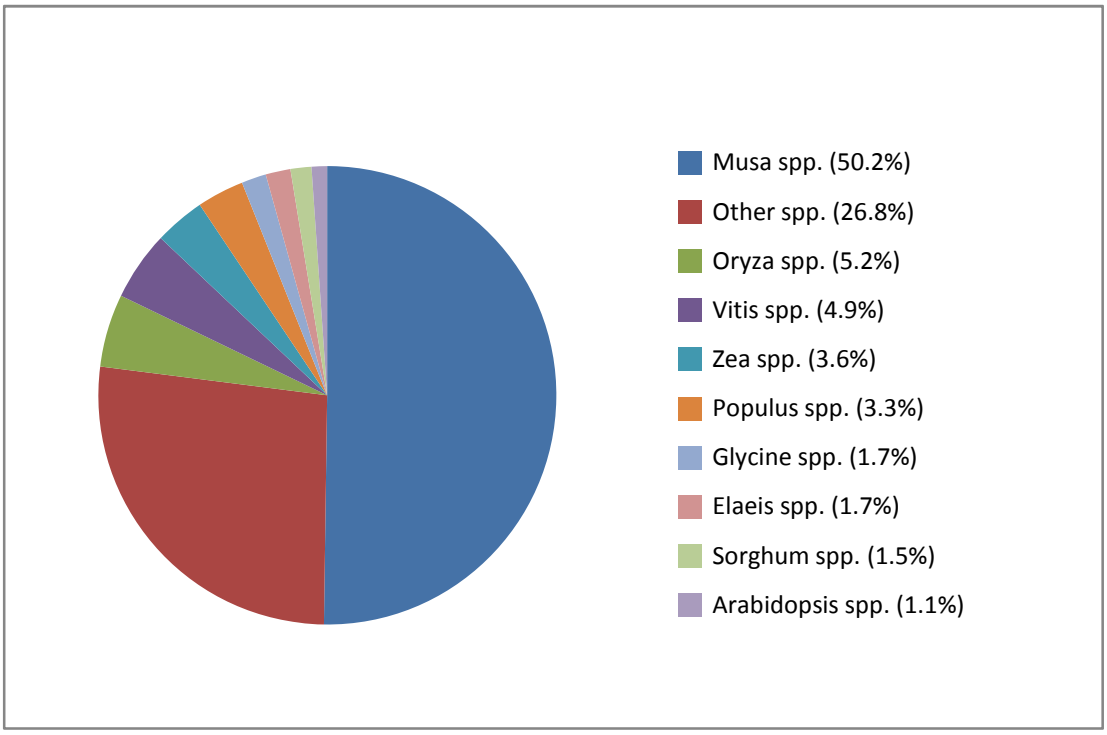


Figure 3.4. Percentage of contigs with homology to other organisms identified 3 and 12 hpi in banana root tissue after infection with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 at 10°C, 28°C and predisposed to 10°C for two weeks and infected at 28°C. The species were taken from the first BLAST hits against the NCBI non redundant protein database or the *Musa acuminata* cv. Pahang database. As *M. acuminata* cv. Pahang was sequenced (D'Hont *et al.*, 2012), 50.2% of the total contigs showed homology to *Musa* spp.

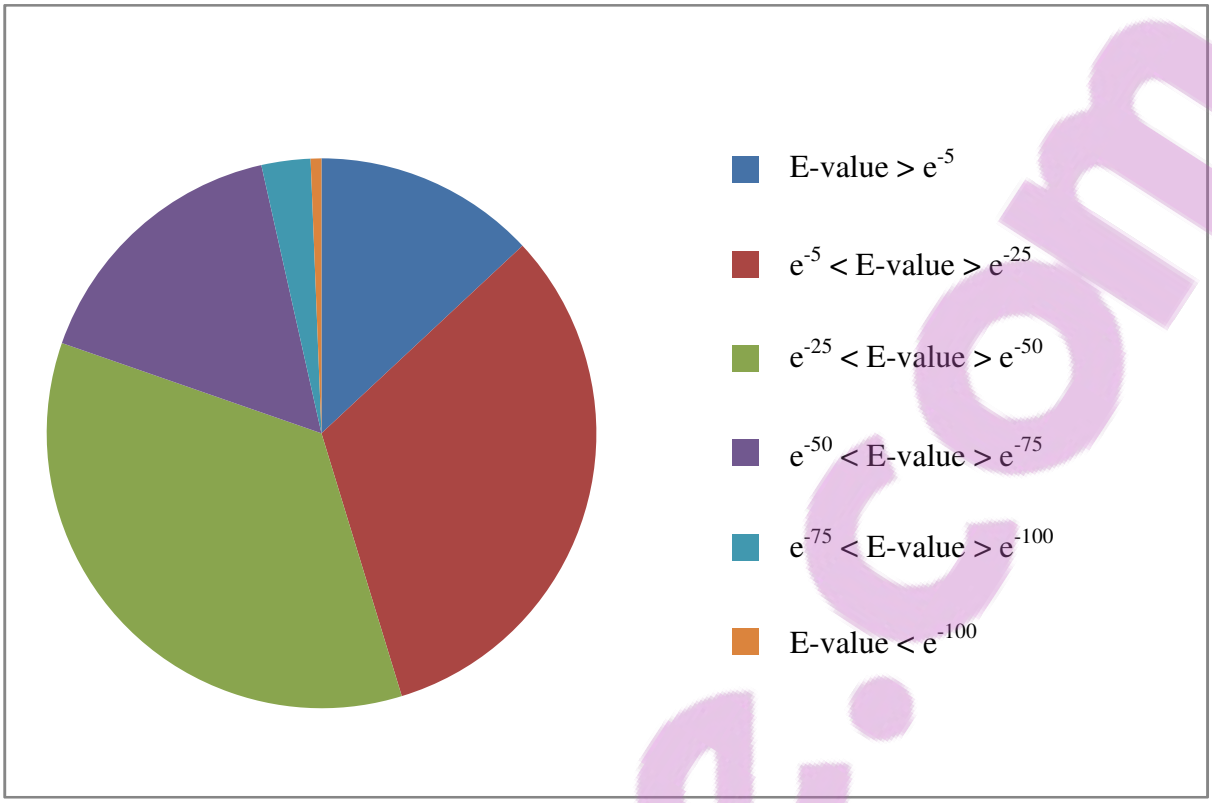


Figure 3.5. E-value distribution maps illustrating the best hits that the contigs identified in banana root tissue after infection with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 had in the NCBI non redundant protein database or *Musa acuminata* cv. Pahang database. A large portion of the contigs had a low e-value, making it unlikely that the sequences in this study aligned to sequences in the NR or *M. acuminata* cv. Pahang database by chance.

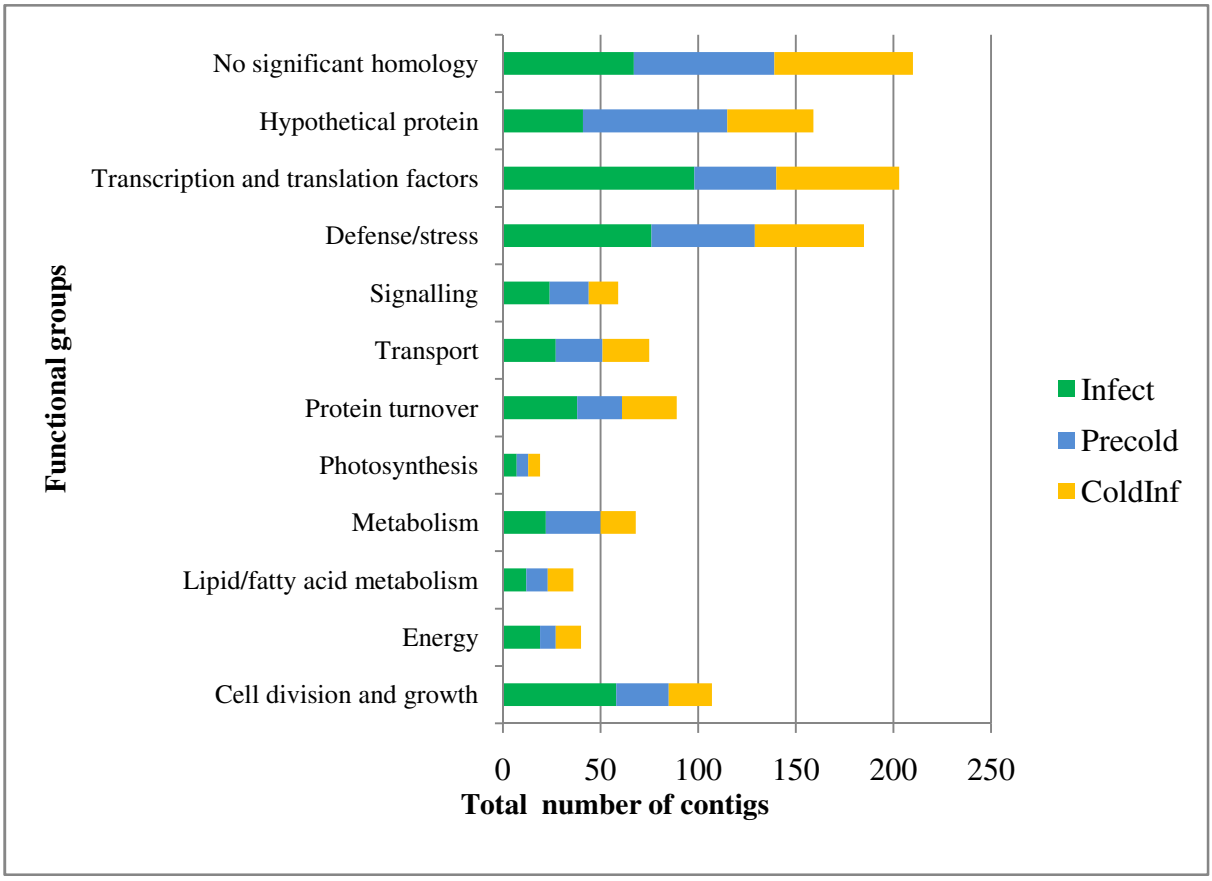


Figure 3.6. Bar charts summarising the annotation of banana transcripts identified in three different cDNA libraries (Infect: Infection at 28°C with *Fusarium oxysporum* f. sp. *cubense* (*Foc*) 'subtropical' race 4 (STR4), Precold: Plants predisposed to 10°C for two weeks and transferred to 28°C and infected with *Foc* STR4 and Coldinf: Plants subjected to 10°C and infected with *Foc* STR4) based on putative identities in the functional categories according to the GO database. The functional groups were identified automatically but it was manually curated and re-assigned if needed.

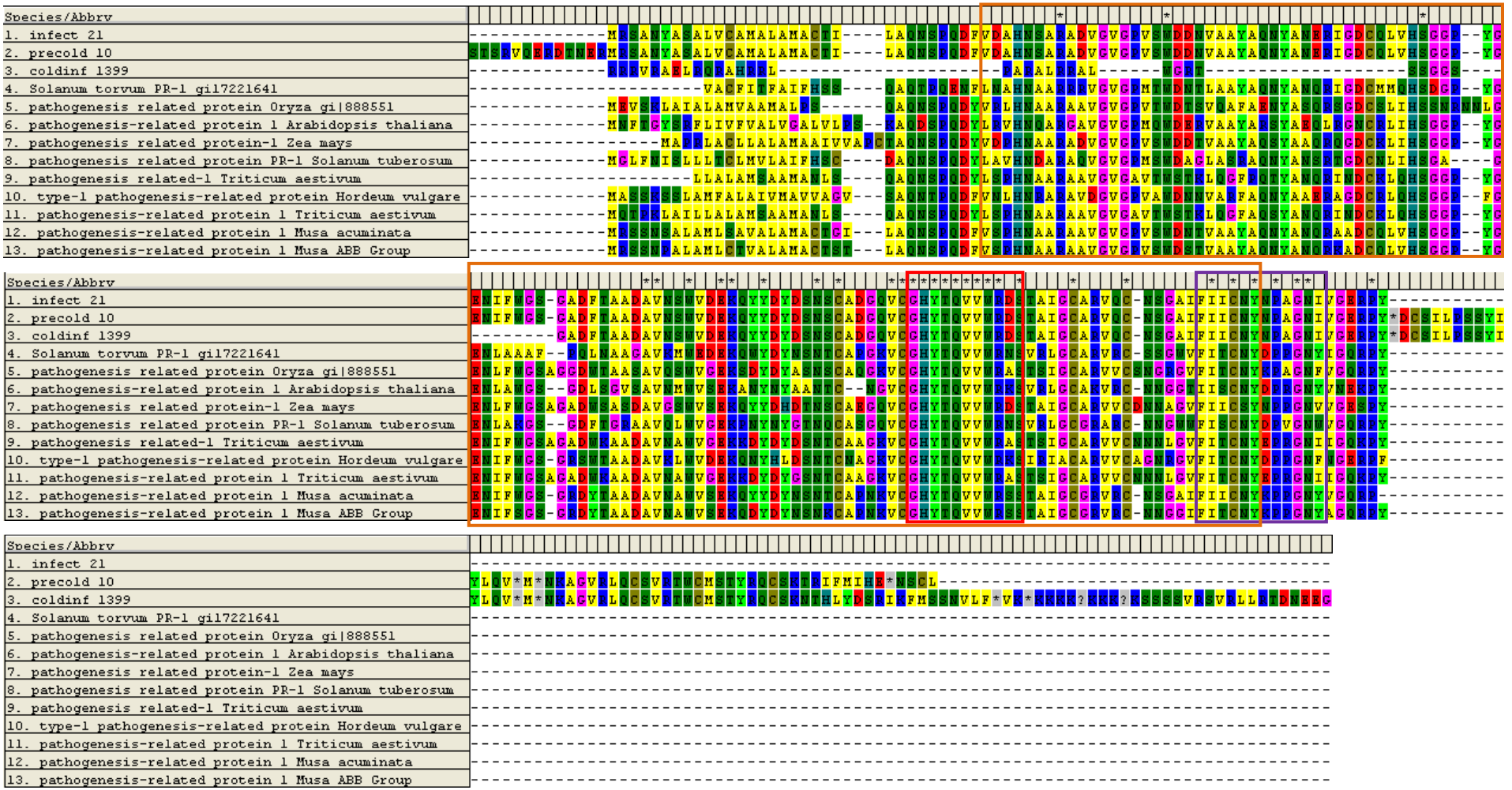


Figure 3.7. Alignment of pathogenisis related 1 (PR-1) amino acid sequences by MUSCLE incorporated in MEGA 5.05 (Tamura *et al.*, 2011). The red box indicates the cysteine-rich secretory proteins (CRISP) family signature 1 motif, the purple box denotes the CRISP family signature 2 motif, while the brown box denotes SCP-like extracellular protein. 1. Infect_21, 2. Precold_10, 3. Coldinf_1399, 4. BAB78476.1 PR-1 [*Solanum torvum*], 5. AAB49685.1 PR-1 [*Oryza sativa* Indica Group], 6. NP_179068.1 PR-1 [*Arabidopsis thaliana*], 7. ABA34055.1 PR-1 [*Zea mays* subsp. *parviglumis*], 8. CAB58263.1 PR-1 [*S. tuberosum*], 9. AAP14676.1 PR-1 [*Triticum aestivum*], 10. CAA88618.1 PR-1 [*Hordeum vulgare*], 11. AAK60565.1 PR-1 [*T. aestivum*], 12. ABK41053.2 PR-1 [*Musa acuminata*] and 13. ADD97801.1 PR-1 [*Musa* ABB Group].

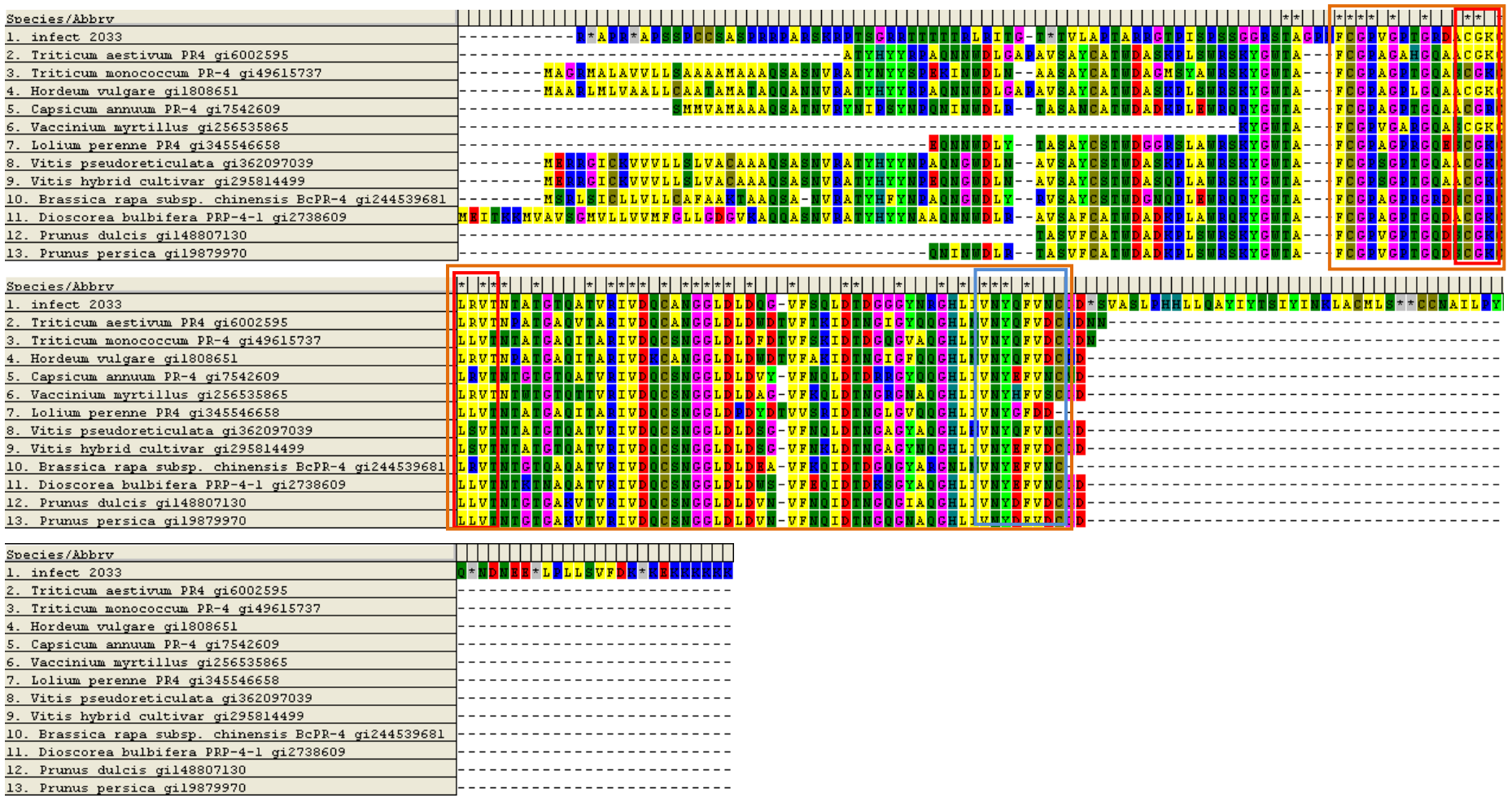


Figure 3.8. Alignment of pathogenisis related 4 (PR-4) amino acid sequences by MUSCLE incorporated in MEGA 5.05 (Tamura *et al.*, 2011). The red box indicates Barwin domain signature 1, the blue box indicates Barwin domain signature 2 and the brown box indicates Barwin family domain (PF00967). 1. Infect_2033, 2. AAF00050.1 PR-4 [*Triticum aestivum*], 3. AAT67050.1 PR-4 [*T. monococcum*], 4. CAA71774.1 PR-4 [*Hordeum vulgare*], 5. AAF63520.1 PR-4 [*Capsicum annuum*], 6. ACU82402.1 PR-4, partial [*Vaccinium myrtillus*], 7. AEO11774.1 PR-4 [*Lolium perenne*], 8. AEW12795.1 PR-4 [*Vitis pseudoreticulata*], 9. ADG35965.1 PR-4 [*Vitis* hybrid cv.], 10. BAH82748.1 PR-4 [*Brassica rapa* subsp. *chinensis*], 11. AAB94514.1 PR-4 [*Dioscorea bulbife*ra], 12. ABR13276.1 putative PR-4 [*Prunus dulcis*] and 13. P83343.1 PR-4 [*P. persica*].

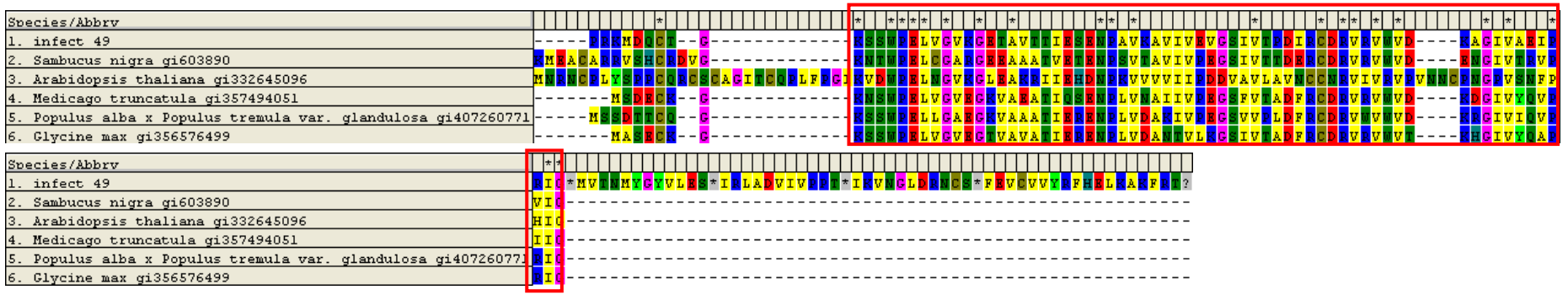


Figure 3.9. Alignment of pathogenisis related 6 (PR-6) amino acid sequences by MUSCLE incorporated in MEGA 5.05 (Tamura *et al.*, 2011). The red box indicates potato inhibitor I family (PF00280). 1. Infect_49, 2. CAA87073.1 PR-6 [*Sambucus nigra*], 3. NP_190571.1 serine protease inhibitor/potato inhibitor I domain-containing protein [*Arabidopsis thaliana*], 4. XP_003617314.1 protease inhibitor [*Medicago truncatula*], 5. AFT92004.1 potato inhibitor I family protein [*Populus alba* x *P. tremula* var. *glandulosa*] and 6. XP_003556368.1 *Streptomyces griseus* protease inhibitor [*Glycine max*].

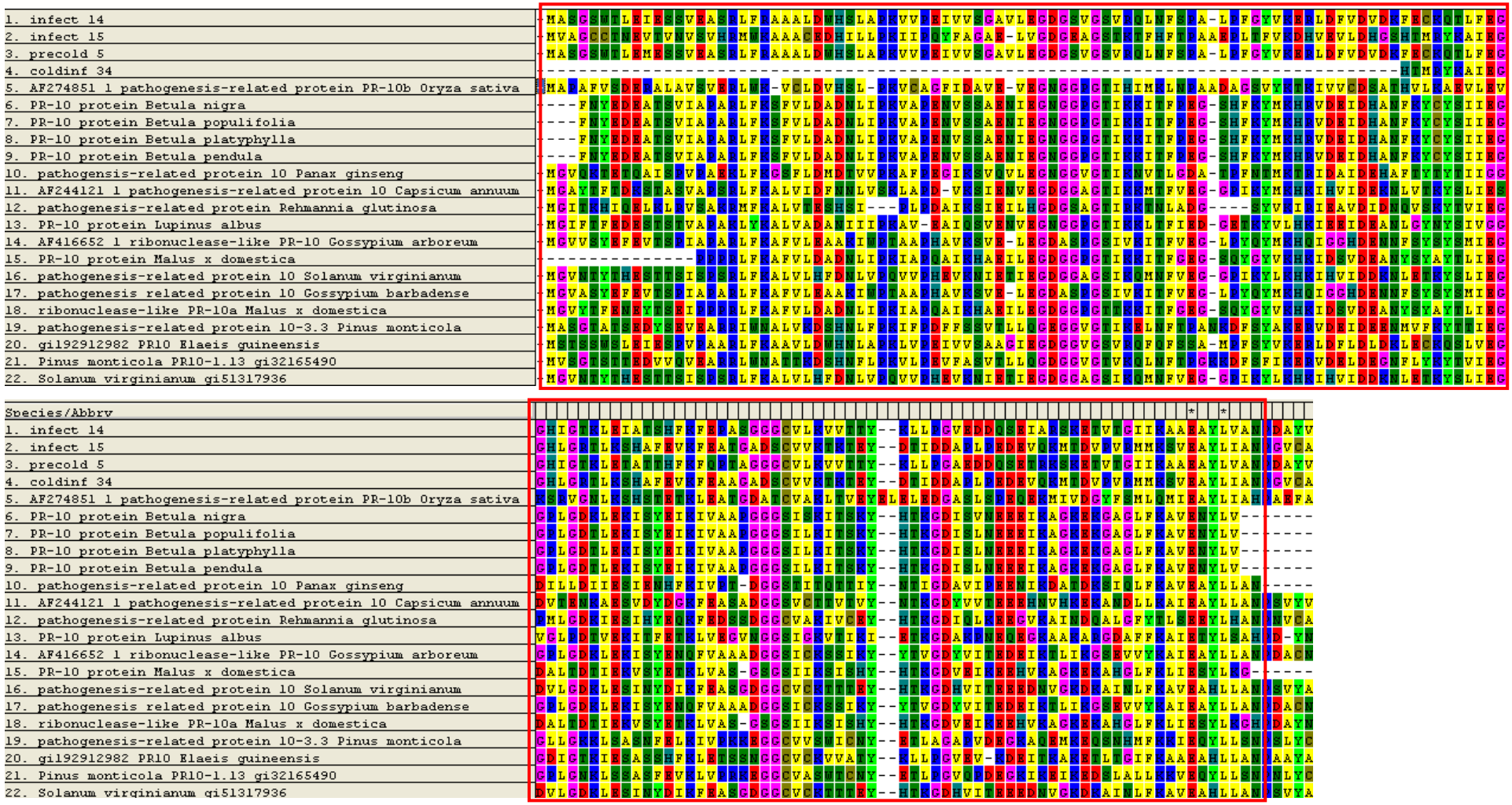


Figure 3.10. Alignment of pathogenisis related 10 (PR-10) amino acid sequences by MUSCLE incorporated in MEGA 5.05 (Tamura *et al.*, 2011). The red box indicates pathogenesis-related protein Bet v I family (PF00407). 1. Infect_14, 2. Infect_15, 3. Precold_5, 4. Coldinf_34, 5. AAF85973.1 PR-10b [*Oryza sativa* Indica Group], 6. ACF75100.1 PR-10 [*Betula nigra*], 7. ACF75092.1 PR-10 [*B. populifolia*], 8. ACF75091.1 PR-10 [*B. platyphylla*], 9. ABC41606.1 PR-10 [*B. pendula*], 10. ACY36943.1 PR-10 [*Panax ginseng*], 11. AAF63519.1 PR-10 [*Capsicum annuum*], 12. ACB12048.1 PR protein [*Rehmannia glutinosa*], 13. CAA03926.1 PR-10 [*Lupinus albus*], 14. AAL09033.1 ribonuclease-like PR-10 [*Gossypium arboreum*], 15. CAT99609.1 PR-10 [*Malus* x *domestica*], 16. AAU00066.1 PR-10 [*Solanum virginianum*], 17. AAP76504.1 PR-10 [*G. barbadense*], 18. AAK13030.1 ribonuclease-like PR-10a [*Malus* x *domestica*], 19. AAU00105.1 PR protein 10-3.3 [*Pinus monticola*], 20. ACF06599.1 PR-10c [*Elaeis guineensis*], 21. AAL50006.1 PR10 protein [*P. monticola*], 22. AAU00066.1 PR-10 [*S. virginianum*].

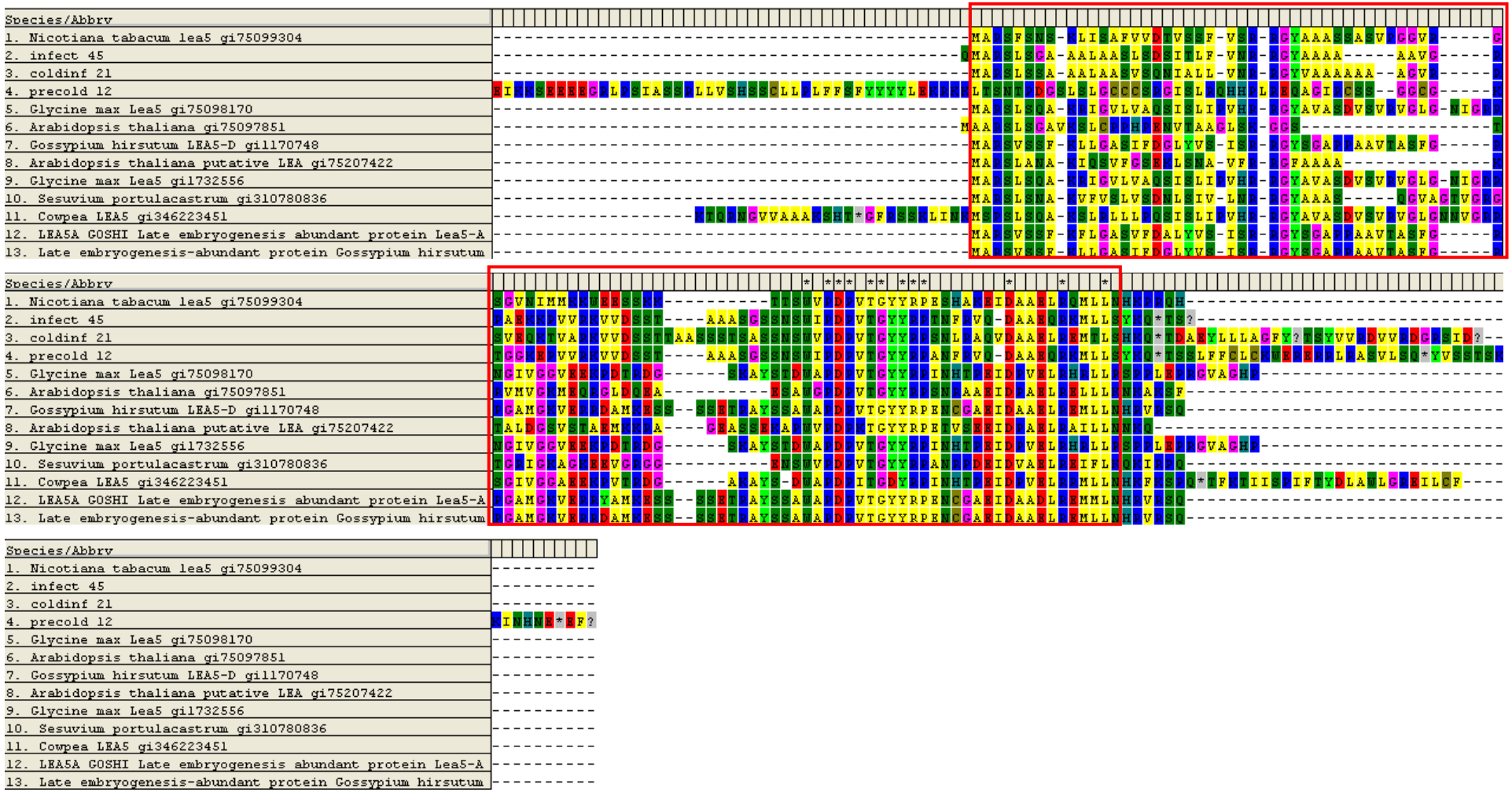
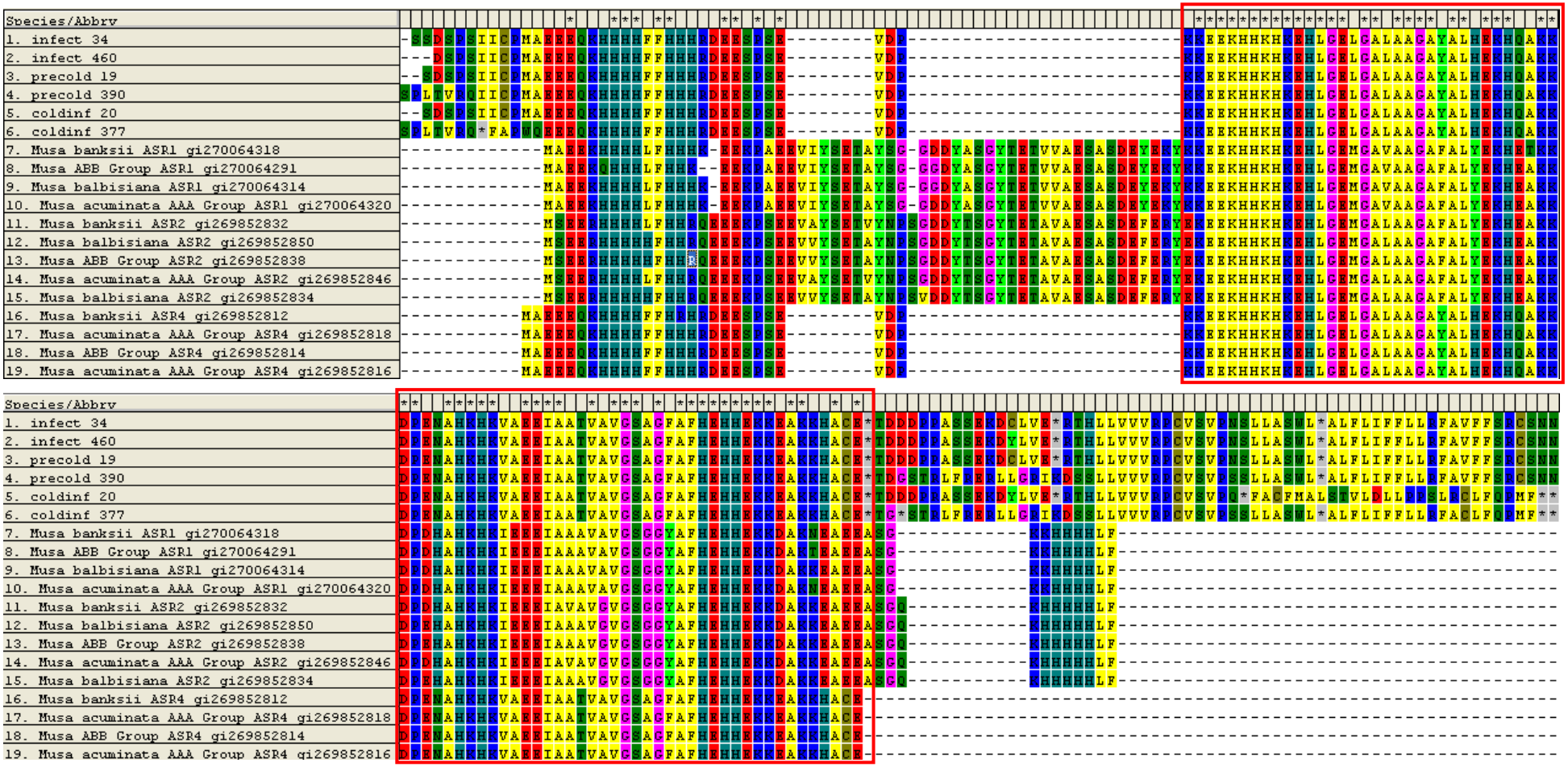


Figure 3.11. Alignment of late embryogenesis abundant (LEA) 5 amino acid sequences by MUSCLE incorporated in MEGA 5.05 (Tamura *et al.*, 2011). The red box indicates LEA class 3 (PF03242). 1. AAC06242.1 LEA5 [*Nicotiana tabacum*] 2. Infect_45, 3. Coldinf_21, 4. Precold_12, 5. O24422 Desiccation protective protein LEA5 [*Glycine max*], 6. O23440 Drought-induced protein like [*Arabidopsis thaliana*], 7. P46522.1 LEA5-D [*Gossypium hirsutum*], 8. Q9SRX6 LEA protein, putative [*A. thaliana*], 9. AAB38782.1 desiccation protective protein LEA5 [*G. max*], 10. ADP23916.1 LEA protein [*Sesuvium portulacastrum*], 11. GR942575.2 LEA5 [*Vigna unguiculata*], 12. P46521.1 LEA5-A [*G. hirsutum*] and 13. P46522.1 LEA5-D [*G. hirsutum*].



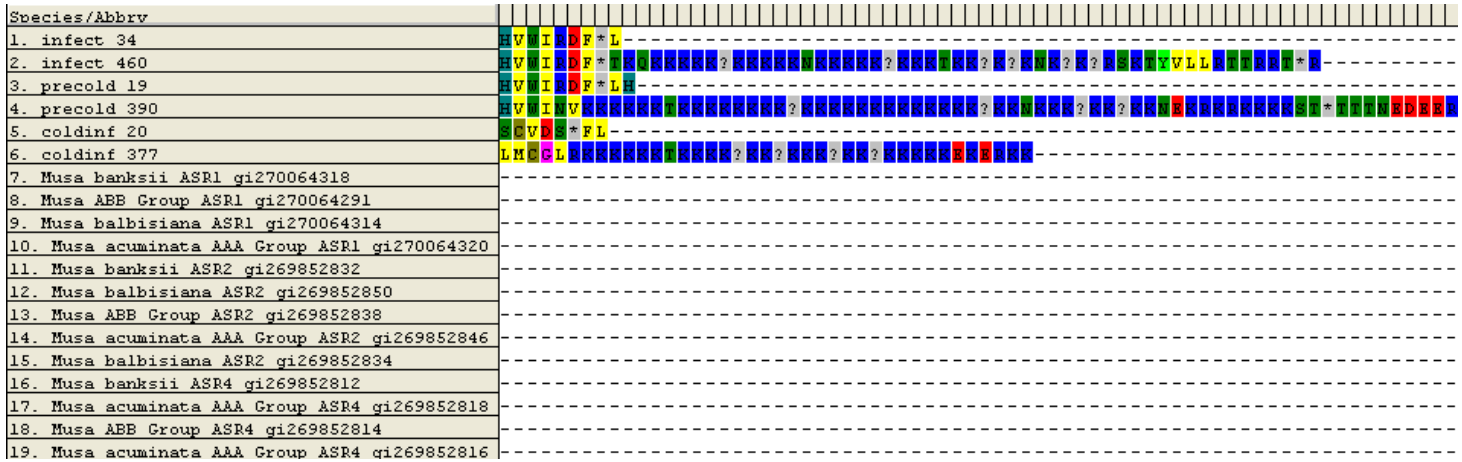


Figure 3.12. Alignment of abscisic stress ripening (ASR) amino acid sequences by MUSCLE incorporated in MEGA 5.05 (Tamura *et al.*, 2011). The red box indicates abscisic acid/water deficit stress (ABA/WDS) induced protein (PF02496.8). 1. Infect_34, 2. Infect_460, 3. Precold_19, 4. Precold_390, 5. Coldinf_20, 6. Coldinf_377, 7. ACZ60137.1 ASR [*Musa banksii*], 8. ACZ60124.1 ASR [*Musa* ABB Group], 9. ACZ60135.1 ASR [*M. balbisiana*], 10. ACZ60138.1 ASR [*M. acuminata* AAA Group], 11. ACZ50743.1 ASR [*M, banksii*], 12. ACZ50752.1 ASR [*M. balbisiana*], 13. ACZ50746.1 ASR [*Musa* ABB Group], 14. ACZ50750.1 ASR [*M. acuminata* AAA Group], 15. ACZ50744.1 ASR [*M. balbisiana*], 16. ACZ50733.1 ASR [*M. banksii*], 17. ACZ50736.1 ASR [*M. acuminata* AAA Group], 18. ACZ50734.1 ASR [*M.* ABB Group] and 19. ACZ50735.1 ASR [*M. acuminata* AAA Group].

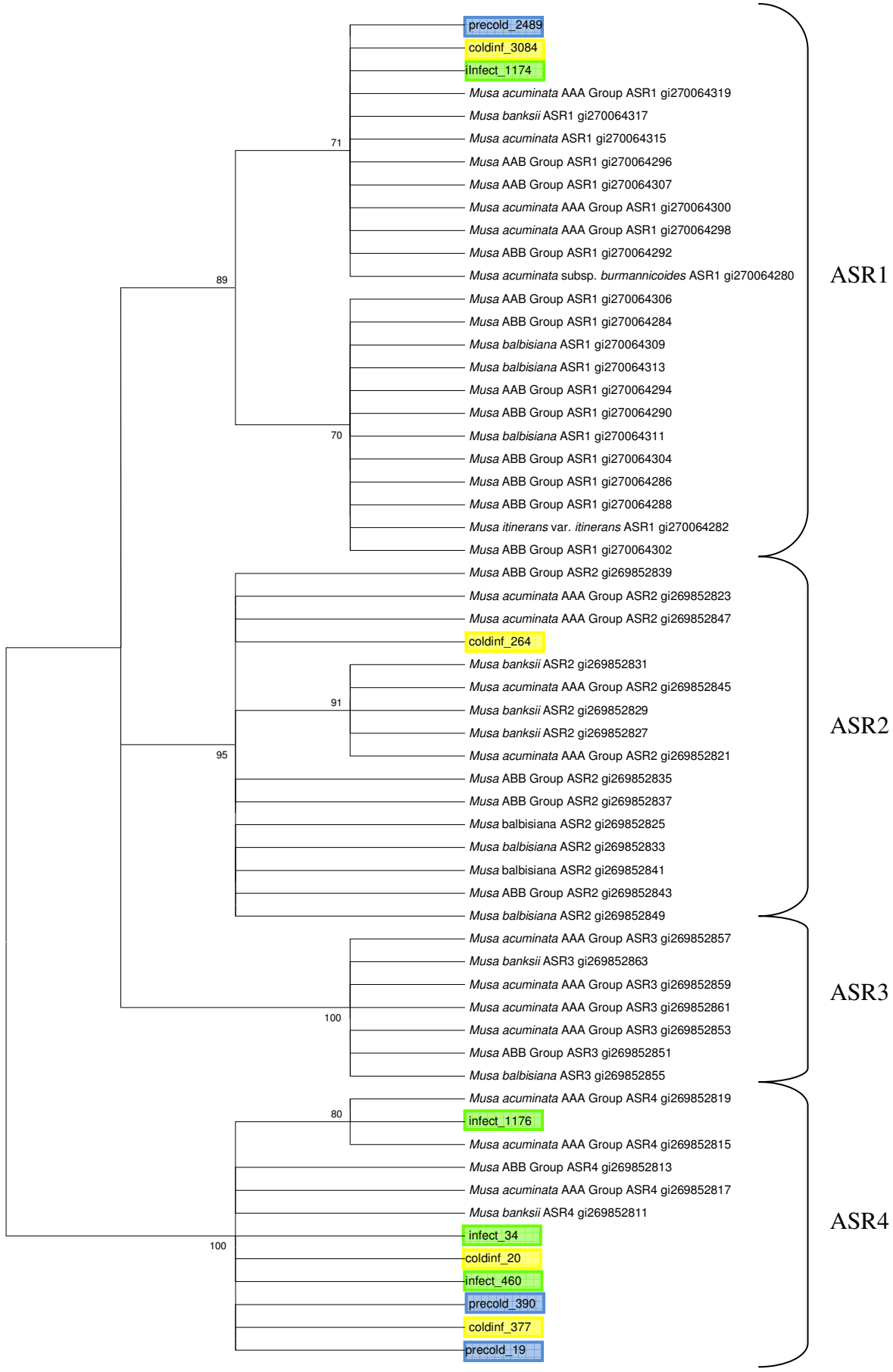


Figure 3.13. A neighbour-joining tree of the abscisic stress ripening (*ASR*) sequences. The nucleotide sequences were aligned using MUCSLE, and the tree was displayed in MEGA5. Bootstrap values out of 1,000 bootstrap resamplings are shown at the nodes to assess the robustness of the tree. Contigs precold_2489, coldinf_3084 and infect_1174 grouped with *ASR1*, coldinf_264 grouped with *ASR2* and infect_1176, infect_34, coldinf_20, infect_460, precold_390, coldinf_377 and precold_19 grouped with *ASR4*.

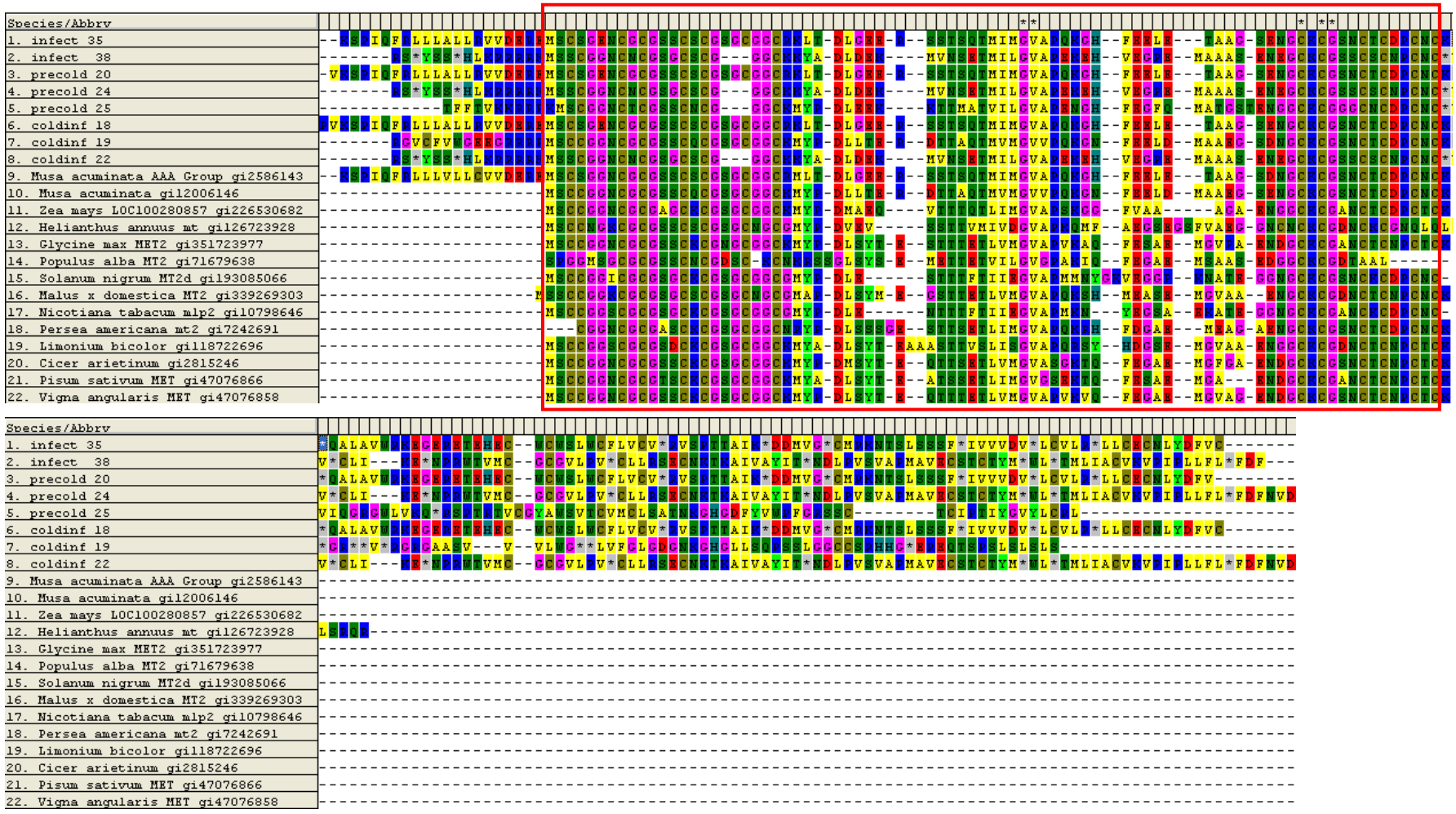
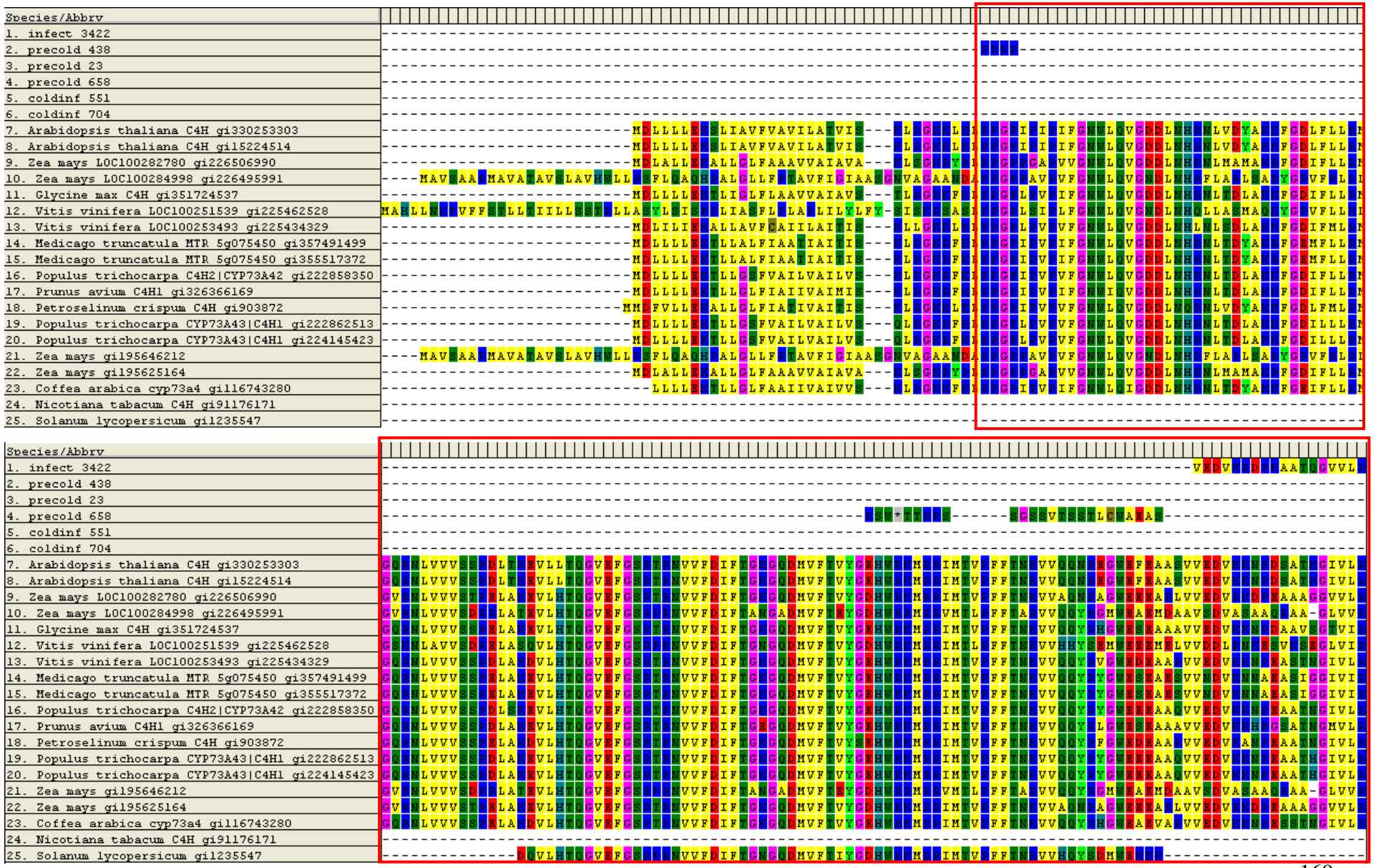
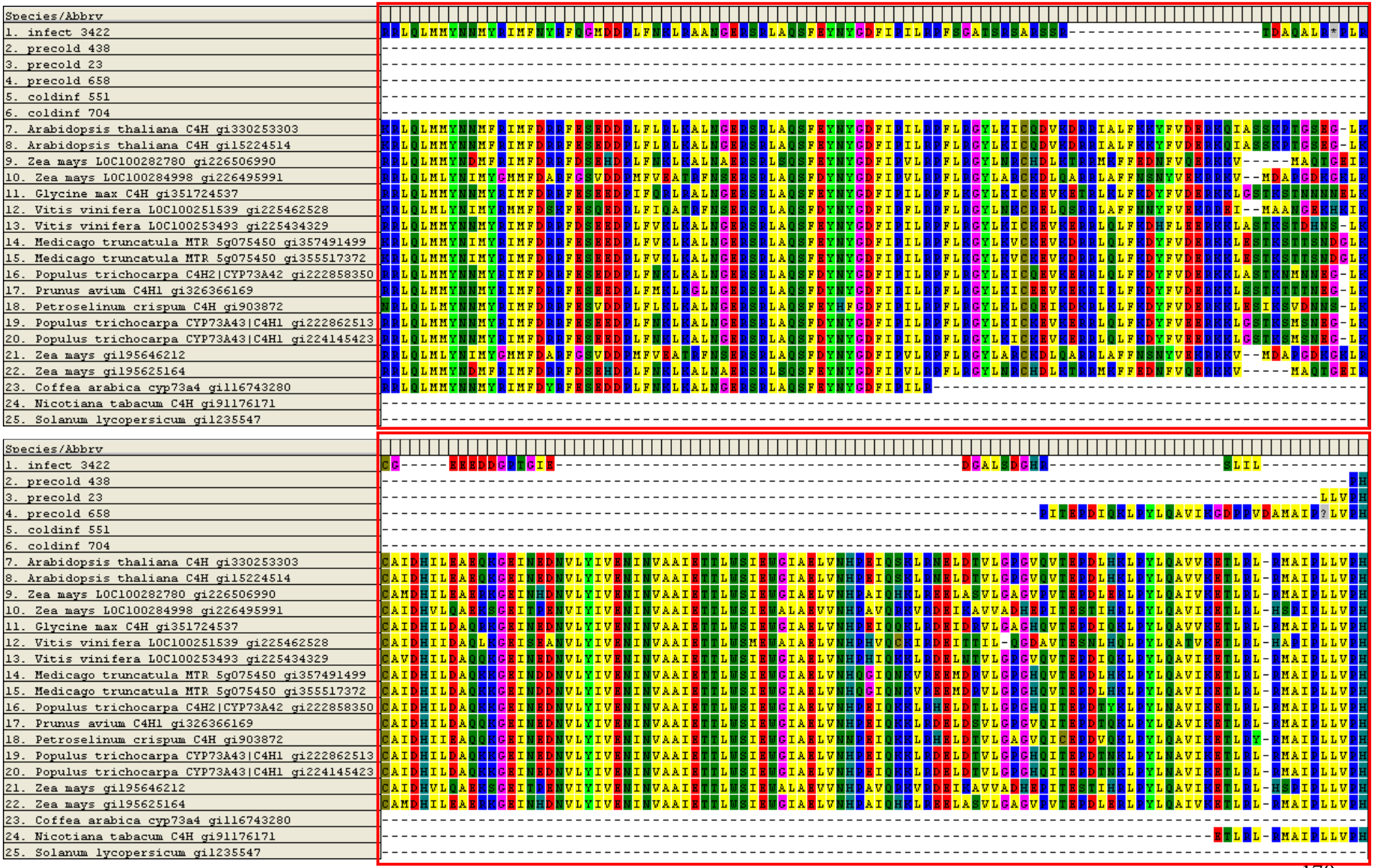
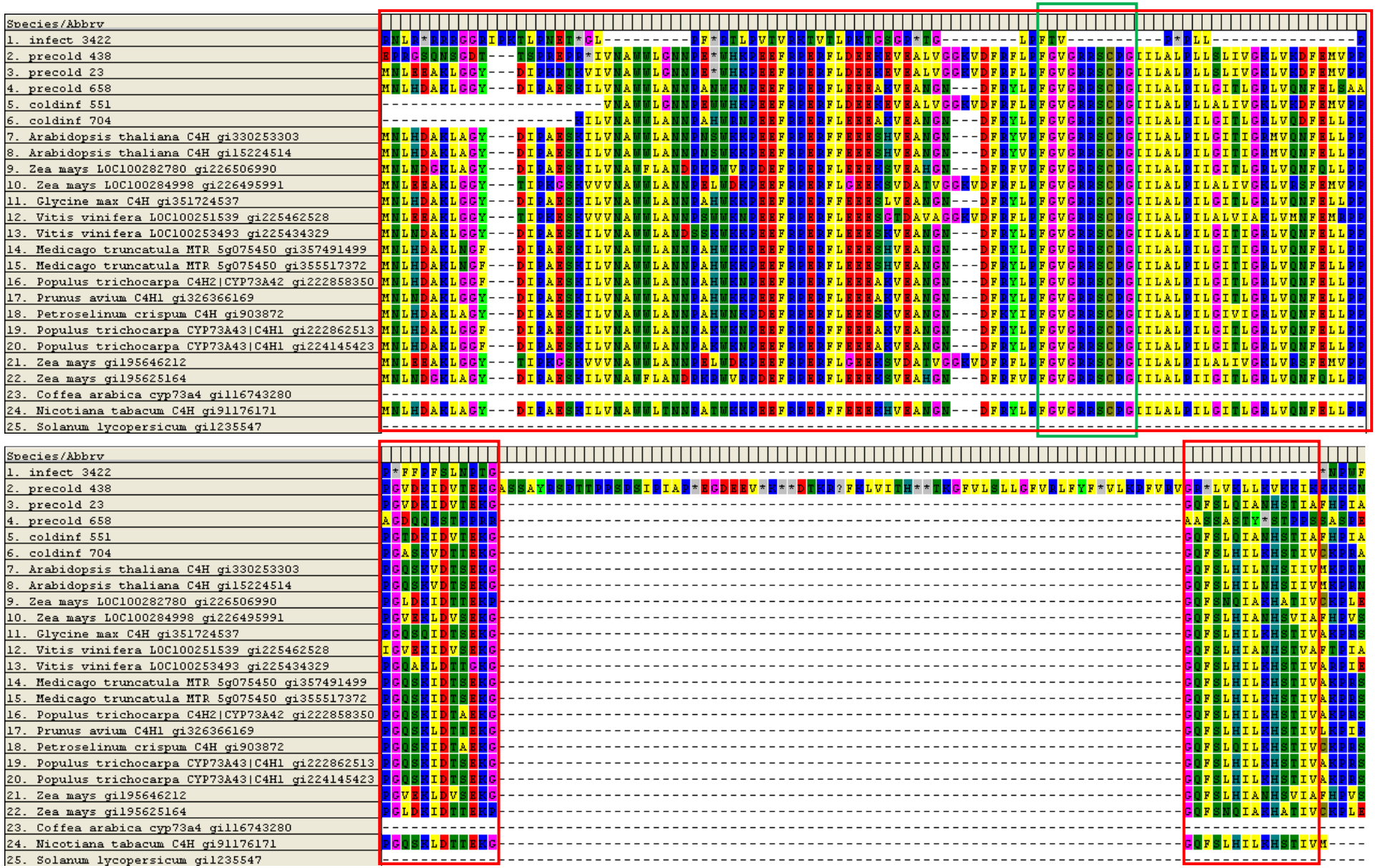


Figure 3.14. Alignment of metallothionein type 2 (MT2) amino acid sequences by MUSCLE incorporated in MEGA 5.05 (Tamura *et al.*, 2011). The red box indicates MT2 domain (PF01439). 1. Infect_35, 2. Infect_38, 3. Precold_20, 4. Precold_24, 5. Precold_25, 6. Coldinf_18, 7. Coldinf_19, 8. Coldinf_22, 9. AAB82774.1 ripening-associated protein [*Musa acuminata* AAA Group], 10. AAG44757.1 metallothionein-like protein [*M. acuminata*], 11. NP_001147249.1 MT2 [*Zea mays*], 12. ABO26877.1 MT2 [*Helianthus annuus*], 13. NP_001235506.1 MT2 [*Glycine max*], 14. AAZ38879.1 MT2 [*Populus alba*], 15. ACF10398.1 MT2 [*Solanum nigrum*], 16. AEJ37038.1 MT2 [*Malus* x *domestica*], 17. CAC12823.1 MT2 [*Nicotiana tabacum*], 18. CAB77242.1 MT2 [*Persea americana*], 19. ABL10086.1 MT2 [*Limonium bicolor*], 20. Q39459.2 MT2 [*Cicer arietinum*], 21. BAD18383.1 MT2 [*Pisum sativum*] and 22. BAD18379.1 MT2 [*Vigna angularis*].







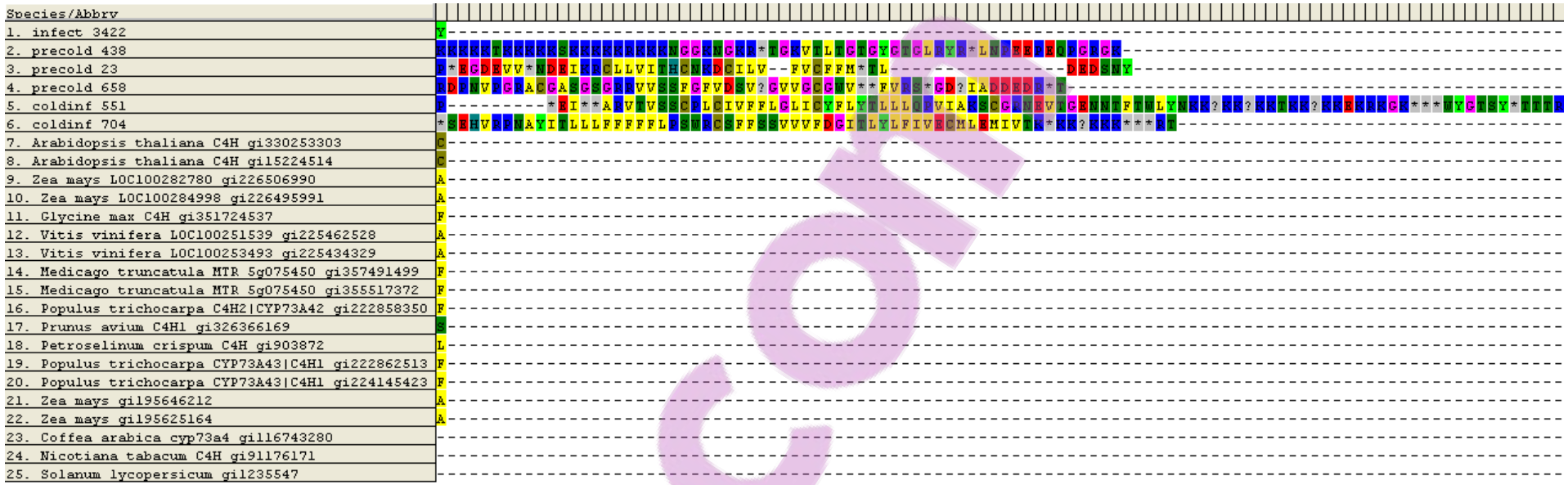


Figure 3.15. Alignment of cinnamate 4-monooxygenase (C4H) amino acid sequences by MUSCLE incorporated in MEGA 5.05 (Tamura *et al.*, 2011). The red box indicates p450 Cytochrome P450 (PF00067) while the green box indicates cytochrome P450 cysteine heme-iron ligand signature. 1. Infect_3422, 2. Precold_438, 3. Precold_23, 4. Precold 658, 5. Coldinf_551, 6. Coldinf_704, 7. AEC08397.1 C4H [*Arabidopsis thaliana*], 8. NP_180607.1 C4H [*A. thaliana*], 9. NP_001149158.1 C4H [*Zea ma*ys], 10. NP_001151365.1 C4H [*Z. mays*], 11. NP_001237317.1 C4H [*Glycine ma*x], 12. XP_002266037.1 C4H [*Vitis vinifera*], 13. XP_002266238.1 C4H [*V. vinifera*], 14. XP_003616037.1 C4H [*Medicago truncatula*], 15. AES98995.1 C4H [*M. truncatula*], 16. EEE95897.1 C4H [*Populus trichocarpa*], 17. ADZ54778.1 C4H [*Prunus avium*], 18. AAC41660.1 C4H [*Petroselinum crispum*], 19. EEF00020.1 C4H [*Populus trichocarpa*], 20. XP_002325638.1 C4H [*P. trichocarpa*], 21. ACG42574.1 C4H [*Z. mays*], 22. ACG34412.1 C4H [*Z. mays*], 23. CAJ41419.1 C4H [*Coffea arabica*], 24. BAE93150.1 C4H [*Nicotiana tabacum*] and 25. CAA94178.1 C4H [*Solanum lycopersicum*].

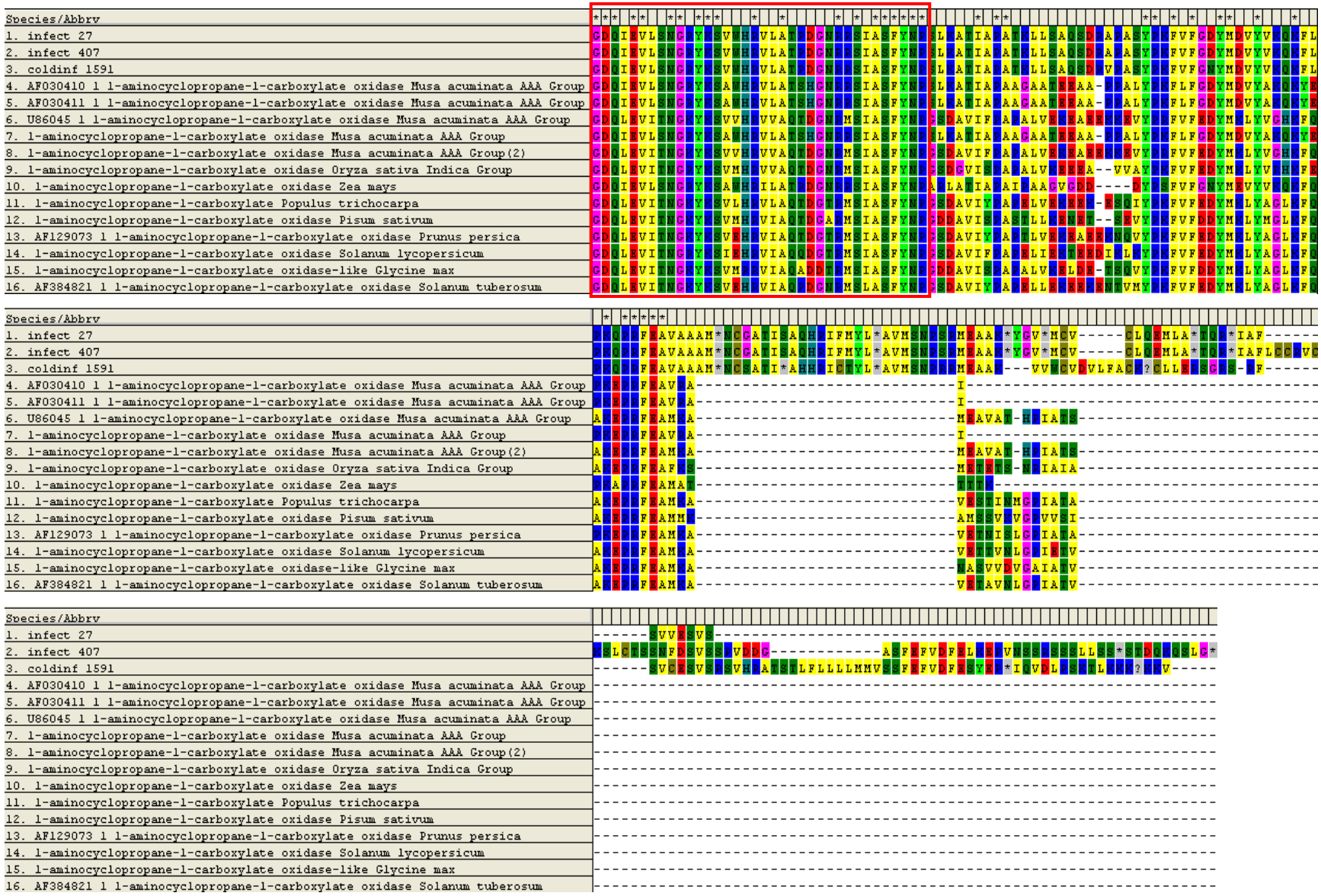


Figure 3.16. Alignment of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) amino acid sequences by MUSCLE incorporated in MEGA 5.05 (Tamura *et al.*, 2011). The red box indicates 2OG-Fe(II) oxygenase superfamily (PF03171). 1. Infect_27, 2. Infect_407, 3. Coldinf_1591, 4. AF030410.1 ACO (*MAO1B* gene) [*Musa acuminata* AAA Group], 5. AF030411.1 ACO (*MAO1A* gene) [*M. acuminata* AAA Group], 6. U86045.1 ACO (*MAO2* gene) [*M. acuminata* AAA Group], 7. AAR00511.1 ACO [*M. acuminata* AAA Group], 8. AAB00556.1 ACO [*M. acuminata* AAA Group], 9. CAA59749.1 ACO [*Oryza sativa* Indica Group], 10. NP_001146957.1 ACO [*Zea mays*], 11. XP_002331528.1 ACO [*Populus trichocarpa*], 12. P31239.1 ACO [*Pisum sativum*], 13. AF129073.1 ACO (*ACO1* gene) [*Prunus persica*], 14. NP_001233867.1 ACO [*Solanum lycopersicum*], 15. NP_001241899.1 ACO-like [*Glycine max*] and 16. AF384821.1 ACO (*ACO2* gene) [*Solanum tuberosum*].

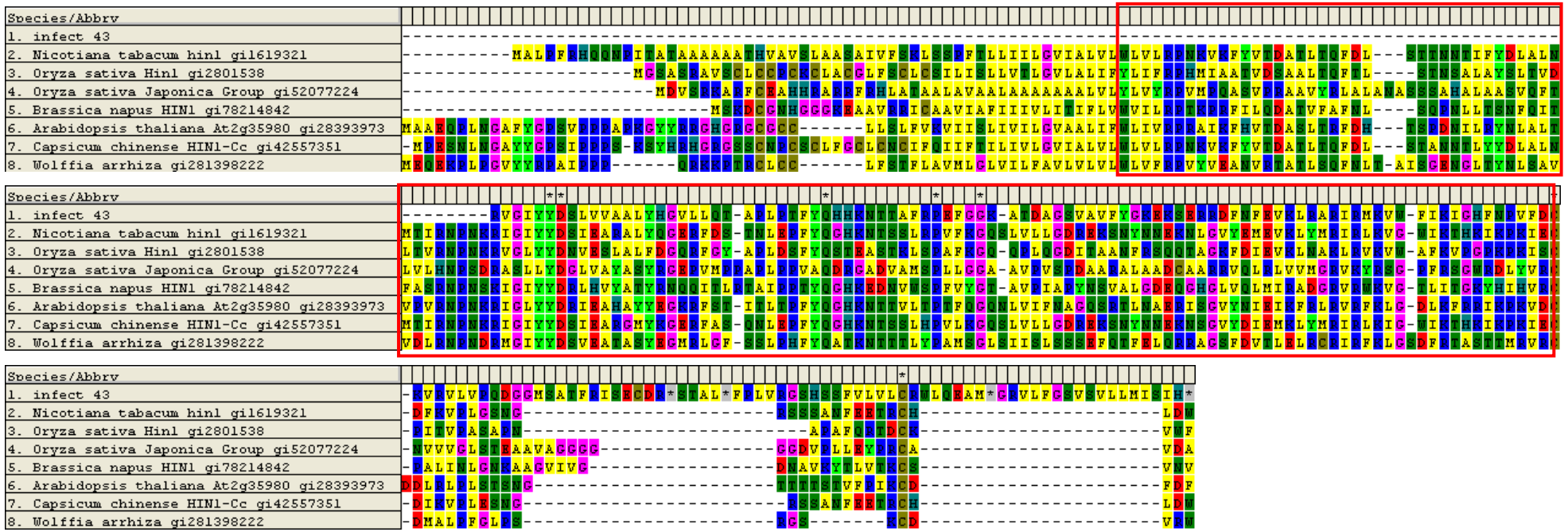
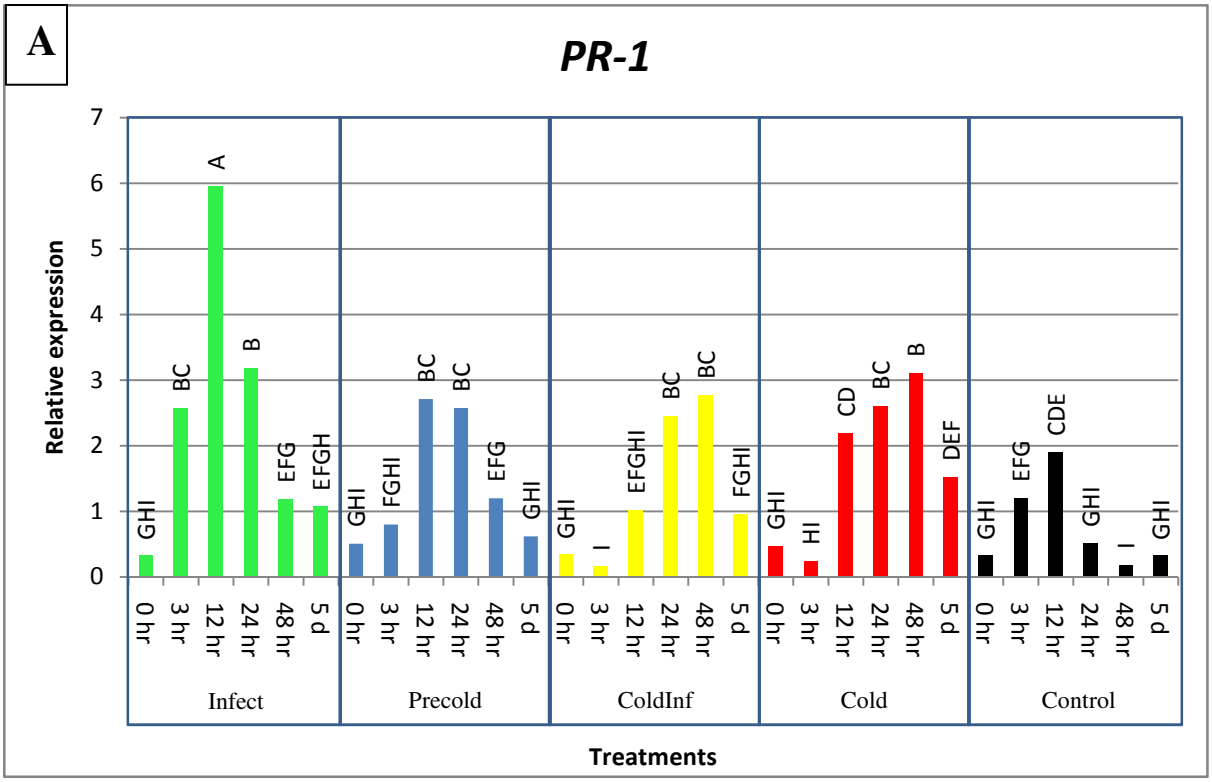
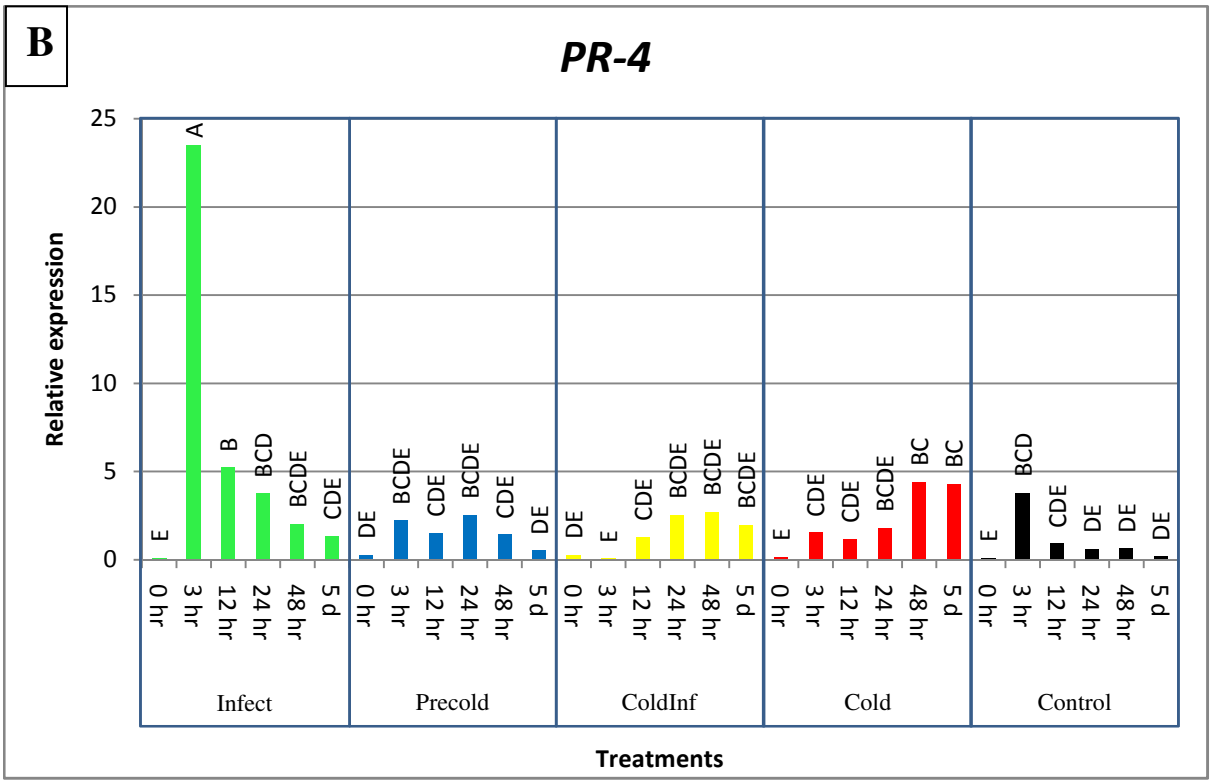
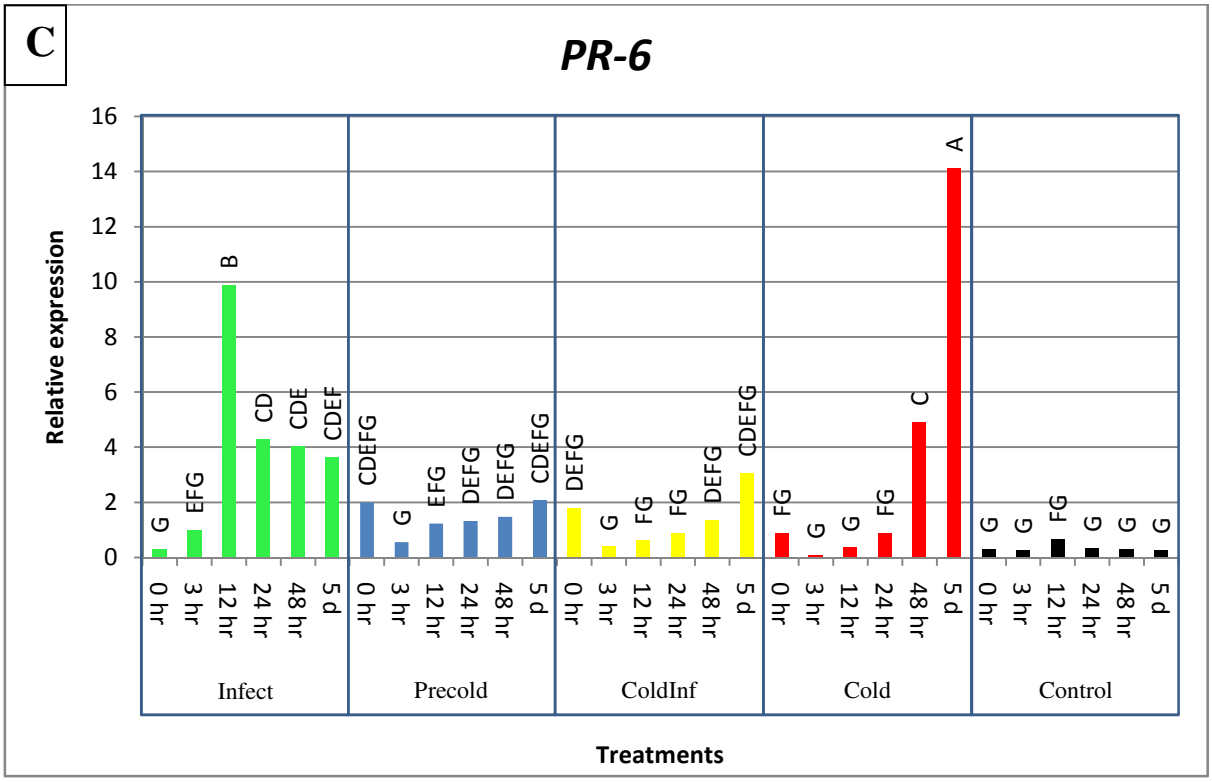
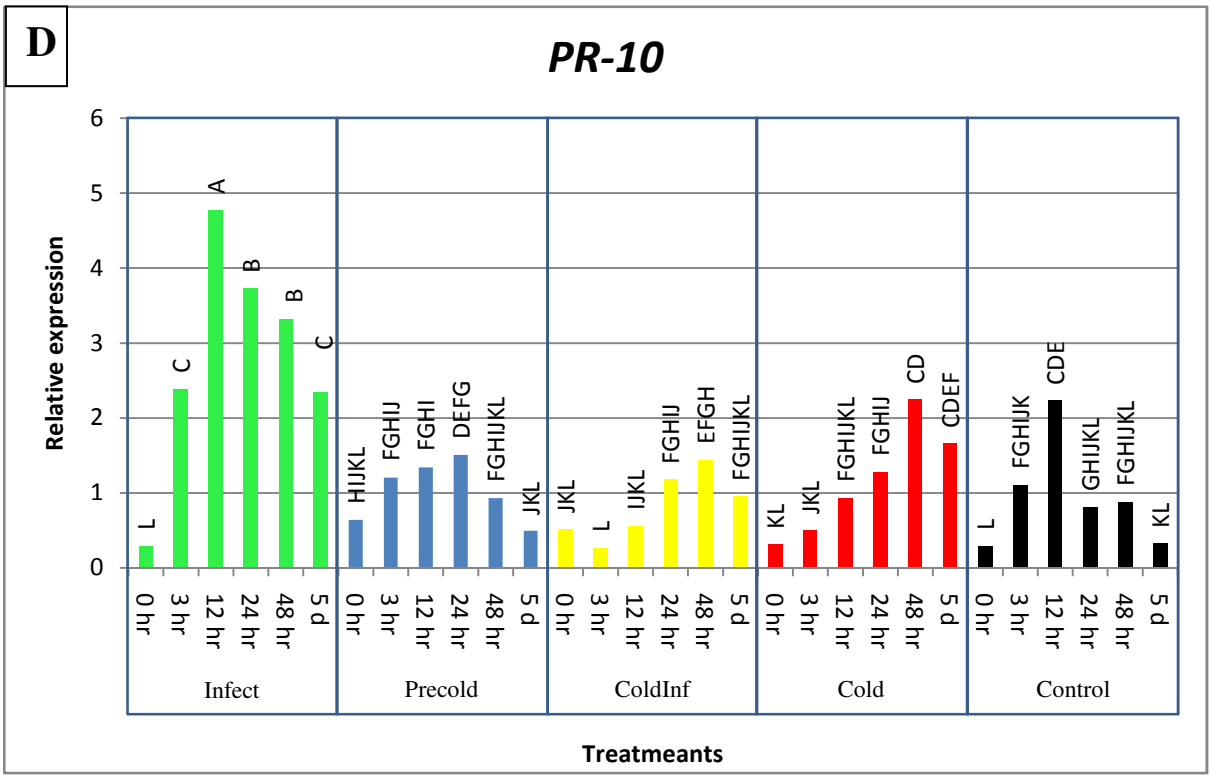


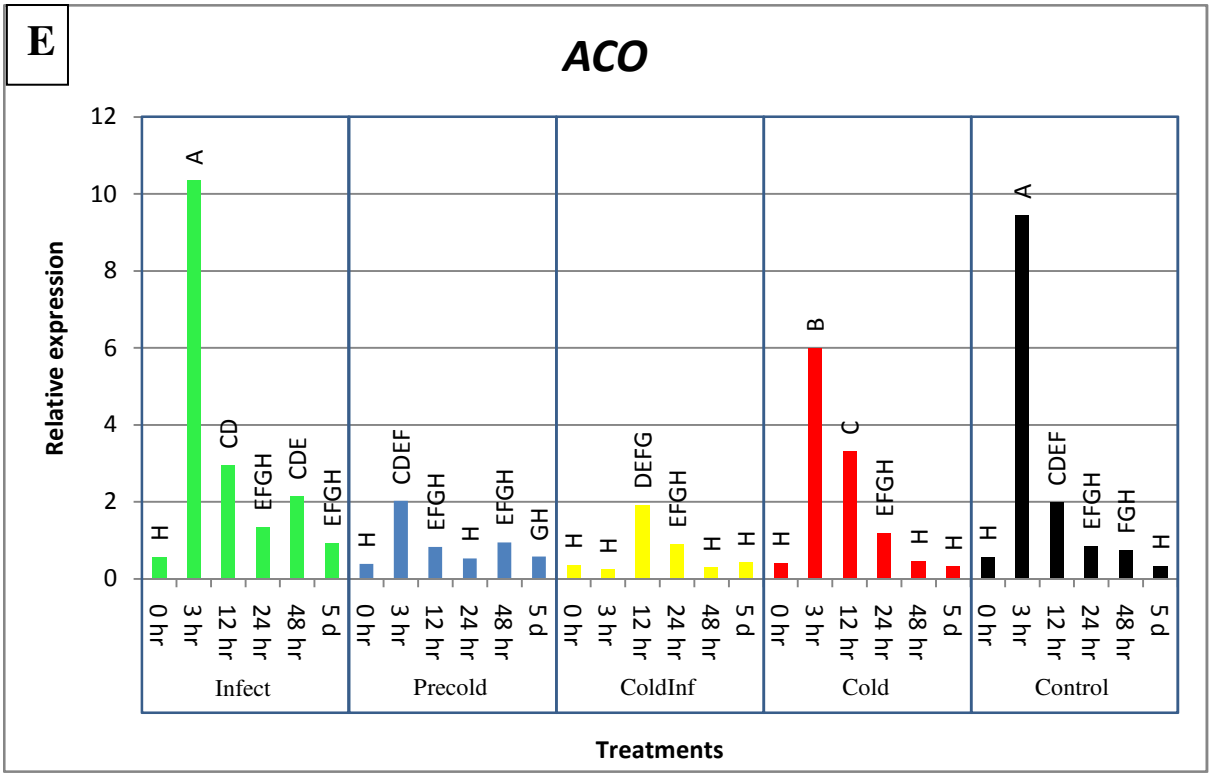
Figure 3.17. Alignment of harpin-induced protein 1 (Hin1) amino acid sequences by MUSCLE incorporated in MEGA 5.05 (Tamura *et al.*, 2011). The red box indicates Hin1 (PF07320). 1. Infect_43, 2. CAA68848.1 Hin1 [*Nicotiana tabacum*], 3. AAB97367.1 Hin1 homolog [*Oryza sativa*], 4. BAD46268.1 Hin1-like [*O. sativa* Japonica Group], 5. ABB36604.1 Hin1-like protein NHL18B [*Brassica napus*], 6. AAO42394.1 putative Hin1 [*Arabidopsis thaliana*], 7. BAD11071.1 Hin1-like protein [*Capsicum chinense*] and 8. ADA67934.1 putative Hin1 [*Wolffia arrhiza*].

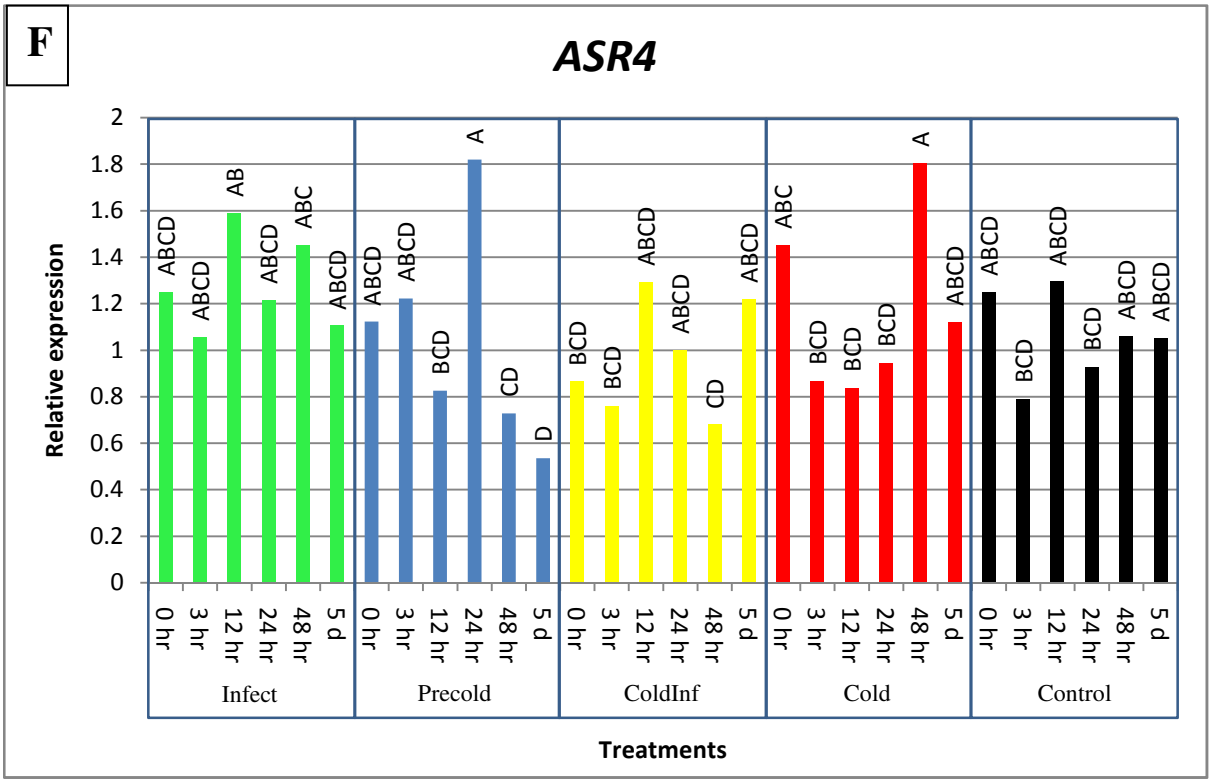


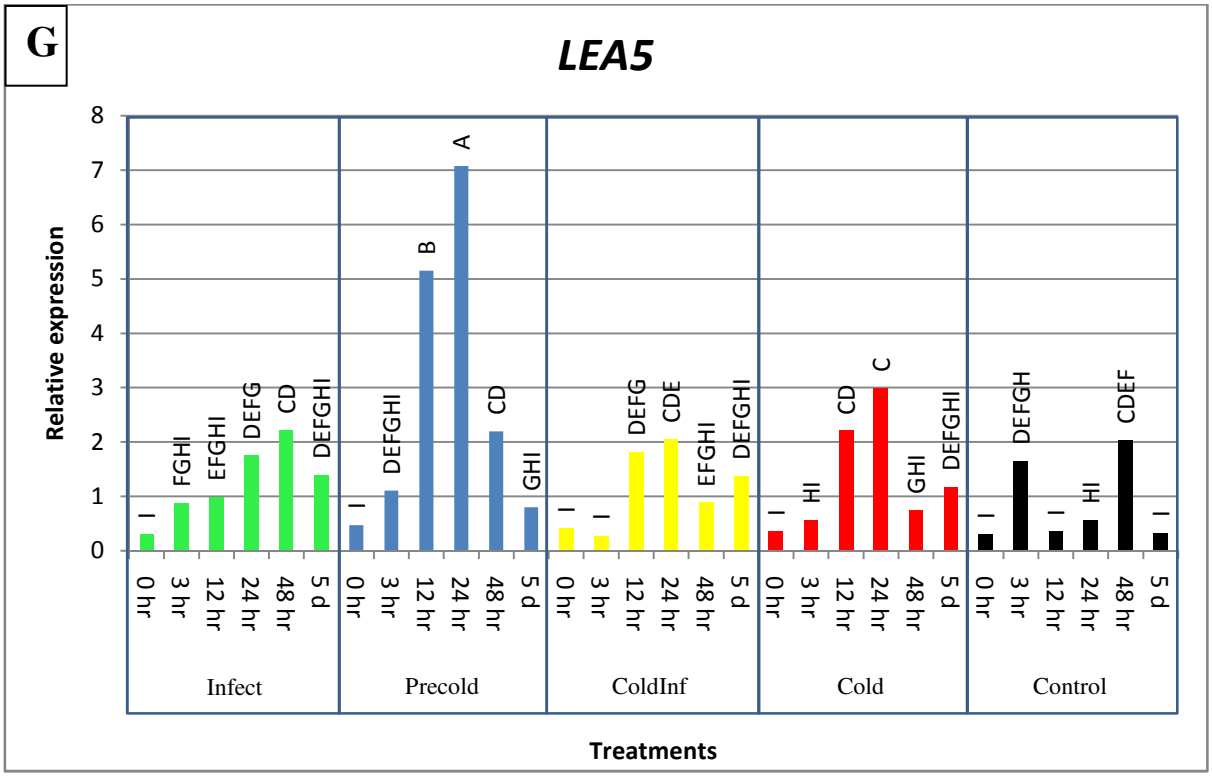


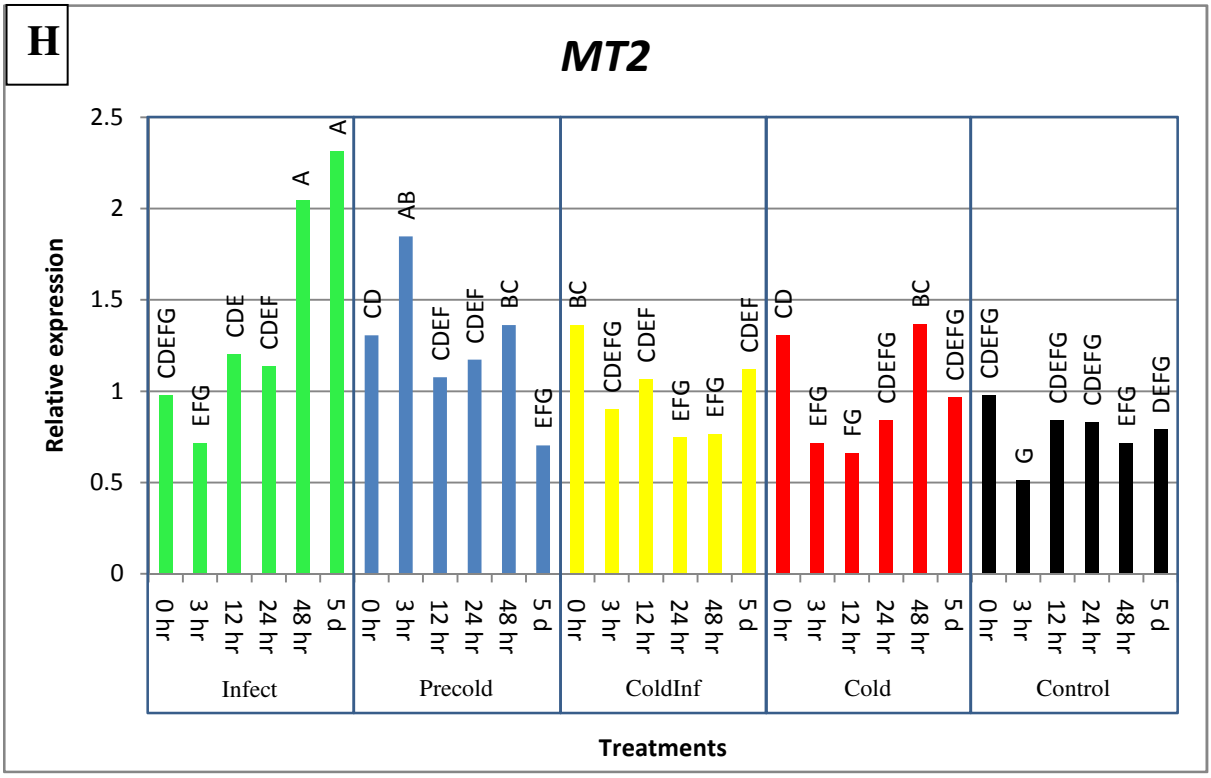


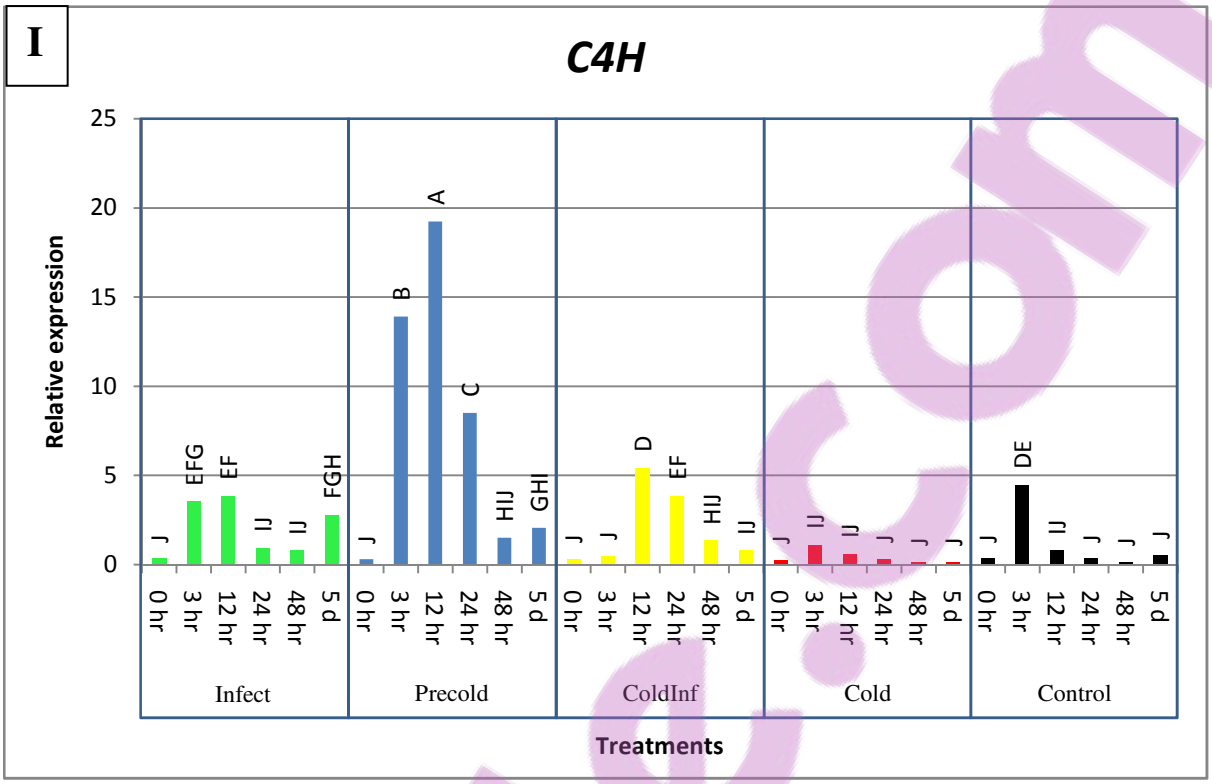












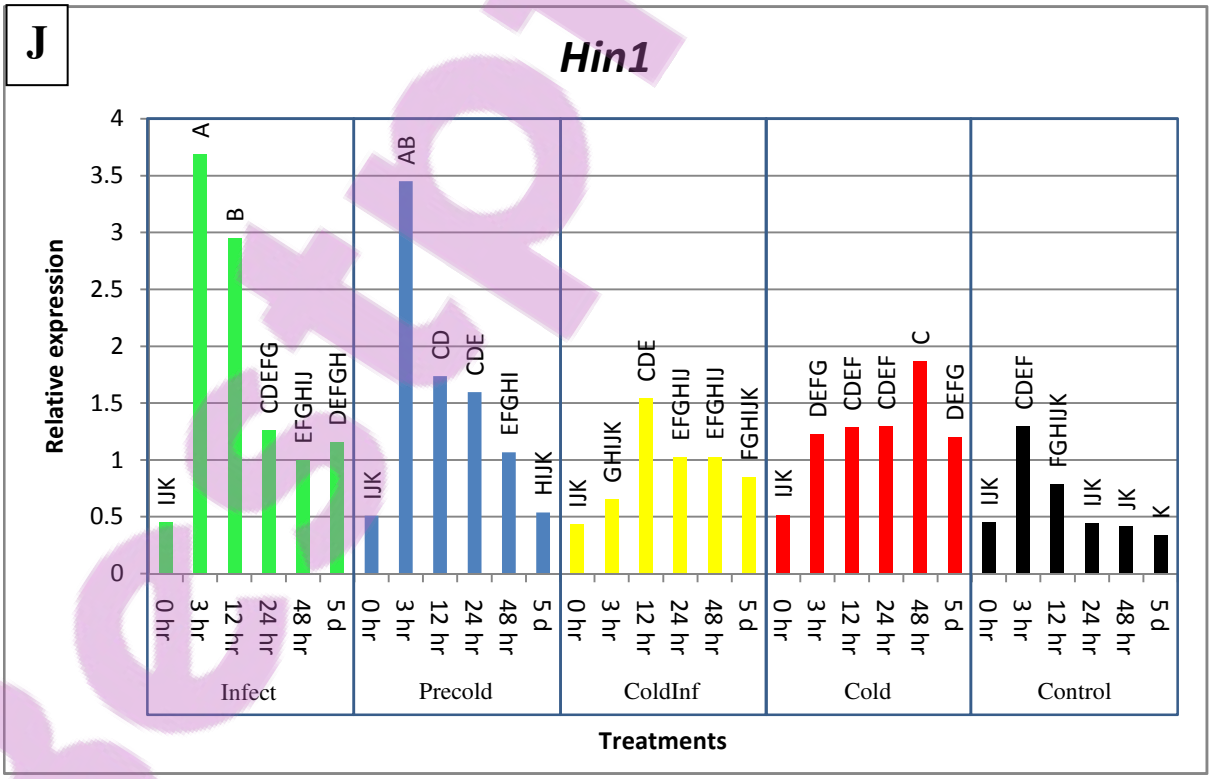


Figure 3.18. Expression patterns of selected defence/stress-related transcripts by relative quantification using qRT-PCR in roots of susceptible Cavendish cv. Grand Naine plants. Treatments included i. Infection at 28°C with *Fusarium oxysporum* f. sp. *cubense* 'subtropical' race 4 (*Foc* STR4) (infected), ii. Plants predisposed to 10°C for two weeks, transferred to 28°C and infected with *Foc* STR4 (precold), iii. Plants subjected to 10°C and infected with *Foc* STR4 (coldinf), iv. Plants incubated at 10°C without infection (cold) and v. Control plants at 28°C without infection (control). Time points investigated included 0, 3, 12, 24, 48 hours and five days post infection. A. Pathogenesis-related protein 1 (*PR-1*), B. Pathogenesis-related protein 4 (*PR-4*), C. Pathogenesis-related protein 6 (*PR-6*) D. Pathogenesis-related protein 10 (*PR-10*), E. 1-Aminocyclopropane-1-carboxylic acid oxidase (*ACO*), F. Abscisic stress ripening 4 (*ASR4*), G. Late embryogenesis abundant protein 5 (*LEA5*), H. Metallothionenin type 2 (*MT2*), I. Cinnamate-4-monooxygenase (*C4H*), and J. Harpin-induced protein 1 (*Hin1*). The expression data were analyzed by JMP® (SAS Institute, Cary, North Carolina) using analysis of variance (ANOVA) test and significant difference values at $p<0.05$ using the Student t-test. Different letters above the bar show significant differences.

CONCLUSIONS

Banana (*Musa* spp.) originates from Malaysia in Southeast Asia, and is grown in many tropical and subtropical countries around the world (Simmonds, 1959). There are more than 1000 banana varieties worldwide, including Cavendish, Gros Michel, Pisang Awak and Pisang Raja (Heslop-Harrison and Schwarzacher, 2007). More than 100 million tonnes of bananas are produced annually, with India being the highest producer (FAOSTAT, 2010). The crop is not only important as a staple food to people in the developing world, but also plays a significant role in export markets. Sustainable production, however, is threatened by a number of diseases of which Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is the most destructive. Cavendish bananas, the cultivar most popular in the export market is highly susceptible to *Foc* race 4.

The molecular mechanisms governing fungal entry of the xylem vessels of Cavendish bananas were investigated (Sutherland *et al.*, 2013). This study suggested that *Foc* race 4 produces cell wall degrading enzymes (CWDE) to enter the xylem vessels and that it expresses *chsV*, *rhoI* and MFS transporter genes to avoid toxic substances produced by the plant. The banana wilt pathogen further appears to control the nitrogen pathway, which is essential for pathogenicity, by expressing *cyp55*. Recently, Meldrum *et al.* (2012) identified *SIX*-genes (secreted in xylem), of which *SIX1*, *SIX7* and *SIX8* occur in *Foc*, with the latter two specific to *Foc* race 4. Knockout mutants can confirm the function of these *SIX* genes during pathogenicity of *oc* to banana.

CWDE, like xylanases, play an important role in pathogenicity in *F. oxysporum* f. sp. *lycopersici* (Michielse and Rep, 2009) and in *Foc* race 4 (Sutherland *et al.*, 2013). If the pathogen can be prohibited from degrading banana roots by either restraining the activity of the CWDE, or by increasing the ability of the plant cell wall to withstand degradation, resistance in bananas to *Foc* STR4 can be achieved. Several CWDE inhibitors have been identified in plants like polygalacturonase inhibiting protein, pectin lyase inhibitor protein, pectin methylesterase inhibitor proteins, endoxylanase inhibitors and xyloglucan-specific glucanase-inhibiting protein (Lagaert *et al.*, 2009). Cell wall strengthening can be achieved by lignification or accumulation of phenolic compounds in the root tissue. Pectin acetyl esterase (*PAE*), also involved in cell wall strengthening, has shown a significant induction in a tolerant Cavendish banana

compared to a susceptible when infected with *Foc* STR4 (Van den Berg *et al.*, 2007). The introduction of genes underlying these proteins by genetic engineering provides an opportunity to hamper CWDE produced by *Foc* and to strengthen root cell walls to prevent infection with *Foc* STR4.

Non-pathogenic *F. oxysporum* strains are morphologically similar to pathogenic *Foc*. Both strains enter the roots via wounds or root tips, but the non-pathogenic *F. oxysporum* strains are restricted to the cortex cells, while the pathogenic *F. oxysporum* strains enter the xylem tissues and colonize the rhizome (MacHardy and Beckman, 1981). The pathogen further blocks the xylem tissue leading to severe wilting and eventually death. Pathogenicity genes identified in *F. oxysporum* strains pathogenic and non-pathogenic to banana were similar, but their expression patterns differed (Sutherland *et al.*, 2013). Therefore, the timing of the expression is vital for the pathogen to infect the root tissue. It has further been demonstrated that pathogenic *F. oxysporum* strains contain a lineage-specific region on chromosome 14 that is specifically involved in pathogenicity (Ma *et al.*, 2010). Dissection of this chromosome, and confirmation of gene function by means of site-directed mutagenesis/gene silencing, may provide novel and highly specific means of pathogen control.

Pathogenicity genes in *Foc* showed enhanced expression *in vitro*, but a better understanding of their transcription *in planta* is required. With the genome sequence of banana (*Musa acuminata* cv. DH-Pahang) available, and that of the pathogen (*Foc* race 4) currently being annotated, transcriptome sequencing at different time points following infection of banana roots is now possible. This provides an opportunity to determine which pathogenicity genes are important during early invasion of the plant material and which defence-related genes are present in banana.

Cold stress plays a significant role in the development of plant diseases, including Fusarium wilt of banana. When greenhouse banana plants were exposed to 10°C and 28°C for ten weeks, disease was significantly increased in plants at the cooler temperature. Several genes involved in the host response were identified, such as genes encoding pathogenesis related (PR) proteins, 1-aminocyclopropane-1-

carboxylic acid oxidase (ACO), abscisic stress ripening (ASR) protein, late embryogenesis abundant protein 5 (LEA5), metallothionein (MT), cinnamate-4-monooxygenase (C4H), harpin-induced protein (Hin) 1, lipid transfer protein, germins, peroxidase and defensins. *PR* genes were highly expressed in roots tissue after infection, which might lower disease severity. Under cold temperatures, the expression of *PR* genes was delayed.

The role of several other defence genes were hypothesized during the study namely *C4H*, *Hin1* and *ACO*. Cold temperature inhibited the production of *C4H*, which is involved in the phenylpropanoid pathway and is important for phytoalexin and lignin production against pathogen infection. Thus, cold temperature may suppress the phenylpropanoid pathway which may give *Foc* the ability to invade the xylem tissue. *Hin1*, one of the transcripts responsible for the hypersensitive response (HR) was suppressed under cold temperature. Thus, cold stress delayed the HR which may play an essential part in banana in defence against *Foc*. Furthermore, *ACO* important in defence through ethylene signalling was suppressed under cold temperature. With low ethylene levels in the roots, defence responses are weakened and the pathogen, *Foc,* can overcome the plant's defence, which results in elevated disease development.

The expression of other transcripts was also investigated during cold stress and infection with *Foc* namely *MT2*, *LEA5* and *ASR4*. *MT2* transcripts were induced in the later stages of infection and may help to scavenge the ROS which was formed in signalling during the early response of the plant's defence. *LEA5* was involved in the cold stress response in Cavendish banana; but was not important in the defence response against *Foc*. *ASR4* did not play a significant role in cold stress nor in plant defence against *Foc*.

Cavendish bananas recognize *Foc* STR4 infection with and without cold temperatures but cold temperature delays and suppresses defence related genes as well as signalling pathways, namely salicylic acid, jasmonic acid and ethylene. Thus, cold stress enables *Foc* STR4 to invade the xylem vessels and contribute to disease development. Therefore, cold stress (10^{o}C) may increase fungal infection, but disease development occurs only at 28^{o}C. We hypothesize that the reason behind the increase in disease

development at higher temperature is the increase in growth rate of the pathogen as well as increase evaporation from the plant, which enhanced transport of *Foc* in the xylem vessels.

Research on the banana plant, its genomics and biology is lacking, compared to other staple food crops, despite its importance. The full genome of *Musa acuminata* (DH-Pahang) has recently been sequenced (D'Hont *et al.*, 2012). This will enable the scientific community to identify genes involved in resistance to diseases, pests and abiotic stresses more rapidly, which will aid in banana breeding and transformation. Developing a banana plant resistant to *Foc* requires a proper understanding of the genetics and genomics of the plant and pathogen, their interaction, and factors influencing this interaction, such as cold temperatures. Genetically modified plants that showed resistance to *Foc* in the greenhouse have been developed (Yip *et al.*, 2011; Mahdavi *et al.*, 2012), but the durability of such resistance under field conditions still has to be established. Negative public perception and acceptability of genetic modification of plants may delay the use of this technology in future. The need to develop banana plants that resist biotic and abiotic stresses will become more important in future, especially with changes in the climate and the rapidly increasing human population.

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SUMMARY

Banana is an important staple food crop however, production of the world's most widely planted variety, the Cavendish banana, is threatened by a devastating fungal disease, called Fusarium wilt. Fusarium wilt of Cavendish bananas in the tropics is caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) 'tropical' race 4 (TR4), while a variant of the fungus causing the disease in the subtropics is called *Foc* 'subtropical' race 4 (STR4). The incidence of Fusarium wilt in the subtropics is usually aggravated after winter, which suggests that the plant is predisposed to *Foc* STR4 during cold temperatures. The objective of this study was to investigate the molecular processes, in both *Foc* and banana, which contribute to Fusarium wilt development under cold stress.

cDNA-AFLP expression profiling was used to elucidate the transcriptome of *Foc* STR4, *Foc* TR4 and non-pathogenic *F. oxysporum* isolates on minimal medium (without carbon source). This resulted in the identification of 229 unique gene fragments which included transcript derived fragments (TDFs) encoding for chitinase class V (*chsV*), GTPase activating protein, Major Facilitator Superfamily (MFS) multidrug transporter and serine/threonine protein kinase (*ste12*) genes. We speculate that those genes play a role in escaping host defence responses, and result in cell wall degradation. Pathogenicity-related genes identified in other *formae speciales* of *F. oxysporum*, such as the sucrose non-fermenting, F-box protein required for pathogenicity genes (*frp1*) and *cyp55*, were significantly up-regulated in *Foc* STR4 and *Foc* TR4, but not in *F. oxysporum* isolates non-pathogenic to banana. We suggest that these genes are important for the Fusarium wilt pathogen to enter the host xylem tissue, as they regulate the abundance of cell wall degrading enzymes. The increase in expression of *cyp55* in pathogenic *F. oxysporum* may give the pathogen the ability to regulate the nitrogen response pathway, which is essential for pathogenicity. This study provided the first identification of genes in *Foc* that potentially contribute to pathogenicity in banana.

Cavendish banana plants subjected to cold temperatures and inoculated with *Foc* resulted in a significant increase in disease severity. Visual symptoms, however, only appeared in inoculated plants after they were transferred to 28°C. Transcriptome analysis showed that several general defence mechanisms are activated in Cavendish

bananas infected with *Foc*. An important finding was that expression of defence-related genes was delayed in cold-treated plants, which enhance disease severity. More specifically, the induction of *PR* genes (*PR-1, PR-4, PR-6* and *PR-10*), *C4H*, involved in phenylpropanoid pathway and thus important for phytoalexin and lignin production, and *Hin1,* involved in the hypersensitive response, was significantly suppressed at an early stage during cold stress. This potentially provides an opportunity to *Foc* STR4 to invade the xylem and progress within the vascular bundles before plant defences are activated. Disease development mainly occurs at 28^{o}C, as the pathogen prefers higher temperatures for optimal growth and sporulation. At this temperature, movement of water through the vascular vessels of the roots and pseudostem is increased, resulting in the more rapid wilting of affected plants. Thus, cold stress may enhance infection of *Foc* STR4.